Distinguishing the Role of the $c-myb$ Proto-Oncogene in Mouse Models of Cardiovascular Disease

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Laboratory Medicine and Pathobiology
University of Toronto

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2016

Abstract

Despite considerable advances in our understanding of disease processes, cardiovascular diseases remain the top cause of mortality and morbidity in society today. Investigating the molecular and cellular mechanisms involved in the pathogenesis of these diseases to discover new therapeutic targets is of utmost importance. While much is known about the specific roles of transcription factor \textit{c-myb} in leukocytes, comparatively little is known about \textit{c-myb} in the context of cardiovascular disease. \textit{c-myb} regulates vascular smooth muscle cell (VSMC) differentiation from embryonic stem cells, as well as the proliferation of mature VSMCs. The literature points toward several areas of opportunity to examine the role of \textit{c-myb} in cardiovascular biology: first, to examine the role of \textit{c-myb} in the regulation of adult vessel-resident VSMC progenitor cell proliferation and differentiation in response to vessel injury and the molecular mechanisms involved in \textit{c-myb}-mediated VSMC differentiation. Second, while transgenic models manipulating \textit{c-myb} have yielded significant insight in regards to homeostasis of the immune system, little is known about a role for \textit{c-myb} in the regulation of baseline function of the cardiovascular system. While \textit{c-myb} regulates VSMC differentiation and pathological VSMC
proliferation, it is not known if it regulates VSMC contractile function and vascular contractility, and more generally, blood pressure homeostasis. Lastly, this dissertation examines the involvement of \textit{c-myb} in experimental atherosclerosis, a disease model involving both leukocytes and vessel-resident cells such as VSMCs. Therefore, work of this dissertation aims to elucidate the role of \textit{c-myb} in these areas and thus further the collective understanding of the mechanisms involved in cardiovascular pathophysiology.
Acknowledgments

To my wife Bethany, I am eternally grateful for your patience and encouragement throughout this process. I cannot even begin to fathom the innumerable ways in which you have helped me get through this arduous process. Your love and support means the world to me, and I hope that it will continue forever more.

To my parents and all the rest of my family, this dissertation is a testament to your continued encouragement and nurturing of my interest in science throughout my life. I am where I am today thanks to your efforts.

To my supervisor, Dr. Mansoor Husain, thank you for all that you have done for me in these past few years as my PhD supervisor. The opportunities you have afforded me through your supervision and mentorship have been tremendous in shaping me into the scientist I am today. The guidance you gave has helped me become a critical thinker, and the freedom to pursue ideas and test hypotheses has given me a breadth of knowledge and opportunity beyond my expectations. I hope that I too may one day become an esteemed scientist with the same breadth of knowledge, sharpness of thought and all around intellect.

To Dr. Clinton Robbins, my committee member, collaborator and also mentor, thank you for all that you have taught me since you joined TGRI. I am grateful to have had the honor to work with you and get so much mentorship along the way. The things I have learned from you will go a long way to shaping my approach to science for years to come.

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<tbody>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Abcg2</td>
<td>Adenosine triphosphate-binding cassette transporter subfamily G member 2</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>Acta2</td>
<td>Alpha smooth muscle actin (αSMA/ASMA)</td>
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<td>AF</td>
<td>Alexa Fluor®</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<td>ApoE</td>
<td>Alipoprotein E</td>
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<tr>
<td>β-gal</td>
<td>beta-galactosidase</td>
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<td>BM</td>
<td>Bone marrow</td>
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<td>BMS</td>
<td>Bare metal stent</td>
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<td>BMT</td>
<td>Bone marrow transplant</td>
</tr>
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<td>BP</td>
<td>Blood pressure</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>Ca^{2+}</td>
<td>Calcium^{2+}</td>
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<td>CArG</td>
<td>CC[A/T]_GG elements</td>
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<tr>
<td>CBP</td>
<td>cyclic adenosine monophosphate response-element binding protein</td>
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<td>CCA</td>
<td>Common carotid artery</td>
</tr>
<tr>
<td>CCR</td>
<td>CC Chemokine receptor</td>
</tr>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>Cdk2</td>
<td>Cyclin dependent kinase 2</td>
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<td>cDNA</td>
<td>complimentary deoxyribonucleic acid</td>
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<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
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<td>cKit</td>
<td>tyrosine-protein kinase Kit (CD117/SCFR)</td>
</tr>
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<td>CM</td>
<td>Cardiomyocytes</td>
</tr>
<tr>
<td>c-Myb</td>
<td>cellular homolog of avian myeloblastosis oncogene (MYB)</td>
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<td>Cnn1</td>
<td>Calponin 1, basic, smooth muscle</td>
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<td>Collagen</td>
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<tr>
<td>DBD</td>
<td>Deoxyribonucleic acid binding domain</td>
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<tr>
<td>DES</td>
<td>Drug-eluting stent</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
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<tr>
<td>DN</td>
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<td>DNA</td>
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<td>Dnase 1</td>
<td>Deoxyribonuclease 1</td>
</tr>
<tr>
<td>DOCA</td>
<td>Deoxycorticosterone acetate</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
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<td>EC</td>
<td>Endothelial cell</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ELISPOT</td>
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<tr>
<td>Eln</td>
<td>Elastin</td>
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<td>ENU</td>
<td>N-ethyl-N-nitrosourea</td>
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<td>Endothelial progenitor cell</td>
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<td>Erk</td>
<td>Extracellular regulated kinase (p42/p44)</td>
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<td>FACS</td>
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<td>Fbln</td>
<td>Fibulin</td>
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<td>Fibrillin</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>Flk1</td>
<td>Fetal liver kinase 1 (VEGFR2/KDR/CD309)</td>
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<td>FoxO</td>
<td>Forkhead box protein O</td>
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<td>gDNA</td>
<td>genomic deoxyribonucleic acid</td>
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<tr>
<td>Gy</td>
<td>Gray</td>
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<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
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<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
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<tr>
<td>Hprt</td>
<td>Hypoxanthine-guanine phosphoribotransferase</td>
</tr>
<tr>
<td>hr</td>
<td>heart rate</td>
</tr>
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<td>HSC</td>
<td>Hematopoietic stem cell</td>
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<tr>
<td>hypo</td>
<td>hypomorphic</td>
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<td>I/M</td>
<td>Intima/media</td>
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<td>Interleukin</td>
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<td>inositol 1,4,5-triphosphate receptor type-1</td>
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<td>K-562 human chronic myelogenous erythroleukemia cell line</td>
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<td>Kruppel-like factor 4</td>
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<tr>
<td>LIF</td>
<td>Leukemia inhibitor factor</td>
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<td>Lineage</td>
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<td>Lldr</td>
<td>Low density lipoprotein receptor</td>
</tr>
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<td>loxP</td>
<td>locux of X-over P1 bacteriophage P1 site</td>
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<td>LV</td>
<td>Left ventricular</td>
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<td>Ly</td>
<td>Lymphocyte antigen</td>
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<td>Lysine</td>
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<td>MBE</td>
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<td>Dominant negative Myb-Drosophila Engrailed</td>
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<td>Term</td>
<td>Definition</td>
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</tr>
<tr>
<td>mESC</td>
<td>mouse embryonic stem cell</td>
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<td>Myocardial infarction</td>
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<td>miRNA</td>
<td>micro ribonucleic acid</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MRTF-A/B</td>
<td>Myocardin-related transcription factor A/B</td>
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<td>MVSC</td>
<td>Multipotent vascular stem cell</td>
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<td>Neural/glial antigen 2</td>
</tr>
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<td>Nitric oxide</td>
</tr>
<tr>
<td>NRD</td>
<td>Negative regulatory domain</td>
</tr>
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<td>OCT</td>
<td>Optimal cutting temperature medium</td>
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<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>Recombination-activating gene</td>
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<td>rotations per minute</td>
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<td>Reverse transcription-polymerase chain reaction</td>
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<td>Sca1</td>
<td>Stem cell antigen 1 (Ly6A/E)</td>
</tr>
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<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
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<tr>
<td>SM</td>
<td>Smooth muscle</td>
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<td>SM22α</td>
<td>Transgelin</td>
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<td>SMC</td>
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<td>Smoothelin-B</td>
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<td>SRF</td>
<td>Serum response factor</td>
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<td>Transforming growth factor-B control elements</td>
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<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<td>U</td>
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<td>UTR</td>
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<td>Abbreviation</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR2</td>
<td>Vascular endothelial growth factor receptor 2 (Flk1/KDR)</td>
</tr>
<tr>
<td>v-myb</td>
<td>avian myeloblastosis viral oncogene</td>
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<tr>
<td>VPC</td>
<td>Vascular progenitor cell</td>
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<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
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CHAPTER 1:

1 Literature Review
1.1 Introduction

Despite considerable advances in our understanding of disease processes, cardiovascular diseases remain the leading cause of mortality and morbidity in society today. Common diseases such as hypertension and atherosclerosis, as well as complications of interventions for cardiovascular disease such as in-stent stenosis all share the involvement of vascular smooth muscle cells (VSMCs) as part of their pathology. VSMC migration and proliferation are hallmarks of atherosclerosis and restenosis, while in diabetes and hypertension, vascular dysfunction contributes to the progression of the disease. Thus further examining both the normal and pathological mechanisms that regulate VSMC physiology and pathophysiology are relevant to understanding the development and progression of a wide variety of cardiovascular diseases and ultimately in designing and implementing treatments against them.

The ability of VSMCs to de-differentiate from a mature, contractile, fully differentiated state to a less-differentiated proliferative (“synthetic”) state underlies their involvement in various vasculopathies. Because of their common involvement in cardiovascular pathologies, understanding the underlying mechanisms of both normal and dysfunctional VSMCs is of paramount importance in the management and treatment of disease. To this end, understanding the role of specific factors that are involved in the differentiation, proliferation, migration and contractility of VSMCs will expand our understanding of normal and pathological functions of VSMCs.

Several studies have shown that inhibiting the proto-oncogene c-myb reduces vessel remodeling following injury [1-6]. The most well characterized role of c-Myb is in the regulation of cell lineage determination and proliferation of specific hematopoietic stem and
progenitor cell populations. Knock out of \textit{c-myb} \((c-myb^{-/-})\) causes embryonic lethality at E15.5 in mice due to the failure to switch to definitive erythropoiesis \([7]\), without any overt cardiovascular phenotypes otherwise. While most cardiac and larger vascular structures have developed fully by E15.5 \([8, 9]\), it is unknown if further development of the cardiovascular system beyond this stage is affected. As such, examining the role of \textit{c-myb} in adult animals has largely precluded examination. While several studies have implicated it as a stem/progenitor cell regulator in multiple tissue compartments \([10-15]\), \textit{c-myb} also regulates the proliferation of adult VSMCs and the differentiation of VSMCs from a specific embryonic stem cell (ESC)-derived hematogenic progenitor cell population \([16, 17]\). However, it remains unknown if the same VSMC progenitor cell exists \textit{in vivo}, and if it persists into adulthood where it might contribute to vascular pathophysiology.

Several studies have attempted to identify if vessel-resident progenitor cells or circulating bone marrow (BM)-derived cells are involved in vessel injury responses. Evidence exists for both populations having a key role in the recruitment and proliferation of cells post-injury \([18-28]\). Different studies of atherosclerosis, endothelial denudation and flow-mediated arterial injury have shown evidence of the recruitment, investment and trans-differentiation of a circulating bone marrow (BM)-derived progenitor cell in response to injury, while other models of injury have not been able to identify BM-derived cells as contributing to VSMCs following injury. However, many of these studies have been performed with different models of vascular injury, on different strains of mice, at different ages and time points, hence drawing conclusions as to the source of cells involved in vascular remodeling remains difficult.

Beyond regulating proliferation and differentiation of VSMCs, \textit{c-myb} has also been implicated in the regulation of progenitor cell homeostasis in the hematopoietic system, colon,
brain and skeletal myoblasts [7, 10, 11, 29-32] indicating that \textit{c-myb} may have an additional role in the regulation of vessel-resident and/or bone-marrow derived vascular progenitor cells. It remains unknown if \textit{c-myb} has a critical role in the proliferation or potential (trans)differentiation of circulating BM-derived vs. vessel-resident cells which contribute to vascular remodeling in response to vessel injury. In addition, it is not known if \textit{c-myb} is critical for the differentiation and proper vasomotor function of VSMCs in the adult animal. Presumably defects in \textit{c-myb} activity will also result in the improper differentiation of VMSCs which will result in defective vasomotor function in mice harboring defects in \textit{c-myb}.

While much is known about the specific roles of \textit{c-myb} in hematopoietic cells, comparatively little is known about \textit{c-myb} in the context of cardiovascular disease. The literature points toward several areas of opportunity to examine the role of \textit{c-myb} in cardiovascular biology: firstly, the role of \textit{c-myb} in the regulation of cardiovascular progenitor cell proliferation and differentiation in response to vessel injury; secondly, and more generally, the role of \textit{c-myb} in other cardiovascular disease models involving VSMCs, such as atherosclerosis. Lastly, while transgenic models manipulating \textit{c-myb} have yielded significant insight in regards to homeostasis of the immune system, little is known about a role for \textit{c-myb} in the regulation of baseline function of the cardiovascular system. Therefore, work of this dissertation aims to clarify the role of \textit{c-myb} in these areas and thus further our collective understanding of the mechanisms involved in cardiovascular pathophysiology.
1.2 Cardiovascular system and vascular smooth muscle cells

The cardiovascular system is composed of the heart, blood vessels and the blood that is transported throughout the tissues of the body. As one of the earliest organ systems of vertebrate embryos to begin functioning, it allows for the expansion of developing tissues by overcoming the limitations of gas diffusion and nutrient distribution. In the adult animal, proper maintenance of vascular structure and function is crucial to the homeostatic function and maintenance of all tissues.

In the large arteries, the structure of the vessel wall allows for the bulk transit of blood to the various organs of the body at high pressures and flow rates. All blood vessels are lined by a monolayer of endothelial cells (ECs) surrounded by a basement membrane that comprises the innermost layer of the blood vessel, the intima. Larger diameter arterioles and arteries are also invested with a medial layer of vascular smooth muscle cells and elastic laminae which give larger diameter vessels strength and rigidity and the ability to constrict or dilate in order to regulate blood flow into capillaries and ultimately to target tissues. Outside of the outer elastic lamina lies the adventitia, which is composed of fibroblasts, pericytes, progenitor cells, mast cells, dendritic cells, adipocytes, lymphocytes, nerves, connective tissue and distinct structures such as lymph vessels and vasa-vasorum. In normal physiological states, VSMCs of the media remain quiescent, fully differentiated cells that maintain vascular tone through contractile functions. The current dogma of VSMC biology is that upon activation, either through injury or other extracellular activation signal, VSMCs are able to undergo de-differentiation and become synthetic cells that are capable of proliferation [33, 34]. This dogma relies on the notion that VSMCs are not terminally differentiated, but rather retain remarkable plasticity, including the
ability to reversibly de-differentiate and become proliferative cells in a process known as “phenotypic switching” [35]. This ability to moderate differentiation state allows for the same cells to regulate vascular contractility, but also allow for vascular repair and the formation of new vessels. However, this propensity for phenotypic modulation also underlies their participation in pathological remodeling responses in restenosis and atherosclerosis. Therefore, understanding the many mechanisms that regulate proliferation and differentiation of VSMCs offers the opportunity to manipulate them for therapeutic gain.

An alternate or complementary possibility, depends on the participation of circulating and or vessel-resident progenitor cells, including progenitors of VSMC, having the capacity to differentiate and participate in both normal vessel homeostasis and pathological vessel remodeling. To date, the role(s) played by c-myb in regulating one or both of these processes, namely the proliferation and differentiation of de-differentiated VSMC versus the proliferation and differentiation of VSMC progenitors have not been parsed.

1.2.1 Embryonic origins of SMCs

During development, VSMCs originate from multiple embryonic sources. The splanchnic mesoderm gives rise to the VSMCs of the gut and respiratory tract [36, 37], while the proepicardium, which originates in the splanchnic mesoderm, gives rise to the coronary arteries [38-41]. The secondary heart field, which has origins in the lateral plate mesoderm contributes VSMCs to the aortic and pulmonary trunks [42, 43], while the neural crest gives rise to VSMCs of the aortic arches, head and meningeal vasculature [42, 44, 45]. The VSMCs of the descending aorta arise from the somitic mesoderm [46], while the splanchnic mesoderm gives rise to VSMCs of the abdominal aorta, and mesothelium gives rise to mesenteric VSMCs of the gut [47]. However, all throughout VSMC populations, there are cells that originate from other
embryonic origins [48]. Remarkably, the boundaries between the various VSMCs of different embryonic origins in the aorta are very sharp, with little overlap except in the root of the aorta where several different populations meet [48-50]. While there are seemingly little differences in VSMC function at baseline, the regional heterogeneity and disease susceptibility and development along the aorta has implicated developmental origin of VSMCs as a source of differential disease susceptibility [48, 51]. Indeed, when tested in vivo and in vitro, it has been found that VSMCs from different anatomical areas and embryological origins respond differently to identical stimuli [52-57]. Homograft transplantation of abdominal and thoracic aorta and abdominal aorta and pulmonary artery showed that merely changing the anatomical location (and thus hemodynamic factors) cannot override the susceptibility to atherosclerosis formation of abdominal aorta, suggesting an intrinsic difference in these sections of aorta [58-60]. While these reports are almost 50 years old, they provide evidence that the origins of VSMCs may imprint differential responses to pathological cues. Although more recent studies have not provided conclusive evidence on how embryological origin confers differential susceptibility to disease, it would appear nonetheless that the developmentally diverse ontogeny of VSMCs has a critical lasting effect on pathological susceptibility.

1.2.2 Fetal SMC differentiation

Vascular development in the developing vertebrate embryo follows two distinct stages, the first being vasculogenesis, the de novo formation of blood vessels, and the latter being angiogenesis, the outgrowth of new blood vessels from existing ones. Fetal liver kinase+ (Flk1; also vascular endothelial growth factor receptor 2 [VEGFR2]; human kinase insert domain receptor [KDR]) hemangioblasts, embryonic precursors to hematopoietic cells, ECs and VSMCs, are initially recruited to form primitive blood islands, wherein they undergo partial lineage
commitment to hematopoietic precursor cells and angioblasts [61, 62]. Flk1 is the earliest marker of hemangioblasts, expressed at E7.0 in the mouse [61], which subsequently differentiate into populations of cells that will give rise to HSCs (Flt1⁺Tal1⁺Runx⁺) and the angioblasts (Flk1⁺Tal1⁻) [63, 64]. These primitive angioblasts will form a network vascular structure of ECs and VSMCs forming the basis for the first blood vessels in the developing embryo.

Angiogenesis is then the successive remodeling, through outgrowth and pruning, of these networks to form capillary networks throughout tissues. Proliferating ECs will secrete platelet derived growth factor-BB (PDGF-BB) to attract PDGF receptor-β⁺ (PDGFR-β) VSMC progenitor cells to developing vessels [65, 66]. In larger vessels, subsequent pericyte and VSMC recruitment to vessels leads to muscularized arteries. A critical role for the VSMCs is to lay down extracellular matrix (ECM), which functions to inhibit EC proliferation and migration, and to also stabilize and provide structural integrity to developing arteries [67]. The consolidation of VSMCs and ECM surrounding these vessels is also known as arteriogenesis, with the amount of VSMC and ECM investment into the new vessel dictated by the functional role of the vessel.

Upon contact between ECs and VSMCs, cell-cell interactions begin to mediate vessel maturation. Transforming growth factor β1 (TGFβ1) causes ECs to stop proliferating and begins to stabilize the nascent vessel; while it causes up-regulation of VSMC genes resulting in the expression of characteristic contractile proteins [36, 68, 69].

1.2.3 Transcriptional regulation of VSMC differentiation

The genes that encode the contractile and cytoskeletal proteins that are used as markers of differentiated SMC are broadly divided as serum response factor (SRF)-dependent and -independent. SRF-dependent genes such as alpha smooth muscle actin (Acta2/αSMA/ASMA), transgelin (SM22α), smooth muscle calponin (Cnn1), smooth muscle myosin heavy chain (SM-
MHC/Myh11) and telokin contain in their promoter regions CArG (CC[A/T]GG) elements which bind SRF. SRF-independent smooth muscle genes, which include smoothelin-B, aortic carboxypeptidase-like protein and focal adhesion kinase-related nonkinase are not regulated by CArG elements.

SRF is a member of the MADS-box family of transcription factors that is expressed ubiquitously, yet it modulates transcriptional activity through the direct binding of lineage-restricted transcription factors [70-72]. In cardiomyocytes (CM) and VSMC progenitor cells, SRF heterodimerizes with myocardin to activate CM- and VSMC-specific genes [73, 74]. Myocardin is essential for VSMC development, as myocd−/− mice die at E10.5 with no VSMC development, yet have normal expression of CM-specific genes [75]. Over-expression of myocardin results in the expression of multiple SRF-dependent markers of differentiated VSMCs, as well as SRF-dependent cardiac and skeletal muscle genes [76-78]. In addition to myocardin, there are also myocardin-related transcription factors (MRTF) A and B that have been shown to activate SMC gene promoters via a SRF-dependent mechanism [79, 80]. While loss of MRTF-A does not result in an overt SMC phenotype [81], loss of MRTF-B causes defects only in the differentiation of neural crest-derived VSMCs [82].

1.2.4 Growth Factor regulation of SMC differentiation

SMC-specific gene expression is also regulated through extracellular cues. TGF-β treatment induces the expression of many SMC differentiation markers [68, 83, 84] through the regulation of TGF-β control elements (TCEs) in SMC gene promoters. Mutation of TCEs have been shown to completely abrogate SMC gene expression, independent of CArG element binding [85], suggesting that TGF-β may regulate SRF binding to SMC gene promoters [86]. In addition to activation of SRF-dependent transcription, TGF-β also modulates the expression of
the Kruppel-like factors (KLFs), which modulate VSMC-specific gene expression [85, 87]. PDGF-BB also regulates VSMC differentiation [68], although its regulation appears to be context specific. Loss of PDGF-B or its receptor PDGFR-β in mice results in embryonic lethality due to SMC hypoplasia resulting in widespread edema and hemorrhage [65, 66, 88, 89]. Additionally, mESC-derived Flk1+ progenitor cells will begin to express SMC genes when treated with PDGF-BB [25, 39, 90]. However, treatment of cultured aortic and other VSMCs is associated with proliferation and rapid down-regulation of SMC related genes [91-95]. *In vivo*, PDGF promotes the proliferation and migration of medial VSMCs during neointimal remodeling in arterial injury models [96, 97]. Thus the role of PDGF signaling may be distinct in embryonic development/progenitor cell differentiation versus adult VSMC phenotypic modulation.

### 1.2.5 Epigenetic control of SMC differentiation

MicroRNAs (miRNAs) are a class of short, 20-25 nucleotide regulatory non-coding RNAs that recognize miRNA-binding sites in the 3’ untranslated regions (UTRs) of target mRNAs to cause repression or degradation of the transcript. Multiple genes have been shown to be regulated by a single miRNA, suggesting that miRNAs may be able to regulate processes such as differentiation through the coordinated regulation of multiple transcripts [98]. miRNA-143 and -145 have been shown to regulate phenotypic modulation of VSMCs, promote the contractile phenotype and suppressing SMC proliferation [99, 100]. During vessel injury and atherosclerosis, miRNA-143/145 expression is also decreased as SMCs de-differentiate and begin to proliferate, and over-expression of miRNA-143/145 limited neointimal formation by inhibiting VSMC de-differentiation and proliferation [99, 101, 102]. In addition to SMC gene suppression, miRNAs may also regulate chromatin structure during SMC differentiation. miRNA-10a targets histone deacetylase (HDAC) 4, and is up-regulated during SMC
differentiation from mESCs [103]. Deletion of miRNA-10a inhibits SMC differentiation, presumably through effects on chromatin remodeling, although acetylation of co-factors that regulate SMC differentiation cannot be ruled out as another potential mechanism of action.

The local chromatin structure around SMC genes will actively influence the binding of transcription factors to these genes. In particular, post-translational modifications of histones, including methylation and acetylation, can provide SMC-specific epigenetic control of SRF-responsive genes [104]. Histone methylation is thought to preserve genetic information regarding cellular identity and mediate cell fate decisions by determining if specific gene loci exist in euchromatin (open to transcription) or heterochromatin (closed) states. In addition, methylation of histones can help assemble and anchor transcriptional protein complexes to gene loci. SRF/Myocd complexes specifically associate with histone H3 that has been methylated at Lys4 and Lys79 during activation of SMC-specific gene expression, which may be essential for anchoring transcriptional machinery at SMC-specific genes [104].

While histone methylation serves as a mechanism for relatively stable changes in chromatin structure, histone acetylation serves as a rapid and reversible mechanism for changes in chromatin structure. The amount of histone acetylation determines the degree of chromatin remodeling, and is fine-tuned by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Like methylated histones, acetylated histones also directly bind to transcriptional complexes. Stem and SMC progenitor cell lines display low levels of acetylation of SMC-specific genes before they are differentiated to SMCs [105-107]. SMCs exhibit increased histone acetylation at SMC gene CArG elements, making them more accessible to SRF/Myocd complexes, which in turn recruit other HATs such as CREB binding protein (CBP) and p300 [105, 106], allowing for further stabilization and expression of SMC-specific genes.
1.2.6 Regulation of VSMC phenotype in disease

Because VSMCs have the potential to shift from a contractile to a synthetic phenotype, many of the mechanisms that govern this shift are the same mechanisms that are operant during the differentiation of VSMC. Klf4, which is normally not expressed in mature contractile VSMCs, is rapidly expressed following injury, where it interferes with SRF binding to CArG elements and recruits repressive chromatin remodeling factors to SM-specific promoters, down-regulating their expression [87, 104]. PDGF-BB and its receptor PDGFR-β expression is increased in vascular lesions. Blocking PDGF-mediated signaling has been shown to reduce neointimal lesions, via the down-regulation of myocardin expression, histone deacetylation of SM-specific genes and disruption of MRTF binding to SRF, all of which are associated with decreased SRF-mediated gene induction. Hence during de-differentiation of VSMCs, the same mechanisms are in operation as when VSMCs are differentiating. Interestingly, SRF is not known to change expression level during phenotypic modulation [86, 108], instead acting as a toggle switch between contractile and synthetic states of VSMCs. In order to modulate transcriptional activation of myocardin-target genes through SRF, myocardin has been shown to recruit chromatin modifying enzymes such as p300 and class II HDACs in order to selectively enhance or suppress the expression of SRF target genes [106].

miRNAs also likely control numerous SM-specific genes coordinately, allowing for the appropriate level of expression of SM-specific genes during vessel injury. miR-21, -221/222, which are PDGF-responsive genes, actively promote VSMC proliferation and neointimal hyperplasia [109-111]. miR-221 has been shown to repress cKit, which in turns leads to the down-regulation of myocardin expression, leading to widespread decreased SM-specific gene expression [110]. In contrast, miRs-143 and -145, which are both highly expressed in mature,
contractile VSMCs, are both down-regulated during vessel injury. It has been shown that they regulate the expression of Elk-1 and KLF4, promoting a proliferative phenotype of VSMCs, both in vessel injury as well as atherosclerosis [99, 112]. Likely, these and other mechanisms are amenable to coordinating the expression level of multiple SM-specific genes simultaneously, allowing for the multiple different signaling pathways to fine-tune the expression level of SM-specific genes during phenotypic modulation.

1.2.7 VSMC heterogeneity in disease

In the healthy adult blood vessel, VSMCs are typically quiescent, exhibiting very low rates of proliferation, low rate of ECM synthesis, and are non-migratory, as their main role is in maintaining vascular tone through their contractile functions [113]. However, during vessel remodeling in response to injury, a subset of VSMCs is recruited in the repair process, while the bulk of VSMCs maintain their normal contractile function [33, 114-116]. Because VSMCs are not terminally differentiated, they are able to undergo a de-differentiation process termed “phenotypic modulation” wherein they down-regulate SMC-specific protein expression (e.g. α-SMA, Acta2, Cnn1, and Myh11), and become “synthetic” [35]. These activated synthetic cells share many hallmarks of fetal VSMC, with typically high rates of proliferation, ECM synthesis and migration [117]. While VSMCs from different anatomical and embryological origins are known to have different responses to identical growth factor stimulation or hemodynamic conditions [52-57] to the same stimuli, it is not known which factors, if any, variably regulate differentiation in one area from another. Likewise, within a given area of VSMCs, it is not currently known how a particular VSMC becomes synthetic, or if all VSMCs have an inherent capacity to modulate their phenotype depending on cues from the local environment. In support of the notion that only a subset of VSMC becomes synthetic, in vitro isolation of human thoracic
aortic VSMCs have revealed some to have a distinct polygonal morphology versus the typical spindle shape [118]. These polygonal VSMCs had differing rates of proliferation, migration and responses to contractile agonists, suggesting that they represent a subset of more synthetic VSMCs. Others have shown that bovine arteries also yield heterogeneous VSMC populations that differ in their response to mitogenic stimuli [118-120], suggesting that these cells may be selected to respond to pathophysiological cues and may represent a pool of VSMCs primed to undergo phenotypic modulation and participate as synthetic VSMCs in response to vessel injury.

1.2.8 Challenging the dogma: alternate VSMC origins?

The above studies somewhat challenge the current dogma that all mature VSMCs have the capacity to de-differentiate to become synthetic proliferating VSMCs, but rather suggest that a small subset of VSMCs are poised to proliferate in response to stimuli. In one example of vessel remodeling, neointima formation has been thought to originate entirely from the phenotypic modulation of vessel-resident medial VSMC that migrate to form neointima [113, 115, 116]. However, emerging evidence now suggests that local progenitor populations residing in various compartments of the vessel wall may in fact be a contributing source of synthetic VSMCs in response to injury [121-124], challenging the dogma that most if not all proliferating/synthetic VSMCs originate from de-differentiated mature VSMCs. Through lineage-tagging experiments, it has been suggested that neointimal VSMCs may also be generated in part by the proliferation and differentiation of vessel-resident medial multipotent stem and progenitor cells [121]. This notion has, in turn, been challenged by evidence that adventitial cells rapidly proliferate and migrate in response to vessel injury and also contribute to neointima formation [123, 125-128]. Adventitial cells negative for SMC markers have been shown to migrate through the vessel wall and contribute SMC marker positive cells to neointima [123], thus demonstrating that at least
some portion of neointimal cells can derive from the adventitia. The discovery that VSMCs can be derived from multipotent stem and progenitor cells, and that these stem and progenitor cell-derived VSMCs can participate in the process of vascular repair and regeneration has led to the re-evaluation of the belief that mature vessel-resident VSMC are the only source of SMCs in response to injury. Any paradigm focused on the phenotypic plasticity of mature VSMCs regulating normal physiology and pathological remodeling now requires reworking, based on the discovery of stem- and progenitor-cell pools within the adult cardiovascular system, and that these cells can participate in the pathogenesis of vascular disease. Lineage tracing studies to determine the definitive ontogeny of VSMCs generated in response to injury will be required to conclusively determine the relative contribution of adventitial VSMC progenitor cells to the vessel injury response.

1.3 The MYB gene family

The proto-oncogene myeloblastosis oncogene (c-myb), which encodes a nuclear transcription factor c-Myb, is involved in the proliferation, differentiation and survival of a variety of cell types. c-myb is the mammalian cellular homolog to an avian myeloblastosis viral oncogene (v-myb) [129, 130]. It was identified through the molecular cloning and sequencing of two transforming/oncogenic viral genomes, revealing v-myb to be a fusion between myeloblastosis-associated virus, type-1 and proto-oncogenic sequences in chicken DNA [129, 130]. This viral oncogene was found to be transformative due to insertional mutagenesis by retroviruses that do not themselves contain oncogenes, but instead caused oncogenic activation of c-myb, leading to acute myeloid and lymphoid leukemia. After initial discovery, it was found
that \textit{c-myb} was also in mouse and human genomes. Through comparative genomics, it has been found that \textit{c-myb} is highly conserved, and present in all vertebrate species, some invertebrates, plant species and even some prokaryotes [131]. In vertebrate species, \textit{c-myb} has been shown to be a potent and pleiotropic regulator of hematopoietic cell growth, differentiation and oncogenic transformation, consequently the bulk of knowledge on the function of \textit{c-myb} is in the context of hematopoiesis. However, it has become known that \textit{c-myb} is also active in a variety of other progenitor cells and tissue compartments, such as colon crypt cells [11, 13, 30, 31, 132-134], adult neural stem cells [10], skeletal myoblasts [32, 135], cardiac myogenesis [135] and skin wound healing [136]. Of particular relevance to cardiovascular diseases, \textit{c-myb} has significant roles in hematopoiesis, but also in the differentiation and proliferation of VSMCs as well as the production of extracellular matrix proteins [136-139].

While c-Myb is expressed abundantly in immature HSCs and hematopoietic progenitor cells, both during development as well as in the adult, its expression declines as cells become terminally differentiated. However, in some cells, c-Myb expression can be re-established in terminally differentiated cells, such as mature B- and T-lymphocytes upon activation and proliferation [140-142]. Similarly, re-expression of c-Myb is also observed in colon epithelium [30, 134] and skeletal myoblasts [32]. Of particular note, while mature contractile VSMCs express very low levels of c-Myb expression, they can rapidly up-regulate expression in response to vascular injury [2]. Thus while c-Myb clearly has role in developmental processes, potentially in a multitude of tissue compartments, it clearly also has a role in regulating homeostasis and pathological responses in adult animals.

1.3.1 Myb structure and function
Through screening a human T-cell cDNA library, it was found that there are two other members in the Myb family, \textit{A-myb} (human MYB-like 1) and \textit{B-myb} (MYBL2) [143]. All three Myb family genes encode similar proteins with similar structure and sequence homology, despite not having overlapping functions or expression patterns. The Myb family proteins have an N-terminus DNA binding domain (DBD), a transactivation domain (TA) and a C-terminus regulatory domain. In A- and c-Myb, the C-terminus region contains a negative regulatory domain (NRD), whereas in B-Myb, the C-terminus contains both positive and negative regulatory domains [144, 145].

The DBD of Myb family proteins consist of a three tandem repeat amino acid structure termed R1, R2 and R3 [146-148] containing tryptophan amino acid (AA) residues [149]. This structure is highly conserved [150, 151], and has been identified in human, rodent, teleost (bony fish), avian, xenopus, drosophila, sea anemone, as well as corn and yeast species [131, 143, 151-155]. Deletion analysis has shown that R2 and R3 are required for DNA binding [146, 156], while the R1 motif is involved in the stabilization of DNA complexing with R2-R3 [157, 158]. The DBD binds specifically to the consensus AA sequence PyAACG/TG [159]. While R2 is highly conserved among Myb family members, there are some distinct differences in the R3 domain, which has been proposed as a mechanism for regulating specificity of Myb proteins despite a relatively short target sequence [160].

The DBD in A-, B- and c-Myb are nearly identical, with most of the sequence divergence occurring in the TA and NRD [161]. While all three proteins can bind to the same promoter sequences in reporter assays [162], owing to the similarity of their DBD, they have differential expression patterns and have distinct biological activities. \textit{A-myb} has been found to be essential for spermatogenesis, as a master regulator of genes involved in meiosis [163] and also mammary
gland epithelial development [164]. B-myb, which is ubiquitously expressed in dividing cells, is essential for cell cycle progression [165, 166], and is required for inner cell mass formation during early embryonic development [167]. Microarray studies in human cells have found that while the three proteins share considerable sequence homology, they activate unique sets of genes [168]. The differential activation of genes is mapped to differences in their transcriptional activation and to their C-terminus NRD [169], which profoundly affects their ability to regulate different genes. While in vitro assays have shown that all three Myb proteins can bind to the same sequences, because they activate different genes and appear to have distinct, non-overlapping roles physiologically, loss of function in one of the Myb family proteins cannot be rescued by other Myb family members, as suggested by loss-of-function studies in transgenic mice [7, 164, 167].

1.3.2 Regulation of c-Myb

The c-myb gene encodes a 75 kDa nuclear protein initially found to be ubiquitously expressed in hematopoietic cells, albeit in lower amounts as cells became further differentiated [170, 171]. An 89 kDa splice variant was also identified in several avian, murine and human hematopoietic cells [172-174]. This splice variant has higher transactivational activity than the p75 form of c-Myb, the p89 splice variant lacking a leucine zipper motif that regulates negative autoregulation through dimerization [175]. Later studies found several other transcript variants formed by alternative splicing of additional exons (8A, 9A/B, 10A, 13A, 14A), all in the C-terminal end of the protein, leaving the DBD and TA unaffected [176]. Alternatively spliced mRNA variants of c-myb have differential activity and lead to quantitatively and qualitatively different proteins which appear to have lineage- and stage-specific expression patterns [176, 177].
c-Myb is phosphorylated at multiple sites \textit{in vivo}, which leads to a decrease in activity or leads to proteolytic degradation of the protein. c-Myb phosphorylation in the N-terminal region by casein kinase-II leads to weaker DNA binding activity [178], and also leads to decreased ability of c-Myb to interact with other transcription factors leading to decreased activity [179]. Phosphorylation by p42 Extracellular regulated kinase (ERK) at multiple sites in the C-terminus of c-Myb also results in decreased transactivation [180, 181], with loss of these phosphorylation sites resulting in 2-7 fold increases in transactivation.

Phosphorylation of c-Myb also leads to its proteasomal degradation. Wnt-1 signalling, via TGF-\(\beta\) activated kinase, homeodomain interacting protein kinase-2 and nemo-like kinase, have been shown to directly bind to the NRD and DBD of c-Myb and phosphorylate it, leading to ubiquitination and proteasome-dependent degradation [182, 183]. c-Myb can also be hyperphosphorylated in the C-terminus NRD, and broken down by the 26S proteasome [184, 185]. Ubiquitination of c-Myb is dependent on the E3 ubiquitin ligase Fbw7, which binds to and targets c-Myb for proteasomal degradation [186, 187].

c-Myb can also be acetylated by histone acetyltransferases (HATs) such as p300 and CBP. Acetylation occurs on five lysine residues in the C-terminal NRD leading to increased binding affinity to p300, as well as enhanced binding of c-Myb to DNA [188, 189]. c-Myb activity has also been shown to be regulated by sumoylation of two sites within the NRD [190, 191], affecting the ability to recruit p300 with corresponding changes to H3 and H4 histone acetylation in c-Myb responsive genes [192]. Association with HATs and HDACs with c-Myb is significant as histone modification altering the chromatin structure and availability of c-Myb-responsive genes could be a potential regulatory mechanism of how c-Myb may regulate a significant number of genes without Myb binding elements in their promoters.
1.3.3 Transgenic c-Myb mouse models

Following the identification, cloning and sequencing of c-myb, numerous in vitro studies yielded considerable insights into the function of c-myb as a regulator of cell proliferation, differentiation and survival of hematopoietic cells. The first demonstration that c-myb has a physiological role was from studies of mice that lack c-myb. In a seminal study by Mucenski et al [7], a transgenic mouse was made with a neomycin cassette inserted into the exon 6 of c-myb, causing a premature stop codon and knockout of c-Myb expression. The resulting homozygous knockout (c-myb⁻/⁻) mice died in utero at embryonic day (E) 15. When necropsied, the embryos were found to be severely anemic and had drastically reduced numbers of both myeloid and lymphoid cells. It was found that in these mice, yolk-sac-derived primitive hematopoiesis occurred without defect allowing embryos to develop normally to a certain point, suggesting that early primitive hematopoiesis is completely independent of c-myb. Early embryonic hematopoiesis is characterized by erythrocytes that originate from the yolk sac that are relatively large and retain their nuclei, much like the erythrocytes of non-mammalian vertebrate species such as fish, amphibians and birds [193]. Embryonic hematopoiesis in mammals is thought to be “primitive” like non-mammalian vertebrates, whereas “definitive” or adult hematopoiesis is initiated intra-embryonically independently of yolk-sac-derived hematopoiesis, eventually replacing most yolk-sac derived blood cells. While primitive hematopoiesis was unaffected in c-myb⁻/⁻ mice, definitive hematopoiesis that begins in the fetal liver and eventually in bone marrow was severely defective in these mice, leading to hematopoietic failure and death of the embryos. While multipotent HSCs formed in c-myb⁻/⁻ mice, they failed to efficiently and properly differentiate causing a failure of definitive hematopoiesis to occur. In vitro examination of c-myb⁻/⁻ ESCs revealed that while HSCs form normally, the appearance of definitive hematopoietic
progenitor cell populations is blocked at an early multipotent stage [194]. With embryos dying at E15, it remained possible to examine the effects of $c\text{-}myb^{-/-}$ on hematopoiesis. However, other organ systems were not examined in the original landmark study, with embryonic lethality precluding the examination of the role of $c\text{-}myb$ in the developing cardiovascular system, as well as the role of $c\text{-}myb$ in adult animals, where $c\text{-}myb$ might have distinct roles in normal and/or pathophysiological states.

It was later found that $c\text{-}myb^{-/-}$ embryos exhibited abnormally thin skin, which was attributed to a complete absence of collagen type-I [136], subsequently shown to be directly transcriptionally regulated by c-Myb [137, 138]. Mouse embryonic fibroblasts (MEFs) established from $c\text{-}myb^{-/-}$ embryos showed defective proliferative and migratory responses, consistent with earlier studies [195]. Consistent with a defect in collagen synthesis, $c\text{-}myb^{+/-}$ mice have impaired wound healing of the skin as compared to WT mice, with thinner weaker scar formation [136]. Of particular relevance to cardiovascular biology, collagen type-I comprises approximately 60% of the collagens in a healthy vessel wall [196]. Defective $c\text{-}myb$ may also result in perturbations in vascular ECM components, which are vital to maintaining the differentiated state of contractile VSMCs [197, 198]. Interestingly, hepatic stellate cells, which are pericytes of the liver that mainly function to regulate liver fibrosis [199], also upregulate collagen type-I expression via c-Myb when stimulated with TGFβ1 [200], demonstrating that the regulation of collagen synthesis by $c\text{-}myb$ does not appear to be restricted to specific cell types or tissues. Given that $c\text{-}myb$ may regulate the proliferation and differentiation of vascular cells as well as their ability to lay down the appropriate ECM, perturbing $c\text{-}myb$ function during cardiovascular development as well as during pathological remodeling may have significant impacts on vessel morphology and function.
In the only model examining over-expression of c-myb, Furuta et al. [135] created a mouse with c-Myb expression under the control of the ubiquitously expressed β-actin gene promoter. They found expression of c-myb in heart, brain, muscle, testis, lung and very strongly in thymus. Somewhat surprisingly, the mouse developed no hematological abnormalities compared with WT mice. However, mice developed severe dilated cardiomyopathy and skeletal muscle degeneration. Interestingly, the dilated cardiomyopathy only occurred in male mice, while skeletal degeneration also occurred in some female mice. Onset of cardiomyopathy was after 3 months, as cardiac weights were not different versus WT at that time point. While this report remains the only one on this line of mice, it is interesting to note that over-expression of c-Myb did not result in hematological abnormalities, whereas it resulted in significant adult-onset cardiac and skeletal myopathy. Although this report contains little to no mechanistic information, it is tempting to speculate whether over-expression of c-myb disrupts normal skeletal and cardiac myogenesis by altering myocardin expression, which is significantly impaired in c-myb<sup>−/−</sup> EBs differentiated towards SMCs [17].

More recently, several targeted mutations and mice generated in forward-genetics screens have yielded further insights into the role of c-myb in hematopoiesis. Because these transgenic mice are all viable, more specific studies in the adult animal were now possible. Of the targeted mutations, there are three independently derived conditional floxed alleles of c-myb (c-Myb<sup>Δex3-6</sup>, c-Myb<sup>Δex6</sup> and c-Myb<sup>Δex2</sup>) that have been used extensively in studies of B- and T-cell lineages [201-206]. These mice have been used to show that c-Myb is required for the normal differentiation and proliferation and function of T-cells, as well as the maintenance of B-cells.

There are also three reported models of c-myb deficiency that were discovered as a result of non-targeted, forward-genetics screens. Carpinelli et al. [207] used a forward-genetics
approach to identify genes that could overcome the loss of thrombopoietin signaling to rescue platelet production, resulting in the discovery of two novel loss-of-function mutants of c-myb.

The two new mutants of c-myb, both of which harbored hypomorphic versions of c-myb, mapped to the DBD of c-myb (c-MybPl1) and to the leucine zipper motif of the NRD of c-myb (c-MybPl4). In another forward-genetics screen, Sandberg et al. [15], used an N-ethyl-N-nitrosourea (ENU) mutagenesis screen to identify novel mutations to model thrombocytosis. They discovered a mutant with significantly elevated platelet counts, and when they sequenced the genome of the mutant mouse strain, a single point mutation was found that mapped to a methionine to valine (M303V) mutation in the TA domain of c-myb. This point mutation was shown in an in vitro binding assay to not affect DNA binding capacity of the mutant c-Myb protein. However, the mutation greatly decreases binding affinity for p300 (by ~50%), one of the main cofactors for c-Myb, presumably leading to defective gene transactivation in vivo. This hypomorphic c-myb (c-MybM303V) had a phenotype consistent with the two other hypomorphic c-Myb mice, with increased megakaryocytes and platelets. The c-mybM303V hypomorph also had numerous hematopoietic abnormalities such as mild anemia, lymphocytopenia, and a lack of eosinophils caused by dysregulation of hematopoietic stem/progenitor cell maintenance and stage- and lineage-specific blocks on differentiation. HSC maintenance was perturbed in the c-mybM303V hypomorph, with an approximately 10-fold increase in the number of HSC. However, despite this greatly expanded number of long-term HSCs, the defect in c-myb limited the differentiation and proliferation capacity of downstream progenitor cells, leading to decreased populations of most terminally differentiated blood cells. This mouse has also been shown to have defective colon crypt cell homeostasis which was attributed to decreased cyclin-E1 and cell cycle dysregulation [11]. However, to date, no cardiovascular characterizations had been
performed in this particular c-myb mutant. It is possible that beyond the regulation of genes that are direct targets of c-Myb, the defective interaction of c-MybM303V with HATs p300 and CBP may figure to be critically important in the regulation of VSMC differentiation, given the role played by histone modifications in the context of VSMC differentiation.

To study the role of c-Myb in vivo in a model of vessel injury using a mouse model of genetically decreased c-Myb function, our lab expressed the dominant negative c-mybMEn with a SM22α gene promoter-directed doxycycline-supressible tetracycline-regulated transcriptional activator (SmTA) to create a SM22α-specific dominant-negative c-Myb mouse (c-MybMEn/SmTA) [1]. The c-MybMEn/SmTA mouse had normal development of SMC-rich areas such as aorta, carotids, lungs, liver and kidney and did not manifest any differences in blood pressure, which would suggest that contractile function of VSMCs was not affected in these mice. When subjected to wire denudation injury of the carotid artery, c-MybMEn/SmTA mice were protected from neointimal remodeling compared to WT and doxycycline silenced c-MybMEn/SmTA mice, suggesting that inhibiting c-Myb function in SM22α expressing cells was sufficient to reduce neointimal remodeling. While this was the first genetic model of c-Myb deficiency specifically targeting VSMCs, there were several questions that could not be addressed by this animal. First, it could not be used to determine what the relative contribution of inhibiting c-Myb function might be in non-VSMCs, such as ECs, fibroblasts, vessel-resident progenitors and cells recruited from the circulation such as inflammatory cells and circulating progenitors. Second, from a differentiation perspective, because the transgene was driven by the SM22α promoter, a marker expressed when cells are maturing and differentiating into VSMCs, the in vivo role of c-myb during de-differentiation of VSMCs or prior to the differentiation of a VSMC progenitor could not be determined.
While the c-Myb<sup>MEs/SMTA</sup> mouse line was unfortunately lost due to transgene silencing, our lab acquired the c-myb<sup>M303V</sup> hypomorph (herein referred to as c-myb<sup>h/h</sup>) mouse in order to pursue further studies into the role of c-myb in the development and pathophysiology of VSMCs. The c-myb<sup>h/h</sup> mouse has several advantages. First, because it is a global loss-of-function mutant, it could be used to examine the role of c-myb in the development of VSMCs <i>in vivo</i>. Second, because the loss-of-function is not targeted, we can also use this animal to examine the role of c-myb in cell types where there are no known markers to drive the expression of tissue specific Cre-mediated recombinations, or in cells with expression patterns that overlap those of other tissues, which would also preclude the use of Cre-mediated recombination. Third, the fact that the mutation is a point mutation affecting only the TA domain, while sparing function of the DBD, may also provide an opportunity to distinguish between genes and processes that require c-Myb-DNA binding versus those that require intact c-Myb transactivation. Knowing that cofactor recruitment is severely affected in the hypomorph, this animal model may uniquely inform us about “c-Myb-dependent” processes that are cofactor-dependent. Finally, because the mouse is viable and has a normal lifespan, it is apt to be used in experimental models that depend on long-term studies, such as bone marrow transplantation, and chronic pathogenesis, such as hypertension and atherosclerosis.

1.3.4 c-Myb as a stem- and progenitor cell regulator

Beyond the regulation of the hematopoietic system and the various stem and progenitor cells that comprise the complex hierarchy of immune cells, c-myb has also been shown to be relevant in the regulation of several other stem- and progenitor cell populations.

In addition to defective hematopoiesis, it was later described that c-myb is also required for the normal development of the colonic crypts. It was earlier shown that colonic crypt cells
(the progenitor cell for colon epithelium) have higher c-Myb expression, which is down-regulated as they differentiate [30]. While the embryonic lethality of c-myb−/− mice did not allow for the examination of colonic development, as it occurs after E15, when colon segments from E14 c-myb−/− embryos were transplanted in WT adult mice, it was found that normal colonic crypts failed to form [31], suggesting that c-myb is essential for the homeostasis of colonic crypt progenitor cells. Consistent with these findings, it was later shown that other c-myb mutant mouse lines had defective colon epithelial development [11]. In normal colon crypt cells, c-myb upregulates cyclin E allowing for the entry into the cell cycle [208], hence disrupting c-Myb function results in defective activation of colon crypt cells and defective tissue homeostasis.

c-myb expression in the embryonic brain has been reported to be very high [134], but declines sharply after birth. However, expression of c-myb has been detected in the adult brain [209]. In adult brain, ependymal cells of the ventricular system in the adult brain are formed from neural progenitor cells. In mice with conditional deletion of c-myb in Nestin+ neural progenitor cells, there was a significant dilation of ventricles due to defective neurogenesis [10]. Neural progenitor cells that lacked c-myb expression had reduced proliferation, and had reduced Sox2 and Pax-6 expression, both genes involved in maintenance of neural progenitor cells.

c-myb has also been shown to be involved in the differentiation of airway epithelium. RNA interference or c-myb gene deletion in mice resulted in the loss of differentiated airway epithelium cell that were either Foxj1+ ciliated cells as well as Scbg1a1*Muc5ac+ secretory cells [210]. c-Myb+ airway epithelial cells were also found in human airways, suggesting that c-myb may also regulate airway epithelium differentiation in humans as well as mice. However, in this study it was not determined if c-myb dependent differentiation defects were due to defective proliferation of the progenitor cell or due to defective differentiation. Nonetheless, the role for c-
myb in multiple progenitor cell compartments highlights the possible role of c-myb as a master regulator of a variety of tissues as an upstream regulator of progenitor cell proliferation and differentiation via cell cycle regulation, cell differentiation and cell survival pathways.

1.3.5 The role of c-myb in VSMC proliferation

The earliest report of a role for c-myb in the proliferation of SMCs was a finding correlating a decrease in the expression level of c-myb to heparin-induced cell cycle arrest of SMCs at the G1 phase of the cell cycle [211, 212]. It was shown that mitogen stimulated proliferation of SMC in vitro caused increases in c-myb expression, which could be prevented using anti-sense oligonucleotides against c-myb, thus inhibiting SMC proliferation. In response to vessel injury, it has been shown that the expression of c-myb rises rapidly within a few hours in the area of the injured artery [2, 3]. c-Myb is highly expressed in all layers of the vessel wall, especially within inflammatory leukocytes at the site of injury, as well as the surrounding media and adventitia [2, 3]. After injury, cells in the adventitia also express c-Myb, although it is not clear as to the identity of these cells [3]. By day 3 post-injury, the beginning of the injury response characterized as having high rates of SMC proliferation [213], c-Myb expression is also markedly increased in VSMCs. Application of anti-sense oligonucleotides to rat carotid arteries in vivo prevented neointimal remodeling after injury [6], further demonstrating that c-myb has a role in promoting the proliferation of SMCs. This study was significant in that it was the first demonstration that oligonucleotides could be applied in vivo to prevent neointimal remodeling without affecting the survival of medial SMCs demonstrating that blocking c-myb could be a therapeutically useful strategy in preventing neointimal remodeling post-vessel injury. Other studies have confirmed that oligonucleotides were effective in other species and other models of vessel injury [2, 5, 214], and using ribozymes against c-myb [215-217]. In addition to studies
that examined the utility of inhibiting *c-myb* activity using exogenous treatments *in vivo*, You *et al.*, as described earlier, used a transgenic mouse model with VSMC-restricted DN-c-Myb

Later studies established that *c-myb* regulated the proliferation of SMCs via calcium-sensitive cell cycle regulation. It was first found that blocking *c-myb* with anti-sense oligonucleotides suppressed *c-myb* expression at the G\(_1\)-S cell cycle transition point, which coincides with an elevation of intracellular Ca\(^{2+}\) levels [195, 218-221]. This is accomplished by decreasing transcription of plasma membrane Ca\(^{2+}\) ATPase-1 (PMCA1) [219, 222, 223] and increasing the transcription of inositol 1, 4, 5-triphosphate receptor type-1 (IP3R1) [224] allowing for the elevation of intracellular Ca\(^{2+}\) required for cell cycle progression through the G\(_1\)-S transition point. A rise in intracellular Ca\(^{2+}\) is required for calmodulin-dependent activation of cyclin E/cyclin-dependent kinase (CDK2) to promote G\(_1\)-S phase progression in VSMCs [225]. Interestingly, in intestinal crypt cells, *c-myb* regulates the transcription of cyclin E [11], allowing for cell cycle progression in these cells, demonstrating further complexity of the regulation of cell cycle progression by *c-myb*.

While these studies firmly establish the importance of *c-myb* in the regulation of cell cycle progression and proliferation of SMCs, they do not establish if *c-myb* also has a role in the development of SMCs. Furthermore, it has yet to be established if *c-myb* regulates the differentiation capacity of vessel resident or circulating putative VSMC progenitor cells.

1.3.6 The role of *c-myb* in VSMC differentiation

While several studies had examined the role of *c-myb* in the regulation of VSMC proliferation, its role in the differentiation and maturation of VSMC had not been previously examined. Kolodziejska *et al.* [17] were the first to examine the role of *c-myb* in SMC differentiation and maturation utilizing *c-myb*\(^{-/-}\) ESC-derived embryoid bodies (EBs)
differentiated with retinoic acid. With loss of *c-myb*, it was found that SMCs did not efficiently form, while the differentiation of CM-like cells was enhanced. During retinoic acid-induced differentiation of EBs, it was found that the induction of SMC genes SMα-actin, SM22α and SM-MHC was significantly elevated before the appearance of SMC-like cell contractions, significantly decreasing as mature contractile SMCs appeared. However, SMC gene expression was significantly reduced in *c-myb*−/− EBs, suggesting that loss of *c-myb* results in an inability of EBs to efficiently differentiate into SMCs. Induction of myocardin, a key regulator of smooth muscle myogenesis [35, 106] began early in WT EBs, whereas in *c-myb*−/− EBs, early myocardin expression failed to rise, presumably leading to the failure to induce the expression of SMC-specific genes. Interestingly, in this EB differentiation model, cardiomyogenesis was unaffected, if not enhanced with the loss of *c-myb*, despite the failure of myocardin levels to rise. While myocardin is required for cardiomyogenesis [226], and it is known that additional factors regulate skeletal myogenesis versus SMC development with myocardin [227, 228], the role of *c-myb* in regulating myocardin and related genes in the differentiation of SMCs versus CMs is not clear.

In the same study, *c-myb*−/− ESCs labeled with a β-galactosidase (β-gal) reporter were mixed with WT ESCs to generate chimeric mice. When chimerism was examined in tissues with prominent SMC populations such as the gut and the aorta, there was paucity in the investment of β-gal+ *c-myb*−/− ESCs compared to tissues such as the heart and the brain [17]. While providing valuable *in vivo* data on the role of *c-myb* in the generation of SMCs, it could not address if *c-myb* was involved exclusively in the differentiation of SMC from progenitor cells, or it was also involved in the proliferation and expansion of SMC progenitors which could have lead to significant differences in the appearance of β-gal+ cells in the chimeric mice.
To more mechanistically examine to role of \textit{c-myb} in the differentiation of SMC from EBs, a follow up study by Ishida et al. \cite{229} examined specific populations regulated by \textit{c-myb} in response to retonic acid stimulation. It was found that there was differential expression of \textit{c-myb} in differentiating populations of ESCs marked by expression of Flk1 and Pdgfr-\(\alpha\), with the highest expression of \textit{c-myb} being in cells that were Flk1\(^+\)Pdgfr-\(\alpha^-\) or Flk1 Pdgfr-\(\alpha^+\). In \textit{c-myb}\(^-\) EBs, there was a complete absence of a Flk1\(^+\)Pdgfr-\(\alpha^-\) SMC\(^-\) progenitor cell. It was shown that the Flk1 promoter is directly regulated by c-Myb, thus loss of \textit{c-myb} resulted in the failure of Flk1 expression, resulting in the loss of the SMC-primed Flk1\(^+\)Pdgfr-\(\alpha^-\) population. This study demonstrated that \textit{c-myb} appears to regulate the appearance of specific SMC progenitor cell populations via regulation of Flk1 expression \textit{in vitro}. While this study added significantly to the mechanism by which \textit{c-myb} regulates EB differentiation \textit{in vitro}, it was not able to address some important questions, namely, the \textit{in vivo} relevance of \textit{c-myb} regulation of SMC progenitor cell differentiation. It did however raise some interesting questions, such as whether a population analogous to the SMC progenitor Flk1\(^+\)Pdgfr-\(\alpha^-\) EB sub-population could be identified in the adult animal, and if \textit{c-myb} has a role in the SMC differentiation of this adult cardiovascular progenitor cell.

While c-Myb has established roles in SMC differentiation and proliferation, as well as in the differentiation and proliferation of various hematopoietic cells, all of which are relevant to cardiovascular diseases, the role of \textit{c-myb} in pathogenesis of cardiovascular diseases is largely unknown. In the context of restenosis, while it is appreciated that \textit{c-myb} regulates VSMC proliferation, the role of \textit{c-myb} in other cell compartments, both BM-derived (hematopoietic) as well as other vessel-resident cell populations is unknown. In the context of atherosclerosis, while \textit{c-myb} has been shown to have roles throughout BM-derived inflammatory cell
proliferation and differentiation, the role of c-myb specifically in the context of atherogenesis is completely unknown. Finally, while c-myb has been shown in various mouse models to have a role in pathological VSMC proliferation, it is unknown what role c-myb has in the steady-state regulation of VSMC function and by extension, the steady-state functioning of the cardiovascular system. Thus, examination of the role of c-myb in the context of different cardiovascular disease settings will allow for greater understanding of the specific roles of c-myb in the context of both steady state and pathophysiological role of c-myb. These insights will not only better inform our understanding of the cardiovascular system, but may one day allow for specifically targeted therapies based on our understanding of the role of c-myb in the cardiovascular system.

1.4 The role of c-myb in vessel injury and restenosis

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide. Atherosclerosis is the leading vascular pathology, and features vascular inflammation and remodeling which begins as fatty streaks in the vessel wall in areas with altered non-laminar flow that are known to be athero-susceptible [230]. The risk factors for developing atherosclerosis include elevated cholesterol and lipid levels, hypertension, and diabetes. Atherosclerosis is thought to be initiated by endothelial activation or damage, resulting in the up-regulation of adhesion molecules leading to the recruitment of circulating immune cells which subsequently extravasate through the endothelium and enter the vessel wall [231, 232]. Circulating monocyte-derived macrophages that are recruited to the vessel wall begin to accumulate lipids, eventually forming lipid-laden foam cells to form a fatty streak lesion [233]. With continued inflammatory stimuli coming from the developing fatty streak, monocyte-
derived macrophages are continually recruited, as well as the local proliferation of macrophages occurring, leading to continued worsening of the fatty streak [234, 235]. As the fatty streak develops, SMCs and SMC progenitors are recruited to the lesion, begin to proliferate and secrete ECM to stabilize the developing atheroma, leading to lesional thickening. Eventually, as lipid-laden foam cells begin to die and more ECM is secreted, a necrotic core forms within the lesion, and the lesion expands into the luminal space, narrowing the artery, leading to further local hemodynamic alterations. If the atheroma continues to grow, it can completely occlude a vessel, leading to infarction, which can be fatal if occurring in the heart or brain. Altered hemodynamics and weakening of the fibrous cap of the atheroma can result in the embolization of atheroma, potentially resulting in occlusion downstream. In addition, acute rupture of the atheroma can cause a local thrombotic response, leading to the occlusion of the artery or embolization of the thrombus leading to infarction downstream of the atheroma. Even without occlusion occurring, the loss of perfusion by gradual narrowing of the vessel lumen can result in significant impairments in tissue function due to inadequate tissue perfusion.

While first-line treatment of atherosclerosis and most cardiovascular diseases involves lifestyle modification and treatment with systemically delivered therapeutic agents aimed at lowering plasma lipids and blood pressure combined with anti-platelet agents to decrease the likelihood of the occurrence of thrombotic events. When arteries become too occluded the next line of therapy involves surgical interventions to restore blood flow to obstructed tissues. Endarterectomy involves the surgical removal of the plaque, but has limited uses. Two interventions that are commonly employed are bypass grafts, where an artery or vein is used to bypass the vessel occlusion, or angioplasty, where an endovascular balloon catheter is used to re-
open a narrowed artery, often followed with the implantation of a permanent stent to keep the vessel open.

While immediate alleviation occurs with the restoration of blood flow to previously under-perfused tissues, complications can arise from angioplasty and stenting. Stents are enlarged with a balloon catheter, and the flexible metal material expands to keep open the narrowed artery. The resulting damage to the continuity of the endothelial cell layer, as well as damage to the vascular smooth muscle component and matrix components caused by the surgical procedure can result in an inflammatory response that can ultimately lead to neointimal hyperplasia and in-stent restenosis, or re-narrowing of the artery. Bare metal stents (BMS) were shown to be effective at reopening narrowed or occluded arteries but resulted in 30 – 40% of cases where restenosis occurred. Studies using polymer coated drug-eluting stents (DES) which are coated to be less reactive, as well as containing a drug which elutes into the surrounding vasculature to prevent the inflammatory response of the stent placement to be superior to BMS [236, 237]. However, many, if not all currently available DES use cytotoxic agents such as paxlitaxcel, sirolimus, everolimus and zotarolimus, which have been implicated in complications with stenting which may be mediated by poor re-endothelization of the stented area. Poor re-endothelization will result in the continued inflammatory response in the intima, resulting in neo-intimal formation through the continued pathological proliferation of VSMC, and restenosis of the stented artery. Thus development and use of laboratory models to study the molecular and cellular mechanisms underlying neointimal remodelling are essential to understanding the underlying pathology and developing therapies to prevent these conditions.

1.4.1 Wire denudation injury in mouse
Immediately after angioplasty, where the procedure has occurred there is considerable endothelial damage, and in parts, complete denudation of the endothelium, exposing the underlying internal elastic lamina. Immediately, platelets adhere to the exposed subendothelial layer, and leukocytes are then recruited to the injury site. By 2-3d post-injury, leukocytes begin to extravasate into the injured vessel wall. At this time, medial smooth muscle apoptosis is observed. Between 3-8d post-injury, the intimal layer begins to thicken. The thrombus that has formed begins to be invaded by VSMCs that have de-differentiated and begun to proliferate and migrate into the site of injury. These synthetic VSMCs also secrete and produce ECM, strengthening the lesion. Endothelial cells are also activated, and begin to re-endothelialize the wounded area from the edges of the wound. In addition, myofibroblasts are also seen to migrate from the adventitia into the neointima. From 10-14d post-injury, re-endothelialization continues to occur, VSMC proliferation slows, but ECM deposition continues. By 28d post-injury, cell proliferation has largely subsided, and the neointimal lesion begins to recede as negative remodeling begins to occur at the site of injury as the vessel wall heals and inflammation abates. Much of the early vessel injury models for neointimal remodeling were performed on larger animals due to the similarities in size and similarity of the pathophysiological events after endovascular surgery. While the use of angioplasty catheters is possible on pig, dog, rabbit and even rats, its use in mouse has seldom been reported in the literature. With the advantages of working in the mouse in mind, Lindner et al. were the first to report a mouse model that simulated the vessel injury that occurs during angioplasty [213]. This model involves the passing of a flexible guide wire through the carotid (or femoral) artery in multiple passes, with the aim of denuding the endothelium from the arterial wall. The mechanical removal of the endothelial layer results in the same pathophysiological events that lead to neointimal
remodeling in humans while preserving blood flow and vessel wall integrity, thus serving as an effective mouse model for human disease. While other models exist in the literature for creating neointimal lesions, they involve complete ligation, partial enzymatic digestion, chemical injury or other mechanisms of injury of the vessel wall that are non-analogous to the human condition. Thus, while these models might offer the same pathological outcome (ie, neointimal remodeling), the mechanisms of disease are presumably different than those seen clinically; thus, wire denudation represents an ideal model of human disease that can be applied in a mouse.

1.4.2 Strain-mediated differences in injury response

Several studies have shown that significant variation of remodelling response exists between different strains of inbred laboratory mouse. The earliest report of strain-based differences in neointimal remodeling showed that C57BL/6 mice develop less neointima following vessel injury compared to mice on a mixed C57BL/6 x 129 background [238], suggesting that the C57BL/6 strain harboured mutations that rendered the strain more resistant to neointimal remodeling. In a comparison of five inbred strains of mice, it was found that C57L/J and FVB/N strains of mice were susceptible to neointimal remodeling following vessel injury, repeatedly forming massive neointimal lesions; conversely, C57BL/6J, 129sv and C3H/He strains were shown to be resistant to neointimal remodeling following wire injury [239].

Consistent with the results of studies comparing the different responses of various strains of mice to wire denudation vessel injury, carotid ligation also produces similar results in terms of strain-based differences in neointimal remodeling. When the carotid ligation model was used to assess strain differences in 11 strains of inbred lab mouse [240], while all consistently showed a response to ligation injury, there was a wide variation in remodeling responses. Again, FVB/N and SJL/J strains have greatest degrees of luminal narrowing. However, different mechanisms
may be responsible, as FVB/N mice had significant neointimal remodeling, whereas SJL/J mice
had very little neointima formation, instead having significant negative remodeling of vessel
diameter in response to ligation injury.

These studies have established that the C57BL/6J strain appears to be least-susceptible to
wire denudation injury, whereas FVB/N mice seem to be particularly susceptible to neointimal
remodeling regardless of vessel injury model. It is interesting to note however, that the when
comparing strains on a diet-induced atherosclerosis model, C57BL/6 strain are susceptible to
diet-induced atherogenesis, whereas the FVB/N strain on the same diet-induced model of
atherosclerosis is resistant to atherosclerosis [239]. The results of the above studies clearly
demonstrate that there are genetic determinants that determine susceptibility to vascular
restenosis, and that these genetic differences may not be the same as those that determine
susceptibility to atherosclerosis. However, it is currently not known what the genetic differences
that account for these strain-based differences in disease susceptibility are. Understanding the
genetic determinants of susceptibility to vascular restenosis may hold the key in understating
why some patients suffer from increased complications post-stenting, while others face minimal
complications post-angioplasty.

1.4.3 Role of leukocytes in vascular response to injury

While the literature on cellular and humoral immunity in atherosclerosis is extensive,
reports on the effects of inflammatory cells on injury-induced vessel remodeling are far fewer.
While the role of EC, VSMCs, and other cells normally resident in the vessel wall is well
appreciated as contributing to vascular homeostasis through the maintenance of structural and
contractile functions, the role of circulating leukocytes in vessel injury appear to be highly
dependent on the model of vessel injury. Lymphocyte recruitment and infiltration has been
shown to be one of the earlier events following wire injury, with leukocytes recruited to the site of injury within one hour, but do not persist in the lesion, as they could not be detected 14d post-injury [241]. It is thought that B- and T-cells limit neointimal formation, as injury of both Recombination-activating gene-1−/− (RAG1−/−) and RAG2−/− mice, which lack B- and T-cells, causes the formation of massive neointimal lesions with increased numbers of proliferating VSMCs within lesions [241, 242]. Thus lymphocytes appear to function to limit neointimal remodeling by suppressing cytokine/chemokine-stimulated VSMC proliferation, however complete understanding of the mechanisms involved are unclear.

In addition to lymphocytes, monocytes are also recruited to the site of vessel injury [241, 243, 244], and are thought to induce intracellular signals associated with cell proliferation and migration [245, 246]. CC chemokine receptor 2 (CCR2) and its ligand monocyte chemoattractant protein-1 (MCP-1) have been shown to be required for monocyte recruitment to injured vessels [245, 246]. CCR2−/− mice fail to recruit monocytes to sites of vessel injury, and have significantly decreased VSMC proliferation and resultant decreases in neointimal remodeling. Targeted disruption of P-selectin, required for leukocyte infiltration of the vascular wall, attenuated macrophage infiltration and reduced neointimal remodeling [247, 248]. The chemokine RANTES, secreted by activated platelets at the site of vessel injury, triggers monocyte recruitment [249]. Deficiency of its receptor, CCR5, results in increased VSMC production of the anti-inflammatory interleukin-10 (IL-10), which results in suppression of monocyte recruitment and decreased neointimal remodeling [250].

Because of the reportedly opposing roles for lymphocytes and myeloid cells in mediating neointimal remodeling following vascular injury, treatments that inhibit the recruitment of all leukocytes may not be the most effective means of preventing restenosis following angioplasty.
An ideal treatment would allow for the recruitment and activity of injury-limiting lymphocytes while limiting the recruitment and activity of monocytes/macrophages which promote neointimal remodeling.

1.4.4 Bone-marrow derived progenitor cells in neointimal hyperplasia

While the role of inflammatory leukocytes has been widely examined in multiple models of vascular remodeling, the role of progenitor cells participating in neointimal hyperplasia is controversial. Early reports focused on the possibility that BM-derived SMC progenitor cells could contribute SMCs to neointimal lesions. Among the earliest reports that circulating SMC progenitor cells contributed SMCs to neointimal lesions came from a report showing that the transplantation of hearts results in recipient-derived cells contributing to coronary artery neointimal hyperplasia in atherosclerosis [251]. They suggested that these recipient-derived SMCs could have originated from circulating (possibly BM-derived) SMC progenitor cells. It was later demonstrated that HSC-derived cells can give rise to αSMA⁺ cells in mouse models of hyperlipidemia-induced atherosclerosis, transplantation/graft vasculopathy and post-angioplasty restenosis [23, 24, 27] through co-staining for HSC-derived cells and αSMA in vascular lesions, leading to the belief that HSCs or HSC-derived cells fractions harbored the ability to be recruited to vascular lesions and transdifferentiate into neointimal SMC-like cells. Interestingly, it has been suggested that the degree of BM-derived SMC progenitor cell recruitment was proportional to the amount of damage to the medial SMC layer, with increasing amounts of medial injury resulting in more circulating SMC progenitor cell recruitment [19]. In agreement with the mouse models demonstrating BM-derived SMC-like cells in vascular lesions, data from in vitro differentiation of human circulating blood cells [25, 252] and histopathological examination of
Atherosclerotic plaques from patients who had undergone BMTs suggested that BM-derived cells could integrate into atherosclerotic lesions and become SMC-like cells [253]. Smooth muscle progenitor cells isolated from whole human blood were shown to be CD34+ Flk1+ Flt1+ cells that were distinct from endothelial progenitor/outgrowth cells [25]. These cells could be differentiated in vitro into ASMA+, MYH11+, CNN1+ SMC-like cells demonstrating that there could be a circulating SMC progenitor cell. When CD34+ cells isolated from human peripheral blood were injected intravenously into immunocompromised mice that had undergone myocardial infarction, these cells could be identified post-infarction, demonstrating the transdifferentiation capacity of these cells [252]. Post-infarction, human leukocyte antigen+ (HLA) CMs, ECs and SMCs in both the infarct and peri-infarct region could be identified histologically [252], suggesting that circulating CD34+ cells isolated from human blood had the capacity to transdifferentiate into CMs, ECs and SMCs in vivo. In agreement with these results, BM-derived CD34+ progenitor cells contributed to EC, SMCs and myeloid cells in neovasculature in a tumor angiogenesis model [254]. Additionally, HSC-derived SP and non-SP cells in skeletal muscle were shown to reconstitute from BM-derived cells that reside in the skeletal muscle [255]. These SP cells were shown to reconstitute local myeloid cells as well as contribute to ECs during skeletal muscle neovascularisation. The BM-derived non-SP cells, which had a mesenchymal phenotype, did not reconstitute blood lineages, but instead contributed to SMCs in skeletal muscle neovascularisation suggesting that there are distinct populations of BM-derived EC and SMC progenitors. While it is unclear what the identity of these non-SP cells is, mesenchymal stem cells (MSCs) have also been shown to give rise to SMCs, which may be the fraction of BM-derived cells that can contribute to SMCs [256]. Thus, these studies and others have shown the in vitro and in vivo transdifferentiation capacity of BM-derived circulating
progenitor cells to contribute to SMCs in various injury models. However, the critical limitation of many of these studies has been the paucity of conclusive evidence to demonstrate that the SMCs that have been identified in these studies are in fact mature SMCs expressing not only αSMA, but also markers such as Cnn1 and Myh11 which would differentiate myofibroblastic cells from true SMCs. In studies where more rigorous SMC phenotyping has been performed as well as those that employed only single SMC markers to identify SMCs, cell fusion events between marked circulating and non-marked vessel resident cells, as well as simple overlapping (false double staining) of cells was not eliminated. Thus it remained unclear if circulating cells could truly differentiate into SMCs in vivo and contribute to SMCs involved in neointimal remodeling.

However, contrary to these results demonstrating the possibility of a circulating SMC progenitor cell that contributed SMCs to neointimal remodelling, several studies found opposing results that suggested otherwise. While studies identified that SMCs in neointimal lesions in transplant arteriosclerosis are recipient derived cells [21, 22], they were not bone marrow derived, suggesting that other circulating progenitors that are non-HSC derived may be SMC progenitors [21]. Additionally, other groups could not identify BM-derived cells as significantly contributing to ECs in transplant arteriosclerosis [20], instead identifying that vessel-resident ECs migrated to transplanted vessels. However, in order to examine if HSCs can contribute to non-hematopoietic tissues, Wagers et al. performed single-HSC transplants into lethally irradiated mice and examined chimerism in these animals that received only single GFP+ HSCs. While it was found that a single HSC could successfully reconstitute all blood leukocytes of recipient mice, no other cell types were found to show chimerism suggesting that adult HSCs have little plasticity or transdifferentiation potential [28]. Alternatively, SMC progenitor cells
could also be derived from non-BM HSCs/progenitors residing in other vascular tissues that home to the site of injury, either via the circulation, or having migrated through the vessel wall, although no evidence to support this notion currently exists.

While early studies demonstrated the possible involvement of BM-derived SMC progenitor cell in neointimal remodeling, technical limitations limited the conclusive identification of these cells. However, later studies that have gone to re-examine the role of BM-derived cells have cast significant doubt as to the transdifferentiation of BM-derived cells into true SMCs that participate in neointimal remodeling. Iwata et al. examined the recruitment and SMC differentiation of BM-derived cells using immunohistological examination of Myh11 expression, as well as mouse reporter lines driven by Myh11 or αSMA in the contexts of wire-injury, hyperlipidemia-induced atherosclerosis or transplant vasculopathy models. It was demonstrated that while BM-derived cells could express αSMA, none of these cells were Myh11+, suggesting that while they expressed αSMA, they were not mature SMCs [257]. Likewise, no Myh11+ BM-derived cells were found in lesions, further supporting the notion that αSMA expression is insufficient to identify cells as SMCs. Interestingly, it was found that αSMA+ cells were also CD115+CD11b+F4/80highLy6Chigh BM-derived cells, consistent with the phenotype of inflammatory monocyte-derived cells. Specific adoptive transfer of CD11b+Ly6C<sup>high</sup> BM cells resulted in αSMA expressing cells in the neointima of injured arteries, which was associated with greater expression of inflammation-related genes [257].

In another study, mice were transplanted with GFP<sup>+</sup> BM and injured arteries were examined using high-resolution microscopy at various timepoints post-wire injury. While BM-derived cells were detected in the lesions, they were found to be extremely rarely αSMA<sup>+</sup> (~0.9% of all αSMA<sup>+</sup> cells in the lesion were eGFP<sup>+</sup>), and none were found to co-express Myh11 or Cnn1. In
agreement with Iwata et al., it was found that the majority of eGFP$^+$ cells in the neointima were of monocyte/macrophage origin [258]. Numerous other studies have shown in the context of vessel isografting or allotransplantation in both normal and hyperlipidemic mice, the failure to conclusively identify BM-derived cells that have differentiated into SMCs [21, 259, 260]. Thus these studies provide evidence that the transdifferentiation of BM-derived cells to SMCs is actually an exceedingly rare or non-occurring event. As opposed to transdifferentiation, it would appear that previous studies that identified αSMA$^+$ BM-derived cells in neointimal lesions had actually identified transient inflammatory monocytes/macrophages and not de novo SMCs.

1.4.5 Stem cell antigen 1$^+$ adventitial progenitor cells

While the long-standing dogma in vascular biology has been that in response to injury, neointimal SMCs are derived from the phenotypic modulation of fully differentiated “contractile” VSMCs that de-differentiate and become proliferative and migratory “synthetic” VSMC that migrate from the media and provide the bulk of neointimal cells [261]. However, the discovery that VSMCs can be derived from multipotent stem and progenitor cells, and that these stem and progenitor cell-derived VSMCs can participate in the process of vascular repair and regeneration has led to the re-evaluation of the idea that the synthetic VSMC is the only source of SMCs in response to injury. The previous paradigm focused on the phenotypic plasticity of mature VSMCs regulating normal physiology and pathological remodeling states now requires some reworking due to the discovery that there are stem and progenitor-cell pools within the adult cardiovascular system, and that these cells can participate in the pathogenesis of vascular diseases.

While circulating endothelial progenitor cells (EPCs) were shown to derive ECs in injury repair processes, the participation of circulating VSMC progenitor cells became more dubious
with more refined approaches in defining the ontogeny of VSMCs that were derived from the circulation. Conversely, over the same period, several groups identified and characterized both murine and human vascular-resident progenitor cells that could give rise to various lineages of cells present in the adult vessel wall.

An alternate source of neointimal cells was identified as originating in the adventitia, but was originally believed to be an adventitial fibroblast [262-264]. However, Hu et al. [123] reported that a subset of adventitial cells that express the marker stem cell antigen-1 (Sca-1; alternatively known as Lymphocyte antigen 6A/E) could be isolated and differentiated into VSMCs. These cells were identified in the adventitia of the aortic wall, and were defined histologically as also possibly expressing the stem cell markers cKit, CD34 and Flk1, and negative for the expression of αSMA, SM22α, Cnn1, and SM-MHC. When these cells were isolated, they retained Sca1 and cKit expression in vitro, were CD45-Lin−, and upon stimulation with PDGF-BB would differentiate into VSMCs. When applied to the outside of a transplanted irradiated vein graft in an ApoE−/− mouse, it was found that these Sca1+ adventitial progenitor cells would migrate into the intima of the atherosclerotic lesions that formed within vessel transplants. BMT and staining of aortic roots of mice receiving β-gal+ BM revealed that there were few BM-derived cells in the adventitia. When Sca1+ adventitial progenitor cells were isolated from chimeric mice, they were all negative for β-gal staining, suggesting that they were not of a BM-origin. While this study identified the Sca1+ adventitial cell as a VSMC precursor, the histological approach to characterizing cell surface expression of cKit, CD34 and Flk1 could not determine if these markers were represented on the same cells, or if they represented different populations. It was also not known if the expression pattern of these putative stem/progenitor cell markers changed during the response to injury. Furthermore, it was not determined what the role of Sca1+
progenitor cells was in an intact blood vessel as opposed to a transplanted irradiated vein graft. Nonetheless, these results, taken together suggest that the adventitial Sca1+ progenitor cell is a VSMC precursor that is vessel-resident.

Following the 2004 study identifying adventitial Sca1+ vascular progenitor cells, Passman et al. [265] identified Sonic hedgehog (Shh) signaling to be critically important in the recruitment or maintenance of Sca1+ adventitial progenitor cells. While Shh signaling is not known to regulate c-myb, the closely related Wnt signaling pathway is known to regulate c-Myb activity and stability [182, 183]. Loss of Shh resulted in poor investment of Sca1+ adventitial cells around the aortic root. It was found that in developing embryos, adventitial Sca1+ cells appeared between E15.5 and E18.5 between the aorta and pulmonary trunk. Sca1+ adventitial cells were not neural crest in origin, as histological analysis of Wnt1-Cre lineage tagged cells did not label adventitial Sca1+. However, histological analysis of aortic root shows abundant staining of adventitial cells, where the authors claim no localization of Sca1 and lineage tagged cells leaving some opportunity to clarify these results.

Upon isolating Sca1+ cells by MACS and performing RT-PCR on isolated cells, they found expression of CD34, PDGFR-β, and no expression of CD45, CD68 or cKit, which was not validated by immunohistochemical staining nor flow cytometry. While CD34 expression was consistent with the histological staining by Hu et al, lack of cKit expression did not match the profile found in the earlier report. Interestingly, Sca1+ cells were found to express of mRNAs for transcription factors known to be essential for regulating VSMC differentiation such as SRF, Myocd, MRTF-A and -B. While Sca1+ cells expressed SRF, they also expressed Msx1, Klf4 and FoxO4, which act as potent inhibitors of SRF-dependent transcription [87, 266], keeping adventitial Sca1+ cells “primed” for VSMC differentiation. When isolated and cultured in vitro,
Scal\(^+\) cells began to differentiate and downregulate the expression of Klf4 and Msx1, allowing for efficient SMC differentiation [265]. When treated to VEGF or BMP2, adventitial Scal\(^+\) cells also formed ECs and osteogenic cells \textit{in vitro}, suggesting a mesenchymal stem cell-like multi-lineage differentiation capacity. At this time, it is not known if Scal\(^+\) cells have the same multipotency \textit{in vivo}, and what relevance they might have to diseases such as arteriosclerosis. While the authors posited that the maintenance of an undifferentiated SMC progenitor phenotype critically depends on the expression of transcription factors that inhibit SMC differentiation by blocking SRF-dependent transcription, it is tempting to consider that \textit{c-myb} may be involved in the activation and differentiation of these Scal\(^+\) VSMC progenitor cells.

In follow-up studies to the 2004 study by Hu et al. [123], the same group identified that much like synthetic SMCs, the differentiation capacity of Scal\(^+\) cells depended on interactions with ECM components, namely collagen IV/integrin interactions [122]. Interestingly, they showed that the anti-proliferative drug sirolimus, which is used in drug-eluting stents to limit restenosis, induces the migration and differentiation of Scal\(^+\) progenitor cells via an epidermal growth factor/ERK/β-catenin mediated signalling pathway [267]. They go on to show that sirolimus favors SMC differentiation versus EC differentiation of Scal\(^+\) progenitor cells. The results of this study raise the prospect that while drug eluting stents may limit pathological remodeling by some cell populations, it may actually promote the negative remodeling mediated by vessel-resident progenitor cells, thus highlighting the need for the development of therapeutics specifically targeted against cell populations or processes that adversely affect vessel remodeling.

\textbf{1.4.6 Other vessel-resident stem/progenitor cells}
In addition to \( \text{Sca}^+ \) adventitial progenitor cells, a separate population of pericyte-like progenitor cells has also been described as residing in the adventitia, closely associated with the adventitia-media border. These pericyte-like cells express the markers neural/glial antigen 2 (NG2), PDGFR-\(\beta\) and CD146 [124], and are \( \text{Sca}^+ \). While there are very few \( \text{NG2}^+ \) cells at baseline, post-wire injury of the femoral artery, there is a significant expansion in the \( \text{NG2}^+ \) cell population in the adventitia. When neointimal cells were examined, \( \text{NG2}^+ \, \text{SMA}^+ \) cells were found in the intima.

In a more recent paper by Tang et al. [121], they show the existence of a multipotent vascular stem cell (MVSC). In contrast to the previously mentioned studies of adventitial progenitor cells, they have shown these MVSCs to be medial-resident cells. These cells were shown to be \( \text{Sca}^- \) (consistent with lack of medial \( \text{Sca}^+ \) expression), Pdgfra\(\text{CD}^+\text{CD}31^-\text{CD}34^-\text{cKit}^-\text{Flk}^-\), demonstrating them to be markedly different than the previously mentioned vascular resident progenitor cell populations. It was shown that there are increased MVSCs in response to vessel injury, and that these cells contributed de novo SMCs to the neointima remodeling. Using lineage tracing experiments with SM-MHC, it was shown that these cells arise from a SM-MHC\(^-\) fraction, suggesting that they are not the result of de-differentiation of VSMCs, but rather derived from a separate pool of cells.

In addition to these medial MVSCs, progenitor cells isolated from the media-intimal isolations of adult mice have also been shown to generate VSMCs \textit{in vitro} [268]. These cells were isolated by flow cytometry as side population (SP) cells, identified by the lack of Hoechst 33342 staining mediated by the expression of the ATP-binding cassette transporter subfamily \text{G} member 2 (\text{Abcg}2). Abcg2 expression was found in the intima-medial border just underlying the basement membrane as well as some staining deeper within the media. Interestingly, these cells
were found to be Sca1$^+$cKit$^{low}$Lin$^{low}$CD34$^{low}$Flk-1$^{low}$. Sca1 expression is restricted to the adventitia and intima [123, 265], so it is interesting that these cells are reported to be Sca1$^+$. These SP cells could generate VSMCs and ECs in vitro, and when cultured in matrigel, formed vascular structures \textit{in vitro}, suggesting their vasculogenic potential.

While not generating VSMCs, there have been several reports that have defined other vascular resident stem- and progenitor cell populations. Naito et al. [269] reported the presence of a vessel-resident, non-hematopoietic (CD45$^-$Lin$^-$) EC SP progenitor cell residing among ECs. Psaltis et al. has demonstrated an aortic-resident hematopoietic progenitor cell that can give rise predominantly to myeloid hematopoietic lineages [270, 271]. This progenitor cell, defined by the markers Sca1$^+$CD45$^+$cKit$^+$Ly6C$^{hi}$CD115$^+$, has been shown to be capable of partial reconstitution of the hematopoietic system. How this aortic-resident progenitor cell participates in cardiovascular pathology remains to be conclusively established.

These findings support an emerging hypothesis that challenges the dogma of VSMC biology, which is that VSMCs that participate in neointimal remodeling arise exclusively from the phenotypic modulation of mature VSMCs. Rather, these results support the notion that small pools of vessel-resident progenitor cells also contribute neointimal VSMCs in response to injury. However, it is not clear why there appear to be different sources of neointimal VSMCs, and what if any differences they may have functionally versus VSMCs obtained through phenotypic modulation of mature VSMCs. Also, as is the case with most of the vessel-resident vascular progenitor cells that have been identified to date, it is not certain what the origins of this vessel-resident progenitor cell are. Certainly, determining the origins of the different vascular-resident progenitor cells and determining if they contribute the regional heterogeneity of VSMCs remains an interesting unanswered question.
1.4.7 **Human vessel-resident VSMC progenitor cells**

Complimentary to studies in mouse vessels, there have also been reports of vessel-resident SMC progenitor cells derived from human vessel walls. Bearzi et al. [272] identified vascular progenitor cells (VPCs) that were resident in the adventitial-medial border of the coronary arteries. These cells were cKit⁺KDR⁺CD45⁻CD34⁻CD31⁻, and could form ECs and SMC but not CMs. Transplantation into dogs showed that these cells could generate large coronary arteries and restore cardiac function after myocardial infarction (MI). Interestingly, these cells have a similar profile to the MVSCs isolated by Tang et al. [121], thus potentially representing the human analog of the murine vascular progenitor cell. It is interesting to note that alongside these VPCs, progenitor cells in the coronary arteries were found that were primed for CM differentiation, which were incapable of producing ECs and VSMCs suggesting multiple vascular-resident progenitor cell populations with predispositions for certain lineage differentiation. While this report seems highly promising, disappointingly, no follow-up studies have been performed to date to further characterize these human coronary vascular progenitor cell populations.

Nonetheless, the description and characterization of vascular-resident VSMC progenitor cell populations in both murine and human vasculature raise the interesting dogma-challenging possibility that not all neointimal VSMCs arise from phenotypic modulation of adult VSMCs, and that these progenitor cell pools may have different regulatory mechanisms to derive VSMCs in response to injury. Elucidating the factors that regulate vascular progenitor cells may have specific therapeutic benefit and allow for the local targeting of therapeutic interventions aimed at quelling progenitor-cell derived neointimal remodeling.
1.5 The role of c-myb in atherosclerosis

Atherosclerosis is a multifaceted, progressive inflammatory disease characterized by the build-up of atherosclerotic plaques composed of lipids, extracellular matrix, inflamed VSMCs and ECs as well as the accumulation of inflammatory leukocytes. Much is known about the roles of various leukocyte populations and their subsets, as well as the role of vessel-resident cells such as ECs and VSMCs; similarly, there is abundant literature on the specific role of c-myb in the regulation of many of these cell types at the steady state. However, little is known about the role of c-myb in the regulation of these cell populations and processes in the context of atherosclerosis. The aim of the following sections are to give brief overviews of the role of various cell populations in atherosclerosis, and the known role and how relevant c-myb may be in these cell populations during atherosclerosis.

1.5.1 Etiology and progression of atherosclerosis

While the exact triggers for atherosclerosis remain incompletely understood, several factors have been associated with the early activation of ECs in atherosclerosis prone regions of large and medium sized vessels. EC activation has been shown to be triggered by altered blood flow, modified low density lipoproteins (LDLs), plasma homocystine levels, and inflammation induced by infection. EC activation causes the enhanced expression of adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM1), which binds monocytes and T-cells, among the first leukocytes to be found in atherosclerotic lesions [232, 273]. Once bound to activated endothelium, chemokines and cytokines such as monocyte chemoattractant protein-1 (MCP-1) and IFN-γ [274, 275] recruit leukocytes to enter the intimal layer into the nascent atherosclerotic plaque. While macrophages are the predominant leukocyte within atherosclerotic plaques, T-cells, B-cells, neutrophils, mast cells and dendritic cells have all been observed within
atherosclerotic plaques and have defined roles for the initiation and progression of inflammation within the plaque [276, 277].

Vessel-wall resident cells also produce inflammatory cytokines that continue to drive the progression of atherosclerotic plaques. ECs and VSMCs not only respond to inflammatory cytokines, but also produce these pro-inflammatory mediators [278, 279], that then recruit inflammatory cells that amplify and sustain inflammation within nascent plaques. Once within the plaques, macrophages are seen to express scavenger receptors and begin to take up lipids and hence initiate the formation of foam cells, forming fatty streaks characteristic of early atherosclerotic lesions [280]. As atherosclerotic lesions grow, macrophage and VSMC-derived lipid laden foam cells begin to undergo apoptosis, contributing to the formation of a necrotic lipid core within the lesion. VSMCs are also responsible for the bulk of ECM produced in atherosclerotic lesions, allowing for the strengthening and stabilization of the atherosclerotic lesion. Thus, through the continued production and subsequent death of foam cells as well as the elaboration of ECM, the atherosclerotic lesion continues to develop.

Continued growth of the atherosclerotic lesion can eventually lead to complete occlusion of an artery, leading to infarction of downstream tissues. Plaques can rupture and form thrombi that can lead to further occlusion, or embolize and cause occlusions in distal vessels. Plaque rupture is characterized by thinning of the fibrous cap overlying a large lipid-laden atheromatous necrotic core [281], with high numbers of immune cells (such as T-cells, neutrophils and macrophages) [282-285] and calcification [282]. In contrast to plaques featuring rupture, plaques can also develop through erosion [282]. These lesions feature less inflammation with small or absent necrotic cores [286], thick fibrous caps [282, 283], and feature large numbers of VSMCs and proteoglycan rich sub-endothelial matrix [282-284]. Eroded plaques feature
endothelial cell loss, which may be mediated by changes in endothelial adherence and endothelial apoptosis [281, 282, 287]. These distinctions in ruptured versus eroded plaques suggest that both primarily inflammatory and endothelial-dysfunction mediated processes are important to lesion development.

While monocytes and macrophages have been shown to be central to the progression of atherosclerosis, heterogeneity of mononuclear phagocytes in atherosclerosis has shown that different subsets of these cells have differing contributions to atherosclerosis. Early studies characterized differences in gene expression in lesion-resident macrophages, finding some to express more pro-inflammatory or anti-inflammatory markers [288]. Hypercholesterolemic mice have particularly high levels of pro-inflammatory monocytes, as characterized by the high expression of the cell surface antigen Ly6C [289, 290]. In these mice, extramedullary hematopoiesis in the spleen yields enhanced production of these pro-inflammatory monocytes [235] which may preferentially give rise to pro-inflammatory macrophages within the atherosclerotic lesion. While in early lesions monocyte-derived macrophages are the predominant source of lesional macrophages, in late lesions, it has been shown that in situ proliferation of macrophages drives the maintenance of the local macrophage pool and continued development of the atherosclerotic plaque [234]. Thus the proliferation, mobilization and recruitment of pro-inflammatory monocytes, as well as the local proliferation of macrophages are critical to the development of atherosclerosis, and thus factors regulating these processes could serve as potential therapeutic targets for treating atherosclerotic disease.

Beside the role of mononuclear phagocytes in the etiology of atherosclerosis, other leukocyte subsets have also been shown to be critical in the progression of atherosclerotic disease. T-cells, which are also recruited to early atherosclerotic lesions have been shown to be
potent activators of the inflammatory response within the vessel wall. Adoptive transfer experiments of CD4\(^+\) T-cells have shown a pathogenic role for T-cells in atherosclerosis [291]. Th1 CD4\(^+\) cells produce IFN-\(\gamma\) within atherosclerotic lesions, driving the expression of class II major histocompatibility complex antigens (MCHII) on macrophages and VSMCs [233, 292], further stimulating immunologic responses from recruited leukocytes. Similar to monocyte functional diversity, T-cell subsets also appear to have differing influences on atherogenesis; while Th1 CD4\(^+\) T-cells have been shown to accelerate atherosclerosis, regulatory T-cells (T\(_{\text{regs}}\)) which produce IL-10 and TGF-\(\beta\), appear to limit the progression of atherosclerosis [293]. B-cells are also found in atherosclerotic lesions, yet their roles also remain incompletely understood. Splenectomy can enhance the formation of atherosclerosis [294], and immunization with putative antigens involved in atherosclerosis such as modified LDLs can protect against atherosclerosis, yet depletion of CD20\(^+\) B-cells has also been shown to limit atherosclerosis[295]. However it is not known if B-cell recruitment to lesions is critical to the pathogenesis of atherosclerosis, or if the humoral immune response to modified LDL and other antigens in lymphoid organs is sufficient to moderate atherosclerosis progression.

### 1.5.2 Regulation of myelopoiesis by c-myb

Much of the early research on c-myb focused on its role in myeloid development, as it was discovered as an oncogene causing myeloid leukemia[296]. Consistent with the oncogenic role of c-myb in myeloid leukemia, over-expression of c-Myb inhibits myeloid differentiation [297]. However, c-myb\(^{-}\) embryos fail to develop myeloid cells; however this failure of myelopoiesis may be due to defects at the HSC level rather than in myeloid differentiation [7, 29, 298]. Mice with defective or reduced c-myb expression or function, which survive to adulthood, interestingly do not have significant defects in myelopoiesis, and have normal numbers of monocytes,
macrophages and neutrophils [15, 299]. In $c-myb^{Ptl4/Ptl4}$ mice, rigorous analyses of myeloid progenitor cell number and function failed to yield any defects in their function, suggesting that myeloid cells are not overly sensitive to the level of $c-myb$ expression during myelopoiesis [300]. However, this may also be due to differences in ontogeny of myeloid cells, as well as differences in fetal vs. definitive adult hematopoiesis. $c-myb^{-/-}$ embryos do not transition effectively from fetal to adult hematopoiesis [7], and thus die in utero. However, mice with defective expression or function of $c-myb$ generally survive to adulthood, albeit with several hematopoietic defects, suggesting that a minimum amount of $c-myb$ allows for the transition to adult hematopoiesis and preserved myeloid differentiation. It is not known if $c-myb$ regulates Ly6C$^{hi}$ or Ly6C$^{low}$ monocyte subsets or macrophage polarization. Interestingly, $c-myb$ is dispensable in the myeloid differentiation of yolk-sac derived myeloid cells [301], many of which become tissue-resident macrophages in the adult animal, including aortic tissue-resident macrophages [302]. While decreased expression of $c-myb$ in myeloid cells appears to spare myeloid differentiation for the most part, over-expression of $c-myb$ in hematopoietic cells may affect the cytokine induced differentiation of macrophages and dendritic cells [303]. Additionally, it is not known what the role of stimuli such as hypercholesteremia has on the proliferation and differentiation of myeloid cells in $c-myb$ mutant mice. While myeloid differentiation may be uncompromised at the steady-state, when stimulated and emergency hematopoiesis is initiated, $c-myb$ may have a role in myeloid cell proliferation and differentiation.

1.5.3 Regulation of lymphopoiesis by $c-myb$

The first direct evidence that $c-myb$ regulates lymphopoiesis of B- and T-cells came from chimaeric studies in which $c-myb^{-/-}$ ES failed to generate mature B- and T-cell subsets [29, 298].
Subsequent analyses of several transgenic mice have demonstrated critical roles for c-myb in the
development of both lymphocyte lineages.

Early T-cell progenitors in the thymus lack the expression of CD4 and Cd8 and are termed double negative (DN). DN T-cell progenitors are subdivided into four stages: DN1, DN2, DN3 and DN4, with DN4 T-cell progenitors subsequently maturing to CD4+ CD8+ double positive (DP) T-cells, which subsequently mature to CD4+ or CD8+ single positive (SP) T-cells. c-Myb expression progressively decreases through each stage of T-cell development [202]. Mice deficient in c-Myb show decreased thymocytes at every stage [296, 304], with a particular defect in the transition from DN3 to DN4. In addition, c-Myb deficient mice have a decreased number of DP T-cells, which is associated with increased apoptosis of DP T-cells [202, 204, 305]. c-Myb also regulates the commitment of DP cells to the CD4+ and CD8+ SP stage, with c-Myb deficient mice displaying more profound defects in CD8+ SP T-cells [202, 205].

B-cell differentiation is also severely affected in c-Myb deficient mice. The earliest B-cell progenitors in the bone marrow are pre-pro-B-cells (common lymphoid progenitor-2/Fraction A) [306]. These cells differentiate into pro-B-cells (Fraction B/C) through the expression of the transcription factor Pax5, terminally differentiating them to the B-cell lineage. Successful rearrangement of the Ig heavy chain locus causes pro-B-cells to proceed to the pre-B-cell stage (Fraction D), where rearrangement of the Ig light chain locus results in the expression of the B-cell receptor (BCR). BCR expression ultimately progresses them to the immature B-cell stage where they then migrate from the BM to secondary lymphoid organs to continue development. c-Myb is highly expressed at the pro-B and pre-B-cell stages, but is downregulated in immature B-cells [206]. B-cell maturation into antibody-secreting plasma cells is completed in peripheral tissues.
The two main subsets of B cells, B1 and B2 cells are developmentally and functionally distinct. B1 cells produce the majority of circulating IgM [307], and are further subdivided into B1a and B1b B cells based on CD5 expression. CD5+ B1a cells produce natural IgM antibodies in response to non-antigenic stimuli, of which a substantial proportion recognise oxidation-specific epitopes, such as those found on oxidised low density lipoproteins, and reduce the development of atherosclerosis [308-310]. IgM antibodies, particularly those directed toward modified lipoproteins, mediate atheroprotection [311, 312], while the role of IgG is less clear [313]. CD5- B1b cells also produce IgM in an antigen-independent and antigen-dependent manner and reduce the severity of atherosclerosis [314]. Marginal zone and follicular B2 cells, which represent the majority of B cells, have been primarily implicated as proatherogenic [315-318].

Upon stimulation, B1 cells undergo immense proliferation and differentiation, become IgM-secreting plasmablasts that form extra-follicular foci within the spleen [319]. These foci are relatively long lived, and remain as a collection of plasmablasts that do not fully mature to plasma cells; involution of these immature plasmablasts is regulated by apoptosis [320]. The origin of these immature plasmablasts has been determined to be from B1b cells [319], despite the initial rarity of B1b cells in the spleen.

The regulation of plasma cell differentiation at the molecular level is primarily achieved by the coordinate down-regulation of Pax5, a transcription factor expressed throughout B cell lineages, maintaining mature B cell identity and function [321, 322]. Pax5 maintains B cell identity through the activation of genes critical for B cell function [321, 323], and represses the expression of genes necessary for plasma cell development and function [324-328]. Opposite to Pax5, Blimp1 (Prdm1) expression is required for plasma cell differentiation and the up-
regulation of genes required for plasma cell function [329-332]. Blimp1 silences B cell genes and promotes the exit from the cell cycle [333], and also represses Pax5 [334, 335], resulting in the de-repression of Pax5 target genes. c-Myb and Pax5 are known to interact and regulate the Rag2 promoter in immature B cells [336, 337], however, it is unknown is c-Myb and Pax5 also interact in regulating genes related to plasma cell differentiation. While there is no evidence that c-Myb and Blimp1 directly interact in the differentiation or function of plasma cells, several factors known to regulate Blimp1 are also known to have interactions with c-Myb. ETS1 is known to interfere with plasma cell maturation via Blimp1; c-Myb and ETS1 are known to interact in the differentiation of myeloid cells [338, 339], thus c-Myb and ETS1 may coordinately work to inhibit Blimp1-mediated plasma cell maturation. Additionally, transcriptional repression by Blimp1 also involves the recruitment of chromatin modifying histone deacetylases [340]. However, c-myb-mediated molecular mechanisms regulating plasmablast and mature plasma cell differentiation and survival remain incompletely understood.

While modulation of the responses of cells resident in atherosclerotic lesions remains a significant target for therapies targeted at halting the progression of atherosclerosis, modulating a systemic humoral immune response may also present a viable therapeutic target in atherosclerosis. Thus investigating mechanisms involved in the activation and activity of various cell types involved in the pathogenesis of atherosclerosis remains an ongoing and active area of research. c-Myb plays a critical role in the development of both vessel-resident cell types as well as leukocytes involved in the pathogenesis of atherosclerosis, yet it is unknown what the particular role of c-Myb is in these cell types in the context of atherosclerosis, hence examination of the role of c-Myb in the pathogenesis of atherosclerosis would yield considerable insights into the mechanisms of atherosclerotic disease progression.
1.6 The role of *c-myb* in blood pressure regulation

Steady-state regulation of blood pressure is complex, involving the coordinate regulation of cardiovascular and neuroendocrine systems to maintain blood pressure homeostasis. Both genetic and environmental factors are known to heavily influence the regulation of blood pressure, adding to the complexity of understanding the mechanisms of blood pressure regulation. While hypertension remains as one of the most prevalent chronic diseases worldwide [341], in many cases, the etiology of elevated blood pressure cannot be determined due to the complex nature of blood pressure homeostasis. Developing a more precise understanding of the cellular and molecular mechanisms that regulate blood pressure homeostasis will likely result in better therapies for managing blood pressure regulation.

Studying the factors that cause hypertension in a clinical setting are challenging due to complex pathophysiological mechanisms that may be masked by further compensatory pathways and confounding effects of diet, lifestyle and other factors. In order to facilitate mechanistic studies, several murine models of hypertension have been developed. Selective breeding has developed rats and mice that have elevated basal blood pressure, salt sensitivity, or both. Hypertension can also be induced through diet and surgical intervention aimed at modifying salt sensitivity. Pharmacological or genetic manipulation of key neurohormonal regulators of blood pressure homeostasis have also yielded useful models to study blood pressure homeostasis. Although these models fail to individually encompass all the factors that combine to produce a clinically-relevant pathological model of hypertension, they nonetheless provide essential insights into individual mechanisms that work in concert to regulate blood pressure homeostasis.
In the characterization of transgenic mouse models manipulating $c$-$myb$, no study has gone on to do cardiovascular physiological characterizations of these mice, nor has the role of $c$-$myb$ in homeostasis of cardiovascular function been examined in any other model. Given the role of $c$-$myb$ in VSMC biology, $c$-$myb$ may have a role in regulating vascular function, and hence have a role in regulating the homeostatic function of the cardiovascular system. Because $c$-$myb$ has pleiotropic roles in hematopoiesis, the recent appreciation that the immune system has a regulatory role in blood pressure homeostasis is also of relevance.

1.6.1 Cardiac and vascular regulation of blood pressure homeostasis

The basic components that determine blood pressure are blood flow and resistance of the arteries. Blood flow depends on cardiac output and blood volume; resistance is primarily determined by the contractile state of arteries throughout the body. While $c$-$myb$ is known to regulate the differentiation of VSMCs from ESCs, it is not known if $c$-$myb$ is critical for the vasomotor function of VSMCs in the adult animal. Presumably defects in $c$-$myb$ activity will result in the improper differentiation of VSMCs which will result in defective vasomotor function in mice harboring defects in $c$-$myb$. $c$-$myb$ is known to repress the expression of PMCA4 [223], which when knocked out causes increased blood pressure due to aberrant calcium handling in VSMCs. Over-expression of PMCA4 increases myogenic tone with a concomitant increase in blood pressure [342], hence, c-Myb may regulate vasomotor function through effects on calcium handling. Vascular fibrosis is often seen with peripheral artery disease (PAD), increasing vascular resistance and contributing to increased blood pressure [343], and is also consistently observed in animal models of hypertension [344-348]. Hypertension induces deposition of collagen in the adventitia, which has generally been attributed to fibroblasts residing in the adventitia. However, other cell types such as VSMCs, BM-derived fibrocytes,
endothelial cells and vessel-resident Sca1^{+} progenitor cells can also produce ECM and contribute to vascular stiffening. In response to hypertensive stimuli, vessel-resident Sca1^{+} progenitor cells, BM-derived fibrocytes and CD31^{+} endothelial cells all elaborated adventitial extracellular collagen matrix [349]. Coronary adventitial fibrosis that occurs in the mdx model of Duchenne muscular dystrophy was also found to be attributable to ECM synthesis by Sca1^{+}CD45^{−}CD31^{−} PDGFRα^{+} adventitial progenitor cells, again demonstrating that adventitial progenitor cells are significantly involved in adventitial fibrosis[350]. c-myb is also known to regulate components of ECM such as elastin and collagen [136-138, 200], thus it is also possible that c-myb may regulate vascular fibrosis.

While no overt cardiac defects were reported in c-myb^{−/−} mouse embryos [7], when c-myb^{−/−} EBs were differentiated, they preferentially formed cells with CM-like contractions, suggesting a propensity for cardiac differentiation [17]. When chimeric mice were generated from c-myb^{−/−} ESCs and Wt ESCs, c-myb^{−/−} cells were seen to incorporate highly into the heart [17]. However, it is not known if any functional differences exist in these mice. Interestingly, constitutive over-expression of c-myb was reported to result in dilated cardiac hypertrophy [135], however it is not known if it was due to intrinsic defects in CMs, or if it was due to other systemic defects. Nonetheless, c-myb may also have a role in mediating cardiac function, although the mechanisms are unclear.

1.6.2 Renal regulation of blood pressure homeostasis

The capacity of the kidney to excrete sodium in response to elevations in blood pressure provides a crucial mechanism for regulating fluid volume in the body and maintaining systemic pressure [351]. Defects in sodium excretion create a permissive environment for sustained high blood pressure, hence targeting sodium-chloride transporters are a particularly effective therapeutic
target in the treatment of hypertension [352]. Evidence that the kidney is primarily responsible for hypertensive phenotypes comes in part from studies using the cross-transplantation of kidneys from spontaneously hypertensive rats into normotensive rats. Transplantation of kidneys from hypertensive rats into normotensive rats caused hypertension; conversely, transplantation of kidneys from normotensive rats into hypertensive rats alleviated their hypertension [353].

Further studies with mice lacking the major angiotensin II receptor AT\textsubscript{1} showed that expression of the AT\textsubscript{1} receptor was crucial in the development of hypertension. Wt mice transplanted with kidneys from AT\textsubscript{1} receptor knockout donor mice did not develop hypertension despite normal AT\textsubscript{1} expression in all other tissues [354, 355]. Specific loss of AT\textsubscript{1} in kidney proximal tubule epithelium was sufficient for attenuating hypertension [356]; over-expression of AT\textsubscript{1} receptors in proximal tubule epithelium resulted in elevated blood pressure [357]. These studies highlight the central importance of preservation of normal kidney function to maintain blood pressure homeostasis. Currently, no studies are known to have examined a role for \textit{c-myb} in regulating kidney function or sodium excretion. While therapeutic interventions targeting kidney function have been particularly effective in the management of hypertension, development of future therapeutics will depend on further refinements into the understanding of the regulators of renal function.

1.6.3 Skin regulation of interstitial sodium

While sodium regulation was thought to be primarily mediated by intravascular and interstitial sodium equilibrium, the skin has also been shown to significantly regulate sodium equilibrium. During increased sodium intake, the skin interstitium accumulated hypertonic concentrations of sodium through binding of proteoglycans [358]. Interestingly, skin macrophages are sensitive to the hypertonic sodium concentrations, triggering the expression of
tonicity-responsive enhance binding protein (TonEBP), a transcription factor that regulates the expression of genes involved in osmotic stress [358]. TonEBP in turn regulates macrophage secretion of VEGF-C, a potent initiator of lymphangiogenesis, resulting in lymphatic vessel hyperplasia in response to increased dietary sodium [358]. Depletion of circulating monocytes with clodronate, or blockade of VEGF-C resulted in enhanced hypertension in response to increased dietary sodium indicating that skin macrophage-mediated increases in lymphangiogenesis play a key regulatory role in maintaining whole-body sodium levels. Because c-myb has established roles in myelopoiesis [301, 359, 360], it is entirely possible that c-myb also has a role in the regulation of skin interstitial sodium homeostasis, and thus regulation of blood pressure homeostasis. While many tissue-resident macrophages have been shown to be c-myb-independent in their development [301], the ability of clodronate to abrogate sodium-dependent increases in lymphangiogenesis suggest that monocyte recruitment and subsequent differentiation into macrophages regulates skin interstitial sodium homeostasis.

1.6.4 Immune regulation of blood pressure homeostasis

While it has long been known that leukocyte accumulation occurs in multiple relevant sites (heart, vessels, brain, kidney, and others) during hypertension, a causal role in regulating blood pressure homeostasis is just beginning to be understood. Among of the earliest studies to show that lymphocytes had a direct causal role in the development of hypertension demonstrated that reconstitution of T-cells into RAG1−/− mice (which lack B and T-cells) restored their previously blunted response to hypertensive stimuli [361]. Reconstitution of T-cells, but not B-cells, was able to rescue this effect, suggesting that T-cells have a key role in the development of hypertension. Reconstitution with T-cells from mice lacking AT1 receptor was shown to blunt the redevelopment of hypertension. However, while RAG1−/− mice lack T-cells, their baseline
blood pressure is not different from wild type mice, suggesting that merely lacking T-cells is not sufficient to decrease blood pressure. Recent evidence has shown that particular T-cell populations are capable of regulating blood pressure by regulating vascular contractility. In particular, specific reconstitution of CD4+, CD25+, FoXP3\textsuperscript{high} regulatory T-cells (T\textsubscript{regs}) have been shown to inhibit the response to hypertensive stimuli [362, 363].

While by no means comprehensive, these examples of lymphoid and myeloid cells regulating blood pressure homeostasis point to a possible regulatory role \textit{c-myb} may play in these hematopoietic cell populations both at baseline and in response to pathogenic stimuli during the development of hypertension. However, relatively little is known about the role of \textit{c-myb} in pathogenic hematopoiesis, and nothing is known about the role of \textit{c-myb} in the context of hypertension and blood pressure regulation. Thus investigation of the role of \textit{c-myb} in the immunological regulation of blood pressure homeostasis may represent an area of significant opportunity.
1.7 Rationale and specific objectives

The involvement of VSMCs in cardiovascular pathologies such as atherosclerosis, in-stent stenosis as well as their involvement in vascular dysfunction in hypertension and diabetes underscores the importance of examining the factors that regulate the differentiation, proliferation and function of VSMCs. To this end, further exploring the involvement of $c-myb$, which has been shown to regulate proliferation of VSMCs \textit{in vivo} and differentiation of EBs into VSMCs \textit{in vitro}, in various models of cardiovascular disease will expand on the role of $c-myb$ in regulating the physiology and pathology of VSMCs. Additionally, while VSMC have been shown to have important roles in the pathogenesis of atherosclerosis as well as maintenance of cardiovascular homeostasis, examination of the role of $c-myb$ in these processes in all cell types $c-myb$ is involved in has not been undertaken. As such, identification of a role for $c-myb$ in any process involved in normal and pathological cardiovascular physiology would represent a novel finding contributing to further mechanistic insights of normal and pathophysiological cardiovascular processes.

As such, the aims of this thesis are to:

1. Distinguish the role of $c-myb$ in BM- and tissue-resident cells in response to vessel injury and define a role for $c-myb$ in the regulation of an adult vessel-resident VSMC progenitor cell.
2. Examine the role of $c-myb$ in atherosclerosis.
3. Examine the role of $c-myb$ in baseline cardiovascular function.

These aims together will address the role of $c-myb$ in various models of cardiovascular disease and will aim to extend the overall knowledge and understanding of cardiovascular physiology and pathology.
CHAPTER 2:

2  c-Myb regulates proliferation and differentiation of adventitial Sca1+ vascular smooth muscle cell progenitors by transactivation of myocardin.

The proceeding chapter has been published in full in the journal Atherosclerosis, Thrombosis and Vascular Biology:

**Full Title:** c-Myb regulates proliferation and differentiation of adventitial Sca1^+ vascular smooth muscle cell progenitors by transactivation of myocardin.

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**Running title:** c-Myb regulates a vascular SMC progenitor

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2.1 Abstract

Objective: Vascular smooth muscle cells (VSMCs) are believed to de-differentiate and proliferate in response to vessel injury. Recently, adventitial progenitor cells were implicated as a source of VSMCs involved in vessel remodeling. c-Myb is a transcription factor known to regulate VSMC proliferation in vivo, and differentiation of VSMCs from mouse embryonic stem cell-derived progenitors in vitro. However, the role of c-Myb in regulating specific adult vascular progenitor cell populations was not known. Our objective was to examine the role of c-Myb in the proliferation and differentiation of Sca1+ adventitial VSMC progenitor cells.

Approach and Results: Using mice with wild-type or hypomorphic c-myb (c-myb<sup>h/h</sup>), BrdU uptake and flow cytometry revealed defective proliferation of Sca1+ adventitial VSMC progenitor cells at 8-, 14- and 28-d post-carotid artery denudation injury in c-myb<sup>h/h</sup> arteries. c-myb<sup>h/h</sup> cKit<sup>+</sup>CD34<sup>-</sup>Flk1<sup>-</sup>Sca1<sup>+</sup>CD45<sup>-</sup>Lin<sup>-</sup> cells failed to proliferate, suggesting c-myb regulates the activation of specific Sca1+ progenitor cells in vivo and in vitro. Although expression levels of TGF-β1 did not vary between wild-type and c-myb<sup>h/h</sup> carotid arteries, in vitro differentiation of c-myb<sup>h/h</sup> Sca1<sup>+</sup> cells manifested defective TGF-β1-induced VSMC differentiation. This is mediated by reduced transcriptional activation of myocardin, as chromatin immunoprecipitation revealed c-Myb binding to the myocardin promoter only during differentiation of Sca1<sup>+</sup> cells, myocardin promoter mutagenesis identified two specific c-Myb-responsive binding sites, and adenovirus-mediated expression of myocardin rescued the phenotype of c-myb<sup>h/h</sup> progenitors.

Conclusions: These data support a role for c-Myb in the regulation of VSMC progenitor cells and provide novel insight into how c-myb regulates VSMC differentiation through myocardin.
Non-standard abbreviations and acronyms:

BM  Bone marrow
BMT  Bone marrow transplant
CCA  Common carotid artery
EB  Embryoid body
EC  Endothelial cell
ESC  Embryonic stem cell
ECM  Extracellular matrix
eGFP  Enhanced green fluorescent protein
Lin  Lineage
MBS  c-Myb-binding site
VSMC  Vascular smooth muscle cell
2.2 Graphic abstract
2.4 Introduction

The vessel wall is known to host tissue-resident stem and progenitor cells capable of forming the various cells that constitute its layers. While the adventitia was long thought to be inert, recent studies have shown the adventitia to be a dynamic tissue playing an active role in the regulation of the vasculature. Various progenitor cell populations in the adventitia can give rise to not only vascular smooth muscle cells (VSMCs)[122, 123, 265, 267], but also endothelial cells (ECs)[122, 269], pericytes[124] and small populations of hematopoietic/myelopoietic cells[270, 271]. Indeed, it has been suggested that the adventitia may be a contributing source of long-lasting neointimal VSMCs following injury[121]. Several potential stem/progenitor cell populations have been identified as residing in the tunica media and/or adventitia[121-124, 265, 267, 271, 364], including stem cell antigen-1+ (Sca1+) CD45+ adventitial macrophage progenitor cells[270, 271] as well as Sca1+CD45− progenitor cells that give rise to endothelial and mesenchymal cell lineages[124, 269]. However, few studies[122, 267, 365] have identified factors that regulate the proliferation and differentiation of these vessel-resident adventitial VSMC progenitor cells.

Hu et al. first identified a Sca1+ cell residing in the adventitia as a VSMC progenitor cell[123]. When transferred to the adventitial side of irradiated vein grafts, up to 30% of neointimal cells in the graft were shown to be derived from transplanted adventitial cells[123]. When isolated and expanded in vitro, Sca1+ cells were found to be largely cKit+ and negative for hematopoietic lineage markers (CD45 Lin−), suggesting that these cells were not derived from the bone marrow (BM), but were vessel-resident vascular progenitor cells. Adventitial Sca1+ progenitor cells differentiated into SMCs expressing the mature VSMC markers α-smooth muscle actin (ACTA2), calponin (CNN1) and smooth muscle myosin heavy chain
Passman *et al.* later identified Sonic hedgehog (Shh) signalling as critically important to the development of adventitial Sca1+ progenitor cells[265]. *Shh*−/− mice had greatly reduced numbers of adventitial Sca1+ progenitor cells, suggesting that Shh signaling was critical to the development or homing of adventitial Sca1+ progenitor cells to the adventitia[265]. The differentiation of adventitial Sca1+ VSMC progenitors has been shown to be regulated by integrin/collagen-IV interactions[122], and through extracellular signal-regulated kinase/β-catenin signaling[267] following sirolimus exposure *in vitro*. Matrix metalloproteinase-8[365] and Stromal-cell derived factor-1[122] also appear to regulate adventitial Sca1+ VSMC progenitor cell recruitment during atherosclerosis or neointimal formation in vein grafts. However, in none of these studies were subsets of Sca1+ cells identified as being relevant to vessel remodeling *in vivo*, nor were their endogenous spatiotemporal patterns of abundance examined.

The hematopoietic transcription factor *c-myb* is known as a stem/progenitor cell regulator in multiple tissue compartments[10-15], and has also been shown to regulate the proliferation of adult VSMCs. We have shown that *c-myb* regulates the differentiation of VSMCs from embryonic stem cell (ESC)-derived embryoid bodies (EBs), specifically by promoting the expansion of a Flk1+PDGFRα+ progenitor cell population *in vitro*[17, 229]. However, it remains unknown if such a VSMC progenitor cell exists *in vivo*, and if it persists into adulthood where it might contribute to vascular pathophysiology. In addition, while *c-myb* was shown to regulate the number of Flk1+PDGFα+ progenitor cells, the mechanism by which it regulates VSMC differentiation from progenitor cells remains unknown.
An in vivo model of c-Myb deficiency was reported by Sandberg et al. [15], where an N-ethyl-N-nitrosourea mutagenesis screen identified a mouse line with a non-lethal point mutation in c-myb. This point mutation mapped to the transactivation domain of c-Myb, resulting in defective recruitment of transcriptional coactivators (such as p300), leading to decreased activity of c-Myb. This hypomorphic (c-myb<sup>h/h</sup>) mouse has several hematopoietic abnormalities caused by dysregulation of hematopoietic stem/progenitor cell maintenance, as well as stage- and lineage-specific blocks on differentiation. Several studies have shown that inhibiting c-myb activity reduces vessel remodelling following injury [1-6], however these studies have not been able to address if c-myb also has effects on vessel-resident VSMC progenitor cells. Given the role of c-myb in other stem and progenitor cell compartments [10-15], as well as evidence for the role of c-myb in the differentiation of VSMC from EBs [17, 229], we sought to more specifically test the role of c-myb in the proliferation and differentiation of adventitial Sca1<sup>+</sup> VSMC progenitor cells. Further defining the role of vessel-resident VSMC progenitor cells, as well as identifying specific factors involved in the proliferation, differentiation and activity of cells recruited during the injury response, may represent a novel approach to modulating vessel injury responses as diverse as neointima formation, atherosclerosis, hypertrophy and aneurysm formation.

2.5 Materials and Methods

Animals used

c-myb<sup>h/h</sup> animals on a C57BL/6 genetic background were derived as previously described [15]. To test the strain independence of injury phenotype, C57BL/6 mice were backcrossed 10 generations onto a FVB/N background (FVB/NCrl; Charles River #207). C57BL/6 c-myb<sup>h/h</sup>
mice were crossed with eGFP\textsuperscript{+} mice (C57BL/6-Tg(UBC-GFP)30Scha/J; Jackson Laboratories #004353) to generate eGFP\textsuperscript{+} WT and c-myb\textsuperscript{h/h} mice. For bone marrow transplantation, 5 wk old recipient mice were lethally irradiated with a single 10Gy dose of radiation (Gammacell 40 Exactor, Best Theratronics). Bone marrow was harvested from the femur and tibias of donors; 2.0 x10\textsuperscript{6} cells were injected intravenously via tail vein injection per recipient. Mice were allowed to recover for 8 weeks. All mice used in the study were male. Mice were housed in a SPF facility with access to water and standard chow \textit{ad libitum}. All protocols were approved by the Animal Resource Centre at the University Health Network (AUP#1032, 1034, 1605).

\textbf{Carotid wire denudation injury}

Carotid wire injury was performed as previously described[1, 366]. Mice were anesthetized, then their left common carotid artery (CCA) was exposed. A temporary ligature on the proximal end of the left CCA and on the internal carotid artery was used to stop the flow of blood. A permanent ligation was made on the external carotid artery 2-3 mm distal to the bifurcation of the left CCA. A small incision was then made in the external carotid artery and a wire inserted to denude the endothelium in three passes. After removing the wire, a second permanent ligation was made proximal to the incision towards the bifurcation of the CCA. Both temporary ligatures were then removed to restore blood flow. The right CCA served as the uninjured control artery. Mice were allowed to recover for 8-, 14- or 28-days following injury, at which time animals were sacrificed and left and right CCA collected for histology, flow cytometry or RNA extraction. To assess proliferation, mice were injected with 125 mg/kg bromodeoxyuridine (BrdU) 7.5-, 13.5- and 27.5-d post-injury.
**Histology**

For Masson’s trichrome staining, ~5mm of the CCA and ~1.5mm of both external and internal carotid arteries were carefully dissected and fixed overnight in 4% paraformaldehyde (PFA) before being processed for embedding in paraffin. Serial 5 μm transverse sections were stained with Masson’s trichrome. Brightfield images were taken on a Leica DMLB microscope, captured with an Infinity2-2 CCD camera (Lumenera). Morphometry was performed for sections that were determined to be 100, 200 and 300 μm proximal to the bifurcation of the CCA. Lumen area (a), area within medial elastic lamina (b), and area within outer elastic lamina (c) were measured with ImageJ software (NIH). Intima area was calculated as b – a; media was calculated as c – b; intima:media (I/M) ratio calculated as (b-a)/(c-b).

For immunofluorescence, tissues were fixed in 4% PFA, rinsed with PBS, then cryoprotected with 20% sucrose in PBS and frozen in OCT. 5 μm transverse cryosections were dried at room temperature for 10 minutes, fixed for 15 minutes in 2% PFA, rinsed 3 times in 1x PBS, then blocked for 1 hour in 2% normal donkey serum in PBS. Sections were then incubated with rat anti-Sca1 (1:100; BD Biosciences 553333) and rabbit anti-Calponin 1 (1:200; Abcam ab46794) overnight. For staining controls, isotype control antibodies for anti-Sca1 (rat IgG2Aκ, 1:100; Abcam Ab18450) and anti-Calponin 1 (rabbit IgG, 1:200; Abcam ab172730) were used.

Sections were then rinsed 3 times in 1x PBS, then incubated donkey anti-rat Cy3 (1:400; Jackson ImmunoResearch 712-165-150) and donkey anti-rabbit Cy5 (1:400; Jackson ImmunoResearch 712-175-152) secondary antibodies for 2 hours at room temperature, protected from light. Nuclei were counterstained with 10 μg/ml Hoechst 33258 in ddH₂O for 10 minutes before slides were mounted with 50:50 glycerol:PBS and stored at -20°C until imaging. Images were captured.
on an Olympus VS120 fluorescence slide scanner. Adventitial Sca1+ nuclei were counted in ImageJ.

**Flow cytometry**

To prepare tissues for flow cytometry, left and right CCA of mice were carefully dissected, and enzymatically dispersed as previously described[234]. Vessels were cut into small pieces and subject to enzymatic dispersion in 450 U/ml collagenase I (Sigma-Aldrich C0130), 125 U/ml collagenase XI (Sigma-Aldrich C7657), 60 U/ml DNase I (Sigma-Aldrich DN25), 60 U/ml hyaluronidase (Sigma-Aldrich H3506) in 20 mM HEPES buffer (Mediatech 25-060-C1) for in a heated shaker at 37°C and 900 rpm for 30 minutes. Aortic arches were partially digested (5 minutes at 37°C and 900 rpm) and adventitia was microdissected away from the medial and intimal layers, then minced and completely digested for another 25 minutes to specifically examine adventitial cell populations. After digestion, vessel samples were washed in 1x PBS and passed through a 40 μm filter. Blood samples were collected in heparinised capillary tubes, lysed with 1x RBC lysis buffer (BioLegend 42301), then washed in 1x PBS. Single cell preparations were stained for 45 minutes before being fixed with Cytofix buffer (BD Biosciences). Antibodies used were as follows: anti-Sca1-PECy7 (eBiosciences 25-5981-82), anti-CD34-A700 (eBiosciences 56-0341-82), anti-Flk1-APC (eBiosciences 17-5821-80), anti-cKit-FITC (eBiosciences 11-1171-82) or anti-cKit-APC (BD Biosciences 553356), anti-CD45-eF450 (eBiosciences 48-0454-82), anti-CD3-PE (BD Biosciences 555275), anti-CD31-PE (BD Biosciences 553373), anti-CD11b-PE (BD Biosciences 55331), anti-Ter119-PE (BD Biosciences 553673), anti-NK1.1-PE (BD Biosciences 553165), anti-B220-PE (BD Biosciences 553090). Cell proliferation was assessed using the FITC BrdU flow kit (BD Biosciences 559619)
according to manufacturer protocol. Apoptosis was determined using annexin V staining (BD Biosciences 563973) according to manufacturer protocol. For CNN1 and ACTA2 staining, cells were fixed and permeabilized using Cytofix/Cytoperm buffer (BD Biosciences 554714) for 30 minutes on ice, and were stained with anti-ACTA2 (1:100) and anti-CNN1 (1:100) for 45 minutes on ice protected from light. Samples were washed 3x with Perm/Wash buffer, then stained with secondary antibodies for 45 minutes on ice, protected from light. Samples were run on a LSR II (BD Biosciences), and then subsequently analyzed by FlowJo software (v.10; Flowjo). Gating was determined through the use of fluorescence minus one (FMO) or isotype controls where appropriate.

**Adventitial cell culture and differentiation**

Mice were anesthetized and flushed with 10 ml ice cold 1x PBS via the left ventricle. Left and right CCA were carefully dissected, and a modified adventitial cell isolation protocol[123] was used. Arteries were digested for 5 minutes at 37 °C in 0.2 U/μl collagenase III (Sigma Aldrich C0255), 0.1 mg/ml elastase IV (Sigma Aldrich E0258), 0.5 mg/ml soy trypsin inhibitor (Sigma Aldrich T6522), 25 mM HEPES pH 7.4, 1mg/ml BSA, 100 U/ml penicillin, 100 μg/ml Streptomycin, 2.5 μg/ml amphotericin B (Sigma Aldrich A2942), 50 μg/ml gentamicin (Sigma Aldrich G1397) in 1x high glucose DMEM (Invitrogen 11995073). Adventitia was then microdissected away from the CCA, cut into small pieces, then completely digested for 25 minutes at 37 °C before being cultured in 1x high glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml Streptomycin, 2.5 μg/ml amphotericin B, 50 μg/ml gentamicin, 100 μM β-mercaptoethanol, and 10 ng/ml leukemia inhibitory factor. Cells and adventitial fragments were allowed to grow to ~80-90% confluence, before magnetic activated cell sorting.
Cells were washed 3x in 1x PBS, then trypsinized, centrifuged and resuspended in FACS buffer (1x PBS, 0.5% BSA). Cells were labelled with anti-Sca1-FITC (BD Biosciences 562058) for 15 minutes on ice, washed in 1x PBS, then resuspended in FACS buffer. Sca1+ cells were then incubated with anti-FITC microbeads (Miltenyi biotec 130-042-701) for 30 minutes on ice, washed in 1x PBS and resuspended in FACS buffer. Sca1+ cells were isolated by positive MACS selection using LS columns (Miltenyi biotec 130-042-401), and were passed through two columns to improve purification. Positively selected cells were then rinsed in 1x PBS and cultured in cell culture media.

For cell differentiation, media was changed to 0.2% FBS in 1x high glucose DMEM supplemented with 10 ng/ml TGFβ-1 (R&D Systems 7666-MB-005) to induce VSMC differentiation.

**RNA isolation and qRT-PCR analysis**

RNA was extracted from cells and CCA using a PicoPure RNA isolation kit (Life Technologies) according to manufacturer protocol. RNA concentration was obtained using a Nanodrop ND100 spectrophotometer. cDNA was synthesized using qScript cDNA SuperMix (Quanta BioSciences) according to manufacturer protocol. Transcripts were then detected using specific primers (see Supplemental Table SI) using SYBR Green Master (Roche) detected on a LightCycler 480 (Roche). B2M was used as the housekeeping gene; values were compared using the $2^{\Delta\Delta Ct}$ method. Samples were run in triplicate and averaged.

**Adenoviral overexpression of myocardin**
Adenoviral constructs to over-express mouse myocardin (Adeno-Myocd; ADV-265349) or a GFP control vector (Adeno-GFP; ADV-1060) were purchased from Vector Biolabs. Isolated WT and c-myb<sup>h/h</sup> adventitial Sca<sup>1</sup> VSMC progenitor cells were infected overnight, and then differentiated in VSMC differentiation media for 7 d prior to RNA isolation and subsequent qRT-PCR analysis.

**HEK cell transfection and luciferase**

c-Myb-dependent activation of a myocardin promoter was determined by co-transfection of a myocardin promoter-<i>luc</i> construct (0.5μg/well), pRL-TK (renilla luciferase 0.025μg/well, Promega) and a mouse p75 c-Myb over-expression vector[229, 367] or empty vector control (0.5μg/well) in HEK293 cells using jetPrime (Polyplus-Transfection) according to manufacturer protocol. Luciferase luminescence was read on a Pherastar FS (BMG Labtech). Data were normalized to renilla luciferase expression and expressed as relative luciferase units (RLU).

**Constructs**

**Hypomorphic (hypo) c-Myb:** To create a hypomorphic mouse c-Myb over-expression vector, a mouse p75 c-Myb over-expression vector was mutated in the transactivation domain of c-Myb (Hypo: TGTTGACATCAGGAGC to TGTTGACAcCAGGAGC) using site directed mutagenesis with primers shown in **Supplemental Table II** and Q5 Site-Directed Mutagenesis kit (New England Biolabs) according to manufacturer protocol. The resultant hypomorphic cMyb vector contains the same mutation as the <i>c-myb</i><sup>h/h</sup> mouse[15].
mR3-3 transactivation mutant c-Myb: An additional mutant c-Myb, mR3-3, (a gift from Dr. Lipsick[368]) known to have defective transactivation of c-Myb target genes was used to confirm that c-Myb transactivates a myocardin-luc reporter.

Site-directed mutagenesis: To delete c-Myb binding sites (MBS) identified within relevant regions of the core myocardin promoter[369], MBS1 (ΔMBS1: TAACTCCC to TccgTCCC), MBS2 (ΔMBS2: GAACAGCC to GccgAGCC), MBS3 (ΔMBS3: CAGCGGTC to CccgGGTC), and MBS8 (ΔMBS8: CAACCTTTT to CccgTTTT) were mutated using primers shown in Supplemental Table II and Q5 Site-Directed Mutagenesis kit (ΔMBS 1 and ΔMBS8) or QuickChange II Site-Directed Mutagenesis kit (Agilent) (ΔMBS2 and ΔMBS3). After mutagenesis, each clone was confirmed by DNA sequencing.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed on Sca1⁺ adventitial progenitor cells using the ChIP-IT express enzymatic kit (ActivMotif 53009) according to manufacturer protocol. Chromatin was collected from Sca1+ cells either pre or 1-d post TGFβ-1 induction of VSMC differentiation. Antibodies against c-Myb (Santa Cruz sc-516), RNA polymerase II (Santa Cruz sc-900) and whole rabbit serum were used for immunoprecipitation. Isolated gDNA fragments were then analyzed by qRT-PCR as described above. Primers used are listed in Supplemental Table I.

2.6 Results

c-myb<sup>h/h</sup> mice have defective vessel remodelling following wire denudation injury.
To examine the effect of c-myb hypomorphism on injury-induced vessel remodelling, groups of 10-12 wk old male mice homozygous for the hypomorphic c-myb allele (c-myb<sup>h/h</sup>) and their wild-type (WT) littermate controls on a C57BL/6 background were subjected to wire denudation injury of the left common carotid artery (CCA), and allowed to recover for 8-, 14- or 28-d. The right CCA served as the uninjured control artery. No differences in medial or intimal area were found between WT and c-myb<sup>h/h</sup> mice at baseline (Fig. 1A). Mice were examined 8-d post-injury, a time point previously characterized as that of maximal medial proliferation[213]. No differences were found in intimal or medial remodelling between injured WT and c-myb<sup>h/h</sup> mice, suggesting that medial proliferation may not be affected in c-myb<sup>h/h</sup> mice (Fig. 1B-D).

Given that neointimal remodelling is known to be greater at later time points, mice were also examined at 14- and 28-d post-injury. At these later time points, c-myb<sup>h/h</sup> mice had decreased neointima formation at 14-d (16.7±0.3 vs. 31.4±5.0x10<sup>3</sup> μm<sup>3</sup>; N=6-7/group; P<0.05), and continued to have decreased neointima area at 28-d post-injury compared to WT (17.3±3.8 vs. 39.6±8.5x10<sup>3</sup> μm<sup>3</sup>; N=5-8/group; P<0.0001) (Fig. 1E-J). c-myb<sup>h/h</sup> mice on a FVB/N background also manifested a similar phenotype, demonstrating that the phenotype is strain-independent (Supplemental Fig. I). Medial remodelling in c-myb<sup>h/h</sup> mice remained no different than that of WT mice (Supplemental Fig. II), indicating a preferential defect in cells involved in neointimal, but not medial, remodelling.

**c-Myb regulates vessel-intrinsic remodelling responses.**

As c-Myb has pleiotropic roles in circulating BM-derived leukocytes as well as vessel-resident cells involved in the vessel injury response, reciprocal bone marrow transplantation (BMT) experiments were performed. To determine if the diminished injury response of c-myb<sup>h/h</sup>
mice was vessel-intrinsic or mediated by circulating BM-derived factors or cells, BM from either WT or c-\textit{myb}\textsuperscript{h/h} donors were transplanted into \textit{c-myb}\textsuperscript{h/h} recipient mice. Importantly, BM from WT donors failed to restore the injury response of \textit{c-myb}\textsuperscript{h/h} recipients, suggesting that the decreased neointimal response was vessel-intrinsic (\textbf{Fig. 2}). In agreement with these data, there are no differences in leukocyte recruitment between WT and \textit{c-myb}\textsuperscript{h/h} injured vessels (\textbf{Supplemental Fig. III}). Illustrating the complexity of the biology of neointimal remodelling, when reciprocal experiments were performed using WT recipients, \textit{c-myb}\textsuperscript{h/h} BM conferred protection from neointimal remodelling as compared to WT BM (\textbf{Supplemental Fig. IV}).

\textbf{c-Myb regulates Sca1\textsuperscript{+} VSMC progenitor cell proliferation in response to vessel injury.}

As Sca1\textsuperscript{+} cells have been shown to participate in vessel remodelling, we next examined if \textit{c-myb} has a role in regulating the formation of these adventitial VSMC progenitor cells. The CCA of WT and \textit{c-myb}\textsuperscript{h/h} mice were found not to differ in the number of adventitial Sca1\textsuperscript{+} cells as assessed by immuonohistology (\textbf{Fig. 3A, B; Supplemental Fig.}), suggesting that \textit{c-myb} may not regulate the development of Sca1\textsuperscript{+} adventitial VSMC progenitor cells. While previous studies have relied heavily on histological identification of Sca1\textsuperscript{+} cells, this method has technical limitations for the identification and quantification of specific sub-populations of Sca1\textsuperscript{+} cells. Accordingly, we also employed flow cytometry to refine the analysis of adventitial VSMC progenitor cell populations and their response to injury. Consistent with previous studies[123, 265], we have defined Sca1\textsuperscript{+} adventitial VSMC progenitor cells as CD45\textsuperscript{−} Lineage\textsuperscript{−} (Lin; CD3\textsuperscript{−}, CD31\textsuperscript{−}, CD11b\textsuperscript{−}, B220\textsuperscript{−}, Nk1.1\textsuperscript{−}, Ter119\textsuperscript{−}) Sca1\textsuperscript{+} cells (\textbf{Fig. 3C; S6}), allowing for the exclusion of hematopoietic cells and ECs from the analyzed cell populations. Following CCA injury, adventitial Sca1\textsuperscript{+} cells were increased at 14-d [5629±327 (54.7±1.7%)] and 28-d post-injury
[6725±321 (55.8±1.4%)]) in WT animals compared to baseline [3796±360 (39.8±0.9%)] (N=3-10/group; P<0.01 for both comparisons) (Fig. 3D, E). However, there were significantly fewer Sca1+ cells in c-myb<sup>h/h</sup> mice at 14-d [4371±436 (49.1±2.1%)] and 28-d post injury [5243±218 (48.6±1.2%)] (N=3-10/group; P<0.05 for both comparisons vs. WT), suggesting that c-myb plays a role in the expansion of Sca1+ cells that occurs following injury. To assess the proliferation of Sca1+ progenitor cells in response to vessel injury, we quantified the incorporation of BrdU post-injury. c-myb<sup>h/h</sup> CD45<sup>-</sup>Lin<sup>-</sup>Sca1+ cells had decreased BrdU incorporation compared to WT cells at 8-d (5.4±0.8% vs. 9.1±0.6%), 14-d (2.4±0.4% vs. 4.9±0.4%), and 28-d post-injury (2.0±0.6% vs. 4.1±0.6%) (N=3-10/group; 8-d: P<0.001; 14- and 28-d: P<0.05), indicating that c-myb promotes the proliferation of CD45 Lin Sca1+ adventitial progenitor cells (Fig. 3F, G).

**Expansion of Sca1+ adventitial cells following injury is derived from vessel-resident and not circulating cells.**

Because Sca1+ adventitial VSMC progenitor cells could potentially arise locally or be recruited from the circulation in response to injury, we injured WT and c-myb<sup>h/h</sup> mice transplanted with eGFP<sup>+</sup>c-myb<sup>WT</sup> BM. This analysis revealed that both at baseline and at 28-d post-injury, over 99% of all carotid adventitial CD45 Lin Sca1+ cells remain host vessel-derived (i.e. eGFP<sup>-</sup>) (Fig. 4A), which was consistent in the aortic arch (Fig. 4B). Excluding the possibility that incomplete or unsuccessful BM reconstitution might explain the above result, over 99% of blood cells were eGFP<sup>+</sup> (Fig. 4C).

c-Myb specifically regulates Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>-</sup>Flk1<sup>-</sup> cells in response to vessel injury.
cKit, CD34 and Flk1 have been identified as potential additional markers of adventitial Sca1+ progenitor cells that can differentiate into VSMCs[123, 265], yet it is not known if one or a combination of these markers identifies VSMC progenitor cell populations that may be involved in the injury response in vivo. Thus, we next examined the expression of cKit, CD34 and Flk1 within CD45-Lin’Sca1+ cells in the uninjured carotid as well as following vessel injury and found that very few CD45-Lin’Sca1+ cells in the uninjured carotid artery are cKit’CD34+ (0.15±0.04%) or cKit+CD34+ (0.38±0.09%), and that these proportions were no different between WT and c-myb+/+ mice (Fig. 5A, B). Of interest, the relative abundance of CD45-Lin’Sca1+ Flk1+ cells in carotid arteries was decreased at 14- and 28-d post-injury as compared to baseline, and did not differ between WT and c-myb+/+ mice (Supplemental Fig. VII). By contrast, we found that at 8- and 14-d post-injury there is an expansion specifically of the cKit+CD34+ population, whereas cKit’CD34+ and cKit+CD34+ cells remained unchanged (Fig. 5B) in WT mice. However, in c-myb+/+ mice, the cKit+CD34+ population failed to expand to the same extent as in WT mice (10.4±0.8% vs. 14.8±1.1%; N=5/group; P<0.05) (Fig. 5A-B). When adventitial Sca1+ cells isolated from carotid arteries were cultured in vitro, there was decreased proliferation of c-myb+/+ Sca1+ cells (6.1±1.5% vs. 21.1±1.1% WT Sca1+BrdU+ cells) (Fig. 5C), while the relative abundance of Sca1+ cells did not differ between genotypes (Supplemental Fig. VIII A). It was also found that there were fewer Sca1+cKit+ cells in cultures isolated from c-myb+/+ carotid arteries versus WT carotid arteries (3.1±0.7% vs. 16.2±0.5%; N=3/group; P<0.05) (Fig. 5D). When the expression of cKit and CD34 was examined on proliferating (BrdU+) Sca1+ cells in vitro, it was found that in both genotypes, proliferating cells were predominantly cKit+ (Fig. 5E).
c-Myb regulates the differentiation of adventitial Sca1⁺ cells into VSMCs

To examine if c-myb is involved in the differentiation of Sca1⁺ adventitial VSMC progenitor cells, cells were isolated from WT and c-myb⁺/⁻ mice and differentiated in the presence of 0.2% FBS and 10 ng/ml TGFβ-1 for 7-d. Unlike WT cells, c-myb⁺/⁻ adventitial Sca1⁺ VSMC progenitor cells did not effectively up-regulate markers of differentiated VSMCs such as CNN1, MYH11, ACTA2 and myocardin (Fig. 6A-C), suggesting defective VSMC differentiation in c-myb⁺/⁻ adventitial smooth muscle progenitor cells. Interestingly, endogenous gene expression levels of several VSMC-specific and ECM genes also differed in the injured carotid arteries of WT vs. c-myb⁺/⁻ mice in vivo (Supplemental Fig. IX), in a pattern mirroring the defects observed in differentiated c-myb⁺/⁻ adventitial Sca1⁺ adventitial smooth muscle progenitor cells in vitro. c-Myb deficiency did not have an effect on adventitial smooth muscle progenitor cell apoptosis at baseline or after induction of differentiation by TGFβ-1 (Supplemental Fig. VIIIIC).

The myocardin gene promoter is bound and transcriptionally activated by c-Myb during VSMC differentiation.

While c-myb has been previously demonstrated to regulate ESC differentiation to VSMCs[17, 229], the mechanism by which c-myb promotes VSMC differentiation remains unclear. When Sca1⁺ VSMC progenitor cells were differentiated, c-myb⁺/⁻ Sca1⁺ VSMC progenitor cells failed to up-regulate myocardin expression (Fig. 6A). Similarly, c-myb⁺/⁻ CCA have lower myocardin expression than WT CCA (Fig. 6D). Adenoviral over-expression of myocardin prior to differentiation restored SMC gene expression in differentiated c-myb⁺/⁻ adventitial Sca1⁺ VSMC progenitor cells (Fig. 6E). c-Myb-dependent activation of myocardin was determined using a heterologous system by co-transfection of a myocardin promoter-
luciferase construct and either a c-Myb expression or empty control vector into human embryonic kidney 293 (HEK) cells. Over-expression of c-Myb resulted in activation of the myocardin promoter (Fig. 7A). Co-transfection of hypomorphic c-Myb resulted in decreased myocardin-luciferase reporter activity (Fig. 7A), as did the co-transfection of a distinct mutant c-Myb (mR3-3), previously shown to have defective c-Myb target gene activation [368] (Fig. 7B).

In silico analysis of the myocardin core promoter [369] revealed 8 putative c-Myb binding sites (MBS) in the core myocardin promoter (Fig. 7C). To determine if c-Myb transcriptionally regulates myocardin expression during VSMC differentiation, chromatin immunoprecipitation (ChIP) was performed using an anti-c-Myb antibody. Maximal induction of c-myb was observed 1-d post-differentiation (Supplemental Fig. VIII D), thus c-Myb binding to the myocardin promoter was examined 1-d post-induction of differentiation. While c-Myb was not found to bind to any region of the core myocardin promoter in undifferentiated Sca1+ VSMC progenitor cells, c-Myb bound two regions of the core promoter upon induction of VSMC differentiation with TGFβ-1 (Fig. 7D). To examine the functional importance of both predicted and ChIP-confirmed MBS in the myocardin promoter, WT and point-mutated promoter assays in HEK cells co-transfected with c-Myb were performed. These experiments revealed that c-Myb-dependent myocardin promoter activity was critically dependent on MBS1 and MBS8 (Fig. 8E). Unexpectedly, mutation of MBS3 resulted in a constitutively over-active myocardin-reporter (Supplemental Fig. X). These results demonstrate direct association of c-Myb with the myocardin promoter during differentiation of Sca1+ VSMC progenitor cells to VSMCs, and its transcriptional activation via specific MBS.

2.7 Discussion
It has been established that \textit{c-myb} regulates the proliferation of VSMCs and the differentiation of VSMCs from ESC-derived progenitors \textit{in vitro}\cite{1, 17, 220, 224, 229}, however it remained unknown if \textit{c-myb} has a similar role in adult vessel-resident VSMC progenitor cells \textit{in vivo}. Here we show that a mouse harboring a point-mutated \textit{c-myb} has defects in vessel remodelling in response to wire denudation injury, and that this is a vessel-intrinsic defect associated with defective proliferation of Sca1\(^+\) adventitial VSMC progenitor cells. While \textit{c-myb} does not appear to have a developmental role in the formation of Sca1\(^+\) adventitial VSMC progenitor cells, our data suggest that \textit{c-myb} regulates the proliferation of CD45\(^-\)Lin\(^-\)Sca1\(^+\) cells in response to wire denudation injury. Moreover, we have identified that cKit\(^+\)Sca1\(^+\) progenitor cells expand in response to wire denudation injury, while CD34\(^+\) and Flk1\(^+\) cells do not. \textit{In vitro} differentiation of \textit{c-myb}\(^{h/h}\) derived adventitial VSMC progenitor cells failed to effectively generate mature VSMCs, demonstrating that \textit{c-myb} also promotes the differentiation of adventitial progenitor cells. Finally, our data demonstrate that \textit{c-myb} regulates VSMC differentiation through the binding and transcriptional regulation of the myocardin gene. These results together demonstrate a role for \textit{c-myb} in regulating the proliferation and differentiation of an adult vessel-resident VSMC progenitor cell.

\textbf{c-Myb regulates Sca1\(^+\) progenitor cells in response to injury.}

In the current study, we have established that adventitial Sca1\(^+\) progenitor cell development does not appear to be regulated by \textit{c-myb}, as no differences in the number of adventitial Sca1\(^+\) cells were found by histology or flow cytometry of uninjured vessels in WT and \textit{c-myb}\(^{h/h}\) mice. However, upon injury there is a significant impairment in the ability of \textit{c-myb}\(^{h/h}\)
Sca1\(^+\) progenitor cells to proliferate as compared to WT Sca1\(^+\) cells. This role for \(c-myb\) in the proliferation of Sca1\(^+\) cells is similar to its already established role in VSMC proliferation, providing evidence that \(c-myb\) not only regulates mature VSMC proliferation, but also the proliferation of adult vessel-resident VSMC progenitor cells.

**Sca1\(^+\) progenitors are vessel-resident and expand following vessel injury.**

Using WT and \(c-myb^{h/h}\) mice lethally irradiated and reconstituted with eGFP\(^+\) BM, we have shown that CD45\(^-\)Lin\(^-\)Sca1\(^+\) cells in the carotid artery and aorta are not BM-derived. In response to wire denudation injury, the Sca1\(^+\) cell population proliferates; when mice reconstituted with eGFP\(^+\) BM were injured, the CD45\(^-\)Lin\(^-\)Sca1\(^+\) cells remained eGFP\(^-\), confirming that Sca1\(^+\) adventitial progenitor cells are not BM-derived cells at baseline or in response to injury, but are in fact adult vessel-resident progenitor cells. In addition, leukocyte recruitment is not impaired in \(c-myb^{h/h}\) injured vessels, suggesting that \(c-myb\) does not regulate inflammatory cell recruitment in response to vessel injury. While reconstitution of \(c-myb^{h/h}\) mice with WT BM was not sufficient to restore their injury response, the reciprocal experiment in which WT mice were reconstituted with \(c-myb^{h/h}\) BM revealed that \(c-myb^{h/h}\) BM had a protective effect on neointimal remodelling, demonstrating the complexity of neointimal remodelling.

Given the various hematopoietic abnormalities caused by \(c-myb\) hypomorphism[15], precise elucidation of which BM-derived cell populations mediate the latter phenotype would require complex stage- and lineage-specific transplantation or genetic ablation models beyond the scope of the current study. Moreover, the contribution of BM-derived protection to the decreased vessel remodelling of \(c-myb^{h/h}\) mice, while noteworthy, is overshadowed in the context of wire denudation injury, given that WT BM is insufficient to restore a normal injury response to \(c-\)
myb<sup>h/h</sup> mice. Taken together, these results show that while <i>c-myb</i><sup>h/h</sup> BM-derived cells also confer some level of protection from neointimal remodeling, the defect in vessel remodelling is intrinsic to the <i>c-myb</i><sup>h/h</sup> vessel-resident cells. Consistent with this observation, when TGFβ-1 expression was examined in WT and <i>c-myb</i><sup>h/h</sup> arteries post-injury, there was no difference in TGFβ-1 mRNA levels. Rather, Sca1<sup>+</sup> cells isolated from <i>c-myb</i><sup>h/h</sup> mice manifest defective responses to TGFβ-1 treatment, suggesting that the observed phenotype was independent of circulating or secreted factors. A limitation of the current study is that other cell compartments such as ECs, pericytes and BM-derived cells from <i>c-myb</i><sup>h/h</sup> mice may also harbour uncharacterized defects, however our BMT and in vitro experiments suggest that the effects observed in Sca1<sup>+</sup> cells are cell-intrinsic and not dependent on other cell types.

c-Myb specifically regulates the expansion of cKit<sup>+</sup>Sca1<sup>+</sup> cells

We have shown previously that <i>c-myb</i> has a role in regulating ESC differentiation into VSMCs through the expansion of a subset of hemogenic VSMC progenitor cells that are Flk1<sup>+</sup>[229]. However, it was not known if <i>c-myb</i> has such a role in adult vessel-resident progenitor cells <i>in vivo</i>. Based on our earlier work, we hypothesized that an Flk1<sup>+</sup> progenitor might persist within the Sca1<sup>+</sup> adventitial progenitor cell population, and this ‘adult’ Sca1<sup>+</sup>Flk1<sup>+</sup> cell population would be regulated by <i>c-myb in vivo</i>. Histological examination of mouse adventitia in a previous report suggested that adventitial Sca1<sup>+</sup> progenitor cells are a heterogenous population expressing CD34 and/or Flk1, however flow cytometric analysis identified adventitial Sca1<sup>+</sup> cells as Lin<sup>+</sup>cKit<sup>+</sup> [123]. A separate study found that freshly isolated adventitial Sca1<sup>+</sup> progenitor cells were cKit<sup>+</sup>CD34<sup>+</sup>CD140b<sup>+</sup>CD45<sup>+</sup>CD68<sup>+</sup> [265], however, these expression data came from qRT-PCR analysis of magnetically-isolated Sca1<sup>+</sup> cells without
lineage exclusion, and were not validated by flow cytometry or immunostaining. In the current study, a flow cytometry approach to analysis of the adventitial Sca1+ progenitor cell populations was used to quantitatively determine markers to identify progenitor cells relevant to vessel remodelling following injury. No baseline differences between WT and c-myb<sup>h/h</sup> Sca1+ cell population expression of cKit, CD34 and Flk1 were found. Following vessel injury, while CD34+ and Flk1+ cells declined, cKit+ cells expanded in response to injury in WT mice. However, in c-myb<sup>h/h</sup> mice post-injury, a defect in the expansion of Sca1+cKit+ cells was identified. Consistent with these results, when adventitial Sca1+ cells were isolated from WT and c-myb<sup>h/h</sup> carotid arteries, a proliferative defect was observed specifically in c-myb<sup>h/h</sup> cKit<sup>+</sup>CD34<sup>-</sup>Sca1<sup>+</sup> cells. It is tempting to speculate that CD34 and Flk1 represent markers of quiescent versus activated Sca1+ VSMC progenitors, and that expression of CD34 and Flk1 are down-regulated by activation of Sca1+ progenitor cells. Although the baseline number of Sca1+ progenitors and CD34+ and Flk1+ subpopulations did not differ between WT and c-myb<sup>h/h</sup> mice, we have not excluded the possibility that the function of these progenitors remains disturbed and may also contribute to the overall vascular phenotype of this mutant mouse. Additionally, it remains unknown if there are differential responses of adventitial progenitor cells based on embryological origins or vessel size, as previous reports examined aortic arch adventitial VSMC progenitor cells, which may be of embryological origins distinct from that of carotid artery adventitial cells.

**c-Myb regulates the differentiation of adventitial Sca1+ VSMC progenitor cells.**

To date there have been few reports of transcription factors that regulate the differentiation capacity of adventitial Sca1+ VSMC progenitor cells. WT adventitial Sca1+
VSMC progenitor cells differentiated into VSMCs as evidenced by expression of VSMC markers Cnn1, α-SMA, Myh11 and myocardin. By contrast, c-myb<sup>h/h</sup> adventitial Sca<sup>+</sup> VSMC progenitor cells incompletely up-regulated expression of these genes, suggesting that they are defective in their ability to differentiate into VSMCs, demonstrating the requirement for functional c-myb in the differentiation of this VSMC progenitor cell population.

c-Myb binds the myocardin promoter during VSMC differentiation

Undifferentiated Sca<sup>+</sup> adventitial VSMC progenitor cells were shown to be primed for VSMC differentiation through the co-expression of serum response factor (SRF) and myocardin, while also having high expression of transcriptional co-repressors of VSMC differentiation Msh homeobox 1 (Msx1), Kruppel-like factor 4 (Klf4) and Forkhead box protein O4 (FoxO4)[265]. In WT adventitial Sca<sup>+</sup> cells the expression of Msx1, Klf4 and FoxO4 decreased over time as cells differentiated, while SRF and myocardin were upregulated[265]. Because myocardin expression was lower in c-myb<sup>h/h</sup> carotid arteries at baseline and following injury, and incompletely up-regulated in response to TGFβ-1 in vitro in c-myb<sup>h/h</sup> adventitial Sca<sup>+</sup> VSMC progenitor cells, we hypothesized that c-myb regulates VSMC differentiation of Sca<sup>+</sup> smooth muscle progenitor cells via transcriptional control over myocardin[17]. Adenoviral over-expression of myocardin rescued the expression of SMC gene and protein in differentiated c-myb<sup>h/h</sup> Sca<sup>+</sup> VSMC progenitor cells. While these data demonstrate that c-Myb regulates VSMC differentiation at least partly through myocardin, we have not excluded the possibility that additional mechanisms may also be regulated by c-myb.

In a heterologous system, c-Myb over-expression was sufficient to activate a myocardin promoter-luciferase construct, further demonstrating that c-Myb activates myocardin expression.
Consistent with our observations from \( c-myb^{b/h} \) Sca1\(^+\) VSMC progenitor cells, transfection of our system with hypomorphic c-Myb did not activate the myocardin promoter reporter construct as effectively as WT c-Myb. Importantly, a second c-Myb mutant (mR3-3), previously shown to have defective transactivation of c-Myb target genes[368], also failed to activate a myocardin reporter. Taken together, these data demonstrate that c-Myb is critical to the transactivation of myocardin expression. Interestingly, the pro-myogenic activity of myocardin is enhanced by p300[106], a histone acetyltransferase known to have defective interaction with the mutant-c-Myb expressed in \( c-myb^{b/h} \) mice[15]. Given the specific defect in the transactivation domain of the \( c-myb \) hypomorph studied, it is also likely that interaction of \( c-myb \) with other transcriptional co-activators is necessary for efficient activation of the myocardin promoter and subsequent differentiation of adventitial Sca1\(^+\) VSMC progenitors into VSMCs.

ChIP with anti-c-Myb in Sca1\(^+\) VSMC progenitor cells demonstrated binding of c-Myb to the myocardin promoter in two regions of the core promoter. Importantly, c-Myb binding to the myocardin promoter could not be detected in undifferentiated cells, demonstrating that there is dynamic and direct regulation of myocardin transcription by c-Myb during differentiation of Sca1\(^+\) adventitial VSMC progenitor cells into VSMCs. Through site-directed mutagenesis, it was determined that MBS2 was not as critical for c-Myb induced promoter activity, while mutagenesis of MBS1 and MBS8 significantly decreased c-Myb-induced myocardin promoter activity. Interestingly, the mutation created in MBS3 (CAGCGGTC changed to CccgGGTC) generated a myocardin-luciferase construct with 8.0x10\(^5\)-fold higher activity than the WT myocardin promoter, suggesting either inadvertent creation of a binding site for another potent transcriptional activator or, more likely, loss of an inhibitory element in the myocardin promoter. This is the first direct mechanistic demonstration of how \( c-myb \) regulates the differentiation of
progenitor cells into VSMCs, and provides insight into how c-myb regulates the expression of multiple VSMC genes simultaneously.

It had been widely accepted that VSMC-like cells involved in the neointimal injury response are derived from the phenotypic modulation of contractile medial VSMCs into proliferative synthetic VSMCs [113, 115, 116]. However, emerging evidence now suggests that local progenitor populations residing in various compartments of the vessel wall may in fact be a contributing source of synthetic VSMCs in response to injury [121-124]. Through lineage-tagging experiments, it has been suggested that neointimal VSMCs may also be generated in part by the proliferation and differentiation of multipotent vessel-resident stem and progenitor cells[121]. We now demonstrate that c-myb regulates the proliferation and differentiation of an adventitial Sca1⁺ VSMC progenitor cell. A limitation of the current study is that we have yet to directly examine the relative contribution of the adventitial Sca1⁺ VSMC progenitor cell to neointimal remodelling. In future studies, progenitor cell recruitment will need to be demonstrated with coordinated spatiotemporal expression of several inducible lineage markers in order to conclusively delineate the contribution from heterogeneous populations of adventitial progenitor cells to vessel remodelling. While the current study cannot clarify the relative contribution of adventitial progenitor cells to vessel remodelling, the results do identify the expansion of a CD45 Lin⁻Sca1⁺cKit⁺ progenitor cell population in response to vessel injury, informing specific markers necessary to define a relevant cell population in future studies.

While c-myb has been shown to regulate VSMC differentiation from ESCs in vitro, we have now demonstrated that c-myb also plays a pathophysiological role in the regulation of adult
vessel progenitor cells that participate in neointimal remodelling. By regulating both the proliferation and myocardin-dependent differentiation of adventitial VSMC progenitor cells, c-myb has a profound role in the functional capacity of adventitial VSMC progenitor cells and thus may represent a novel approach to modulating injury responses of vessels. While current therapies for many vascular diseases includes systemic administration of therapeutic factors, understanding the functional role of local vessel-resident stem and progenitor cells and the factors that regulate their activity may represent novel refinements to the therapeutic success of vascular interventions.

2.8 Acknowledgements

The authors wish to thank Dr. Joseph Lipsick for kindly providing the mR3-3 c-Myb activation domain mutant and WT control plasmid.

Some equipment used in this study was supported by The 3D (Diet, Digestive Tract and Disease) Centre funded by the Canadian Foundation for Innovation and Ontario Research Fund, project number 19442 and 30961.

2.9 Sources of Funding

This work was supported in part by Canadian Institutes of Health Research (CIHR) operating grant MOP-136850 (to M.H.). M.H. is a Career Investigator of the Heart and Stroke Foundation of Ontario (CI5503). E.A.S was supported by a CIHR Small Health Organization Partnership Program Priority Area: Hypertension Doctoral Research Award, Ontario Graduate Scholarship and a Meredith & Malcolm Silver Scholarship in Cardiovascular Studies.
2.10 Disclosures

The authors declare no conflicts of interest.

2.11 Highlights

- It has long been thought that mature vascular smooth muscle cells (VSMCs) de-differentiate, proliferate and provide the bulk of cells in vascular pathologies as diverse as neointimal remodelling, atherosclerosis and aneurysm formation. More recently, a role for vessel-resident stem and progenitor cells is being appreciated.

- *c-myb* regulates the proliferation of CD45^−^Lin^−^Sca1^+^cKit^+^ vessel-resident adventitial VSMC progenitor cells *in vivo* in response to vessel injury.

- *c-myb* regulates the differentiation of adventitial VSMC progenitor cells into VSMCs *in vitro*.

- VSMC differentiation of adventitial VSMC progenitor cells is mediated by reduced transcriptional activation of myocardin, as chromatin immunoprecipitation revealed c-Myb binding to the myocardin promoter only during differentiation of Sca1^+^ cells. Adenoviral over-expression of myocardin rescued the phenotype of *c-myb*/*h* progenitors.
2.12 Figure legends

Figure 1: c-myb<sup>h/h</sup> mice have reduced neointimal remodelling following wire-denudation injury to the common carotid artery.

10-12 wk old male mice were subjected to wire denudation injury of their left common carotid artery, and allowed to recover for 8- (B-D), 14- (E-G) and 28-d (H-J). The right common carotid served as the uninjured control artery. c-myb<sup>h/h</sup> mice have decreased neointimal area at 14- and 28-d post-injury. Sections are stained with Masson’s trichrome. Representative 10x images are shown; scale bar represents 100 μm. Two-way repeated measures ANOVA were performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 5-8/group. WT injured: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 vs. Hypo injured.

Figure 2: c-Myb regulates vessel-intrinsic injury remodelling responses.

BMTs were performed on 5 wk old c-myb<sup>h/h</sup> mice to determine the role of c-Myb in the relative contribution of BM-derived and vessel-resident cell responses to vessel injury. Mice were transplanted with WT or c-myb<sup>h/h</sup> BM and allowed to recover for 8 wks before being subjected to wire denudation injury of their left carotid artery, and allowed to recover for 14-d. The right carotid artery served as the uninjured control artery. WT BMT could not restore the injury response to c-myb<sup>h/h</sup> vessels. Sections are stained with Masson’s trichrome. Representative 10x images are shown; scale bar represents 100 μm. Two-way repeated measures ANOVA were performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 5-8/group.

Figure 3: c-Myb regulates Sca1<sup>+</sup> cells in response to injury.
WT and *c-myb*<sup>h/h</sup> carotid arteries (A) were immunostained for Sca1 (yellow pseudocolour), calponin (red), nuclei (blue) and autofluorescent elastic lamina (green). Histological examination showed no differences in the number of Sca1<sup>+</sup> adventitial cells (B). Flow cytometry analysis, gated on CD45<sup>-</sup>Lin<sup>-</sup> cells, also found no differences in adventitial Sca1<sup>+</sup> cells (C, D). When mice were subject to wire injury, it was found that there was a decreased percentage of Sca1<sup>+</sup>CD45<sup>-</sup>Lin<sup>-</sup> cells at 14- and 28-d post-injury in *c-myb*<sup>h/h</sup> mice (E). Injured carotid arteries from *c-myb*<sup>h/h</sup> mice had decreased BrdU<sup>+</sup> incorporation in Sca1<sup>+</sup>CD45<sup>-</sup>Lin<sup>-</sup> cells at 8-, 14- and 28-d post-injury (F,G). A: Images taken at 20x magnification; scale bar represents 50 μm. L – lumen. B, D: Student’s t-tests were performed. Error bars represent SEM. N = 4-9/group. C: Representative flow plots are shown. E,G: Two-way ANOVA was performed with Bonferroni post-hoc tests. N = 3-10/group. *p<0.05, ***p<0.001 vs. Hypo injured. U – uninjured. F: Representative flow plots from 8-d post-injury.

**Figure 4: Sca1<sup>+</sup> cells are vessel-resident cells, not BM-derived.**

WT and *c-myb*<sup>h/h</sup> mice were lethally irradiated and reconstituted with eGFP<sup>+</sup> WT BM and allowed to recover 8 weeks before being subjected to wire injury. Gating on CD45<sup>-</sup>Lin<sup>-</sup> events from carotid arteries, Sca1<sup>+</sup> cells pre- and 28-d post-injury were found to be eGFP<sup>-</sup> (A). Adventitia was microdissected from aortic arches following partial enzymatic digestion. Gating on CD45<sup>-</sup>Lin<sup>-</sup>Sca1<sup>+</sup> events, aortic arch adventitial Sca1<sup>+</sup> cells were also found to be eGFP<sup>-</sup> (B). Chimerism in the blood (C) confirms complete reconstitution. N = 6 mice/group. Representative flow plots are shown.

**Figure 5: c-Myb specifically regulates Sca1<sup>+</sup>cKit<sup>+</sup> cells.**
WT and c-myb<sup>h/h</sup> mice were subjected to wire injury, and Sca1<sup>+</sup> cells were examined for CD34 and cKit expression. Cells were gated on CD45<sup>-</sup>Lin<sup>-</sup>Sca1<sup>+</sup> cells. Representative flow plots are shown (A). During injury cKit<sup>+</sup>CD34<sup>-</sup> cells expanded up to 14-d post injury. c-myb<sup>h/h</sup> mice failed to efficiently expand the Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>-</sup> population (B). Adventitial Sca1<sup>+</sup> VSMC progenitor cells were isolated from WT and c-myb<sup>h/h</sup> carotid arteries and maintained in growth media. There are fewer proliferating (BrdU<sup>+</sup>) Sca1<sup>+</sup> c-myb<sup>h/h</sup> VSMC progenitor cells (C). While there are fewer c-myb<sup>h/h</sup> Sca1<sup>+</sup>cKit<sup>+</sup>CD34<sup>-</sup> cells (D), the proportion of Sca1<sup>+</sup>BrdU<sup>+</sup> cells that are cKit<sup>+</sup>CD34<sup>-</sup> remains unchanged in c-myb<sup>h/h</sup> Sca1<sup>+</sup> cells (E). A: Representative flow plots are shown. B, D, E: Two-way ANOVA was performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 3-10/group. *p<0.05 vs Hypo cKit<sup>+</sup>CD34<sup>-</sup>. U – uninjured. C: Student’s t-tests were performed. Error bars represent SEM. N = 3/group. *p<0.001 vs WT.

**Figure 6: c-Myb regulates Sca1<sup>+</sup> VSMC progenitor cell differentiation into VSMCs.**

Adventitial Sca1<sup>+</sup> VSMC progenitor cells were isolated from WT and c-myb<sup>h/h</sup> carotid arteries and differentiated with 10 ng/ml TGFβ-1 and 0.2% FBS for 7-d. VSMC gene (A) and protein expression (B, C) in differentiated c-myb<sup>h/h</sup> Sca1<sup>+</sup> VSMC progenitor cells is defective. Myocardin expression is also lower in c-myb<sup>h/h</sup> carotid arteries at baseline (D). Adenoviral overexpression of myocardin (E) rescues VSMC gene expression in differentiated c-myb<sup>h/h</sup> adventitial Sca1<sup>+</sup> VSMC progenitor cells. A: Representative flow plots are shown. A, C: Two-way ANOVA was performed with Bonferroni post-hoc tests. D: Student’s t-test was performed. E: One-way ANOVA was performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 3-6/group. *p<0.05; **p<0.01; ****p<0.0001.
**Figure 7: c-Myb transcriptionally regulates myocardin during VSMC progenitor cell differentiation.**

c-Myb-dependent activation of a myocardin promoter was determined by co-transfection of a myocardin promoter-*luciferase* construct and a c-Myb expression (WT) or control vector (Ctrl) in HEK cells (A). Co-transfection of hypomorphic c-Myb (Hypo) results in decreased activation of the myocardin-luciferase reporter. Co-transfection of a unique mutant c-Myb (mR3-3) with defective transactivation also showed decreased activation of the myocardin-luciferase reporter (B). *In silico* analysis revealed that the mouse myocardin core promoter contains 8 putative c-Myb binding sites (MBS) (C). Differentiation of Sca1+ VSMC progenitor cells results in c-Myb binding to two regions of the myocardin promoter shown by ChIP and subsequent quantification by real-time qPCR (D). Mutation of MBS1 and MBS8 within the myocardin promoter reduced c-Myb activation of myocardin-luciferase reporter constructs in HEK cells (E). A, E: One-way ANOVA was performed with Bonferroni post-hoc tests. B: Student’s t-test was performed. Error bars represent SEM. N = 3-6/group.*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.
Figure 2.13: c-myb<sup>h/h</sup> mice have reduced neointimal remodelling following wire-denudation injury to the common carotid artery.
Figure 2-2: c-Myb regulates vessel-intrinsic injury remodelling responses.
Figure 2-3: c-Myb regulates Sca1^+ cells in response to injury.
Figure 2-4: Sca1+ cells are vessel-resident cells, not BM-derived.
Figure 2-5: c-Myb specifically regulates Sca1\textsuperscript{+}cKit\textsuperscript{+} cells.
Figure 2-6: c-Myb regulates Sca1+ VSMC progenitor cell differentiation into VSMCs.
Figure 2-7: c-Myb transcriptionally regulates myocardin during VSMC progenitor cell differentiation.
### 2.14 Supplemental Tables

#### 2-1: Supplemental Table I: Primers used

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2-2: Supplemental Table II: Mutagenesis Primers used

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2.15 Supplemental Figure Legends

**Supplemental Figure I: Injury phenotype is strain independent.**

C57BL/6 c-myb<sup>h/h</sup> were backcrossed 10 generations to the FVB/N strain to assess the strain specificity of injury response. FVB/N c-myb<sup>h/h</sup> mice were injured and assessed for remodelling 14-d post injury. Data at 100 μM bifurcation is shown for both strains. Two-way ANOVA were performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 3-7/group. *p<0.05 vs WT. ###p<0.001, ####p<0.0001. I/M – Intima/Media.

**Supplemental Figure II: Medial remodelling is unaffected in c-myb<sup>h/h</sup> mice.**

10-12 wk old male mice were subjected to wire denudation injury of their left common carotid artery, and allowed to recover for 8- (A), 14- (B) and 28-d post-injury (C). Medial remodelling is not different between WT and c-myb<sup>h/h</sup> mice in response to carotid wire injury. Bone marrow transplants were performed on 5 wk old c-myb<sup>h/h</sup> mice to determine the role of c-Myb in the relative contribution of BM-derived and vessel-resident cell responses to vessel injury. Mice were allowed to recover for 8 wks before being subjected to wire denudation injury of their left carotid artery, and allowed to recover for 14-d (D). The right common carotid served as the uninjured control artery. Lumen area (a), area within medial elastic lamina (b), and area within outer elastic lamina (c) were measured with ImageJ software (NIH). Intima area was calculated as b – a; media was calculated as c – b; intima:media (I/M) ratio calculated as (b-a)/(c-b). Two-way repeated measures ANOVA were performed with Bonferroni post-hoc tests. N = 5-8/group.

**Supplemental Figure III: Leukocyte recruitment is unaffected in c-myb<sup>h/h</sup> mice post-injury.**
WT and c-myb<sup>h/h</sup> carotid arteries were isolated after wire injury and assessed for leukocyte recruitment by flow cytometry. CD45<sup>+</sup> and Lin<sup>+</sup> cells were identified as a fraction of all single cells. No difference in leukocyte recruitment in injured arteries was found between WT and c-myb<sup>h/h</sup> vessels at any time point post-injury. Error bars represent SEM. Two-way repeated measures ANOVA were performed with Bonferroni post-hoc tests. N = 5-8/group.

**Supplemental Figure IV: Hypomorphic bone marrow protects WT mice from injury.**
BMTs were performed on 5 wk old WT mice to determine the role of c-Myb in the relative contribution of BM-derived and vessel-resident cell responses to vessel injury. Mice were transplanted with WT or c-myb<sup>h/h</sup> BM and allowed to recover for 8 wks before being subjected to wire denudation injury of their left carotid artery, and allowed to recover for 14-d. The right carotid artery served as the uninjured control artery. c-myb<sup>h/h</sup> BM conferred protection from remodelling following vessel injury in WT vessels (A-D). Sections are stained with Masson’s trichrome. Representative 10x images are shown; scale bar represents 100 μm. Two-way repeated measures ANOVA were performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 5-8/group. WT>WT injured: ****p<0.0001 vs. WT>Hypo injured. I/M – Intima/Media.

**Supplemental Figure V: Immunofluorescent stain and isotype controls.**
WT carotid arteries were immunostained for Sca1 (yellow pseudocolour), calponin (red), nuclei (blue) and autofluorescent elastic lamina (green). Isotype antibodies for Sca1 (rat IgG<sub>2a</sub>) and calponin (rabbit IgG) were used for determining primary antibody specificity. Images taken at 20x magnification; scale bar represents 50 μm.
Supplemental Figure VI: Gating schemes and staining controls for flow cytometric analyses.

Carotid arteries were dissected and enzymatically digested and stained for flow cytometric analysis. Gating schemes and fluorescence minus one (FMO) staining controls are shown. For BrdU staining, a FMO (no antibody) control was run, as well as a sample without BrdU (-BrdU) but stained with anti-BrdU antibody to demonstrate negative staining control. For Annexin V staining, a FMO (no conjugated Annexin V) and positive staining control (permeabilized cells) were used for staining controls. For CNN1 and ACTA2 staining, isotype antibodies for CNN1 and ACTA2 were used for gating.

Supplemental Figure VII: Flk1\(^+\) cells are reduced during vessel injury.

WT and c-myb\(^{h/h}\) mice were subjected to wire injury, and Sca1\(^+\) cells were examined for Flk1 expression. Cells were gated on CD45\(^-\)Lin\(^-\)Sca1\(^+\) cells. Sca1\(^+\)Flk1\(^+\) cells were reduced post-injury. No differences were found between WT and c-myb\(^{h/h}\) mice. A two-way ANOVA was performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 3-10/group.

Supplemental Figure VIII: Sca1\(^+\) smooth muscle progenitor cells upregulate c-myb in response to TGF\(\beta\)-1.

Adventitial Sca1\(^+\) cells were isolated from WT and c-myb\(^{h/h}\) carotid arteries and cultured in growth media supplemented with 10% FBS and LIF. Smooth muscle progenitor cells were differentiated in media supplemented with 10 ng/ml TGF\(\beta\)-1 and 0.2% FBS for 7-d. Sca1 expression is down-regulated during smooth muscle directed differentiation (A). Low serum
conditions (0.2% FBS) were sufficient to prevent proliferation of Sca1+ cells during differentiation of adventitial smooth muscle progenitor cells (B). *-myb does not regulate Sca1+ smooth muscle progenitor cell apoptosis at steady state or in response to TGFβ-1 treatment (C). *-myb expression peaks at 1-d of TGFβ-1 treatment (D). A, C, D: Two-way ANOVA was performed with Bonferroni post-hoc tests. B: Student’s t-test was run; Error bars represent SEM. N = 3-6/group. *p<0.05; ***p<0.001.

**Supplemental Figure IX: Injured *-myb**/h arteries fail to upregulate SMC and ECM-related genes.**

10 – 12 week old male mice were subjected to wire denudation injury of their left CCA, and allowed to recover for 14-d. The right CCA served as the uninjured control artery. Mice were sacrificed and carotid arteries were collected and snap frozen in liquid nitrogen. RNA was isolated using the Picopure RNA isolation kit (Life Technologies). cNDA was synthesized using qScript cDNA supermix (Quanta BioSciences), and differences in mRNA levels were quantified using SYBR green dye on a Roche LightCycler 480 and specific primers. B2M was used as the housekeeping gene; values were compared using the 2^-ΔΔCt method. Two-way mixed measures ANOVA with Bonferroni post-hoc tests were performed. N = 3-6 arteries/group. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

**Supplemental Figure X: Mutation of MBS3 creates a constitutively active Myocardin-luciferase reporter.**

Mutation of MBS3 (ΔMBS3) unexpectedly created a myocardin-luciferase reporter with 8.0x10^5-fold increase activity over WT-myocardin-luciferase. Co-transfection with WT or
hypomorphic c-Myb had relatively modest (<2.0 fold) effects on the activity of the ΔMBS3-luciferase reporter, with its activation remaining >4.0x10^5-fold higher than the WT-myocardin-luciferase. One-way ANOVA was performed with Bonferroni post-hoc tests. Error bars represent SEM. N = 4/group.****p<0.0001 vs WT-Myocd.
Figure 2-8:
Supplemental Figure I: Injury phenotype is strain independent.
Figure 2-9:
Supplemental Figure II: Medial remodelling is unaffected in c-myb<sup>h/h</sup> mice.
Figure 2-10:
Supplemental Figure III: Leukocyte recruitment is unaffected in c-myb<sup>h/h</sup> mice post-injury.
Figure 2-11:
Supplemental Figure IV: Hypomorphic bone marrow protects WT mice from injury.
Figure 2-12: Supplemental Figure V: Immunofluorescent stain and isotype controls.

1° antibody:
Anti-Sca1 (1:100)
Anti-Cnn1 (1:200)

2° antibody:
Anti-rat Cy3 (1:400)
Anti-rabbit Cy5 (1:400)

1° antibody:
Anti-IgG2aκ (1:100)
Anti-IgG (1:200)

2° antibody:
Anti-rat Cy3 (1:400)
Anti-rabbit Cy5 (1:400)
Figure 2-13: Supplemental Figure VI: Gating schemes and staining controls for flow cytometric analyses.
Figure 2-14:
Supplemental Figure VII: Flk1+ cells are reduced during vessel injury.
Figure 2-15:
Supplemental Figure VIII: Sca1+ smooth muscle progenitor cells upregulate c-Myb in response to TGFβ-1.
Supplemental Figure IX: Injured c-myb<sup>h/h</sup> arteries fail to upregulate SMC and ECM-related genes.
Figure 2-17:
Supplemental Figure X: Mutation of MBS3 creates a constitutively active Myocardin-luciferase reporter.
CHAPTER 3:

3  c-Myb regulates blood pressure homeostasis
Full Title: Blood pressure homeostasis and normal renal function depends on normal c-Myb activity in bone marrow-derived cells.

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Running title: c-Myb regulates blood pressure homeostasis

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3.1 Abstract

Objective: We showed that the proto-oncogene *c-myb* regulates proliferation and differentiation of vascular smooth muscle cells (VSMCs) from specific adult vessel-resident progenitors and embryonic stem cells, however it was not known if c-Myb also regulates contractile function of VSMCs and integrated cardiovascular physiology.

Approach and Results: Mice homozygous for a hypomorphic *c-myb* allele with compromised c-Myb activity (*c-myb*<sup>h/h</sup>) were compared to wild-type (WT). Hemodynamic assessments revealed decreased systolic (104±2 vs. 120±1 mmHg; P < 0.0001) and diastolic (71±2 vs. 83±1 mmHg; P < 0.001) blood pressure (BP) in *c-myb*<sup>h/h</sup> compared to WT mice, which was confirmed by radiotelemetry in awake mice. Echocardiography found no differences in cardiac structure or function, and perfusion myography showed no defects in contractile function of *c-myb*<sup>h/h</sup> arteries. Differences in plasma cytokines and biochemistry were observed. While plasma keratinocyte chemokine (KC) was elevated in *c-myb*<sup>h/h</sup> mice, osmotic pump infusion of KC did not modulate BP. 24-h metabolic cage studies found increased urine output, altered creatinine clearance and fractional excretion of urea, suggesting altered renal function in *c-myb*<sup>h/h</sup> mice. Flow cytometry revealed decreased renal B220+ B-cells in *c-myb*<sup>h/h</sup> mice, with no differences in myeloid or T-cells. Since *c-myb*<sup>h/h</sup> mice carry multiple hematological abnormalities, reciprocal bone marrow (BM) transplantation with WT and *c-myb*<sup>h/h</sup> mice was performed. Reconstitution of WT mice with *c-myb*<sup>h/h</sup> BM (*h/h>*WT) decreased BP of WT recipients to *h/h* levels; conversely *WT>*<sup>h/h</sup> mice had BP equivalent to WT mice. Renal function of *h/h>*WT mice also mirrored that of *c-myb*<sup>h/h</sup> mice, including abnormalities in plasma and urine biochemistry. When challenged with DOCA-salt hypertension, *c-myb*<sup>h/h</sup> mice had lower BP responses vs. WT mice.
Conclusions: The reduced c-Myb activity of $c-myb^{h/h}$ mice does not alter their vessel contractile function or cardiac structure or function. BMT experiments demonstrate that low BP and altered renal function of $c-myb^{h/h}$ mice is due to defective c-Myb activity in BM-derived cell populations. Renal flow cytometry suggests that B220+ B-cells may be implicated in this phenotype. Together, these data suggest that $c-myb$ regulates BM-derived cell populations involved in kidney function and BP homeostasis.

3.2 Non-standard abbreviations and acronyms:

BM Bone marrow  
BMT Bone marrow transplant  
BP Blood pressure  
DOCA Deoxycorticosterone acetate  
ESC Embryonic stem cell  
ECM Extracellular matrix  
eGFP Enhanced green fluorescent protein  
VSMC Vascular smooth muscle cell
3.3 Introduction

Steady-state regulation of blood pressure is complex, involving the coordinate regulation of cardiovascular and neuroendocrine systems. Both genetic and environmental factors are known to influence the regulation of blood pressure, adding to the complexity of understanding mechanisms underlying both normal and pathophysiological blood pressure regulation. Fundamental components determining blood pressure include blood flow (i.e. cardiac output) and arterial resistance, which is in turn derived from vessel stiffness and contractile tone. Hence, factors that regulate vascular contractility are known to regulate blood pressure homeostasis.

The proto-oncogene c-myb has been shown to regulate the differentiation of VSMCs from embryonic stem cell-derived embryoid bodies (EBs) and vessel-resident VSMC progenitor cells [17, 370]. c-myb−/− EBs differentiated towards VSMCs had decreased contractile protein expression, and functionally displayed a marked absence of SMC-like contractions [17]. While c-myb has also been demonstrated to regulate the proliferation of VSMCs [1, 6, 219], and has known roles in the regulation of intracellular calcium levels [218-220, 222-224, 371], it is unclear if c-myb has a role in the regulation of vascular contractility in vivo. c-myb is known to repress the expression of PMCA4 [223], the loss of which causes increased blood pressure due to aberrant calcium handling in VSMCs. Over-expression of PMCA4 increases myogenic tone with a concomitant increase in blood pressure [342], hence c-myb may regulate vasomotor function through effects on calcium handling and VSMC differentiation.

Given the above roles of c-myb in VSMC biology, we posited a role for c-myb in regulating vasomotor function, and hence a role in regulating blood pressure. However, as c-myb has pleiotropic roles in hematopoiesis, the recent appreciation that the immune system has a regulatory role in blood pressure homeostasis is also of relevance. While it has long been shown
that hypertension is associated with tissue inflammation and recruitment of leukocytes to areas such the vessel wall, heart, kidneys and brain, a causative role for leukocytes in the pathogenesis of hypertension is only recently becoming clear. Macnthinik et al demonstrated that macrophages in skin are osmosensitive, detecting increases in interstitial sodium and subsequently increasing secretion of VEGF-C to induce lymphangiogenesis to normalize skin interstitial sodium tonicity [358]. Guzik and colleagues demonstrated the necessity of T-cells for the development of experimental hypertension [361], as Rag1−/− mice lacking functional B- and T-cells did not develop hypertension. Reconstitution specifically with T- but not B-cells restored their response to hypertensive stimuli [361]. After documenting that T-cells infiltrating perivascular adipose tissue and the adventitia of vessels were CD3+CD4+CD8− [361], subsequent studies showed that CD4+CD25+FoxP3+ regulatory T-cells blunt the response to hypertensive stimuli [362, 363].

These studies and others demonstrate not only that tissue inflammation and leukocyte recruitment during the pathogenesis of hypertension, but also highlight a regulatory role for leukocytes play a regulatory role. While c-myb is known to have pleiotropic roles in the maintenance of hematopoietic cells, the role of c-myb in the regulation of tissue and organ homeostasis by immune cells is poorly understood. While differentiation and proliferation are well characterized processes regulated by c-myb, its role in the functional regulation of those tissues and organs remain less clear.

The reduced c-Myb activity of c-myb+/− mice does not alter their vessel contractile function or cardiac structure or function. BMT experiments demonstrate that low BP and altered renal function of c-myb+/− mice is due to defective c-Myb activity in BM-derived cell populations. Renal flow cytometry suggests that B220+ B-cells may be implicated in this phenotype. Together, these data suggest that c-myb regulates BM-derived cell populations involved in
kidney function and BP homeostasis. Given the role of \( c-myb \) in regulating VSMCs and leukocytes, and that of both of these cell types in regulating vasomotor function, our novel findings regarding a role for \( c-myb \) in the regulation of an integrated physiological parameter such as blood pressure may have been predictable.

3.4 Materials and methods

C57BL/6 mice were obtained from Jackson Laboratory (000664; Jackson Laboratory, Bar Harbor, USA). \( c-myb^{+/+} \) animals on a C57BL/6 genetic background were derived as previously described [15]. FVB/N \( c-myb^{+/+} \) mice were derived as previously described [370]. For bone marrow transplantation (BMT) experiments, 5 week old recipient mice were lethally irradiated with a single 10 Gy dose of radiation (Gammacell 40 Exactor, Best Theratronics). Bone marrow was harvested from the femur and tibias of donors; \( 2.0 \times 10^6 \) cells per recipient were injected intravenously via tail vein. Mice were allowed to recover for 8 weeks. All mice used in the study were male. Mice were housed in a specific pathogen free facility with access to water and standard chow \textit{ad libitum}. All protocols were approved by the Animal Resource Centre at the University Health Network (AUP#1032, 1034) in accordance with the guidelines of the Canadian Council on Animal Care.

**Invasive hemodynamics**

Invasive aortic sinus and left ventricular hemodynamics were performed on mice under 1% isoflurane anaesthesia using a 1.4F Millar pressure-transducing catheter introduced via the right common carotid artery as previously described [372]. Aortic root and left ventricular (LV) pressure measurements are collected in triplicate for each animal. Data are averaged across the
three measurements for each parameter. Heart rate is the average derived from the aortic and LV BP recordings.

**Radiotelemetric ambulatory blood pressure measurement**

10 - 11 wk old $c$-$myb^{WT}$ and $c$-$myb^{h/h}$ mice were surgically implanted with pressure and activity monitors (Data Sciences International) and allowed to recover for 1 wk before recording. Unrestrained, unanesthetized mice were continuously monitored for 10 s every 10 min for 72 h. Data were averaged across the same time period during a 12 h day/night period.

**Histology**

10 - 12 wk old $c$-$myb^{WT}$ and $c$-$myb^{h/h}$ mice were sacrificed, perfused with ice-cold PBS, and then perfusion fixed with 10 ml ice-cold 4% PFA. Common carotid arteries (CCA) were carefully dissected from PFA-perfused mice and prepared for paraffin embedding and sectioning. 5 μm-thick transverse sections were stained with haematoxylin and eosin (HE), Massons trichrome and picrosirius red (PSR) and imaged on a brightfield microscope Leica DMLB microscope, captured with an Infinity2-2 CCD camera (Lumenera). PSR stained arteries were also imaged under polarized light. Lumen area (a), area within medial elastic lamina (b), and area within outer elastic lamina (c) were measured with ImageJ software (NIH). Medial area (VSMCs) was calculated as $c - b$.

**RNA isolation and qRT-PCR analysis**

RNA was extracted from CCA using a PicoPure RNA isolation kit (Life Technologies) according to manufacturer protocol. cDNA was synthesized using qScript cDNA SuperMix
(Quanta BioSciences) according to manufacturer protocol. Transcripts were then detected using specific primers [370] using SYBR Green Master (Roche) detected on a LightCycler 480 (Roche), using β-2-microglobulin as a housekeeping gene. Samples were run in triplicate and averaged.

**Tensile strength testing**

Mice were euthanized, perfused with 10 ml ice-cold 1x PBS and thoracic aortas were rapidly dissected. Aortas were clamped to force transducers and force was applied until tissue failure occurred.

**2-D Echocardiography**

Echocardiography was performed on mice under 1% isoflurane anesthesia using a 15-MHz linear ultrasound transducer (Vivid7; General Electric). M-mode measurements were made on short-axis views of the LV at the level of the papillary muscle and averaged over three beats.

**Perfusion myography**

Mice were euthanized and mesenteric arteries were rapidly dissected and prepared for perfusion myography as previously described [373, 374]. Second–order mesenteric arteries were carefully dissected free from fat under a dissecting microscope and cannulated. Concentration-dependent vasoconstriction/dilation relationships for phenylephrine and acetylcholine were determined. Sodium nitroprusside was used to determine endothelial cell nitric oxide (NO)-independent vasodilation. At the completion of each experiment, a Ca^{2+}-free buffer was used to record passive diameter (dia_{passive}). Vessel tone was calculated as: (dia_{passive}-dia_{response})/dia_{passive}, where
dia response is the diameter at a given drug concentration. To assess the myogenic response, transmural pressure was increased in 20 mmHg steps from 20 to 120 mmHg. Vessel diameter (active) was measured at each pressure step once a steady state was reached (5 to 10 minutes). At completion, passive diameter (dia passive) was recorded for each pressure step. Myogenic tone at each step was calculated as: \((\text{dia}_{\text{passive}} - \text{dia}_{\text{active}})/\text{dia}_{\text{passive}}\).

**Bio-plex mouse cytokine/chemokine assay**

Blood was collected by cardiac puncture from 10-12 wk old \(c-myb^{WT}\) and \(c-myb^{h/h}\) in 200 μl EDTA microvettles, and spun at \(10^5\) g for 10 min to collect plasma. BioRad Bio-Plex assay for mouse cytokine/chemokines was performed by the OCI Genomics Center.

**24-hour metabolic cage urine collection**

To determine 24-hour urine output, mice were housed singly in metabolic cages to collect urine. Mice were supplied with standard chow and water *ad libitum*. After 24-hr urine collection, mice were sacrificed and blood was collected by cardiac puncture for analysis of plasma and urine biochemistry.

**Plasma biochemistry**

Plasma samples were run on a Hemavet complete diagnostic profile panel (Drew Scientific) by the Animal Resource Center, University Health Network. Urine and plasma biochemistry was examined on a Beckman AU480 Biochemistry Analyzer at the Toronto Center for Phenogenomics. Creatine clearance was calculated as
((Creatinine_{urine}/Creatinine_{plasma}) \times (Volume_{urine}(ml)/Time\ (min)).\ \text{Fractional\ excretion\ of\ urea\ was}\ \text{calculated\ as}\ \frac{(Creatinine_{plasma} \times Urea_{urine})}{(Urea_{plasma} \times Creatinine_{urine})}.

**Keratinocyte Chemoattractant ELISA**

Plasma keratinocyte chemoattractant (KC) concentration was determined from serum samples using the mouse CXCL1/KC Quantikine ELISA kit (SMKC00B, R&D Systems) according to manufacturer’s protocol.

**Flow cytometry**

To prepare kidneys for flow cytometry, mice were sacrificed and perfused with 10mL ice-cold PBS. Kidneys were carefully dissected and enzymatically dispersed as previously described [234]. Kidneys were weighed and cut into small pieces and subject to enzymatic dispersion in 450 U/ml collagenase I (Sigma-Aldrich C0130), 125 U/ml collagenase XI (Sigma-Aldrich C7657), 60 U/ml DNase I (Sigma-Aldrich DN25), 60 U/ml hyaluronidase (Sigma-Aldrich H3506) in 20 mM HEPES buffer (Mediatech 25-060-C1) for in a heated shaker at 37°C and 900 rpm for 30 minutes. After digestion, kidney samples were washed in 1x PBS and passed through a 40 μm filter and RBCs were lysed with 1x RBC lysis buffer (BioLegend 42301), then samples washed in 1x PBS. Single cell preparations were stained for 45 minutes before being fixed with Cytofix buffer (BD Biosciences). Samples were run on a LSR II (BD Biosciences), and then subsequently analyzed by FlowJo software (v.10; Flowjo).

**Osmotic pump infusion**
Osmotic pumps (4317, Alzet) were loaded with recombinant KC (453-KC-050, R&D Systems) to deliver 0, 100 or 1000 ng/kg/min for 14 d and implanted subcutaneously in C57BL/6J mice according to manufacturer protocol. Invasive hemodynamic measurement was performed 14 d after pump implantation.

**Deoxycorticosterone acetate-salt model of hypertension**

To induce experimental hypertension, *c-myb<sup>WT</sup>* and *c-myb<sup>b/h</sup>* mice underwent unilateral nephrectomy and were implanted with a 50mg deoxycorticosterone acetate (DOCA) 21-day slow release pellet (M-121, Innovative Research of America) [375] and given 1% saline drinking water. Control animals underwent a sham operation and were implanted with a control pellet (C-111, Innovative Research America) and given regular drinking water. Water and standard chow were given *ad libitum*. Invasive hemodynamic measurement was performed 21 d after operation.

**Statistical analysis**

Where appropriate, Student’s t-tests, one-way ANOVA or two-way repeated measures ANOVA with Bonferroni post-hoc tests were performed. Data are expressed as mean±SEM.

**3.5 Results**

**c-myb mice have decreased blood pressure**

Invasive hemodynamic assessment of BP was performed. Compared to *c-myb<sup>WT</sup>* mice, *c-myb<sup>b/h</sup>* mice had decreased aortic systolic (120.2±1.1 vs. 103.6±2.3 mmHg; N = 14/group; P < 0.0001), diastolic BP (83.2±1.3 vs. 70.9±2.3 mmHg; N= 14/group; P < 0.001) (**Fig. 1A**) as well as LV systolic pressure (120.1±1.6 vs. 112.4±2.7 mmHg; N = 14/group; P < 0.05) and heart rate
(532±10 vs.500±10 beats per minute; N = 14/group; P < 0.05) (Fig. 1B). To exclude the possibility of differences in anesthetic sensitivity, mice were surgically implanted with radiotelemetric monitors to determine unanesthetized ambulatory BP. Consistent with anesthetized invasive hemodynamic measurements, radiotelemetry demonstrated that unanesthetized unrestrained c-myb<sup>h/h</sup> mice have lower BP than c-myb<sup>WT</sup> mice (Fig. 1C, D).

**c-myb<sup>h/h</sup> mice display fibrosis and stiffening of arteries**
To determine if the BP phenotype of c-myb<sup>h/h</sup> mice was due to perturbations in VSMC content or gene expression, histology of carotid arteries from c-myb<sup>WT</sup> and c-myb<sup>h/h</sup> mice was performed. Carotid arteries from c-myb<sup>h/h</sup> mice showed distinct differences in the level of collagen staining by Movat’s pentachrome, Massons trichrome and picrosirius red staining (Fig. 2A), suggesting increased collagen deposition in c-myb<sup>h/h</sup> arteries. No differences in medial (VSMC) area (Fig. 2B), or cellularity (Fig. 2C) were found, nor were differences in markers of contractile VSMCs detected (Fig. 2D). When thoracic aorta were isolated and tensile strength tested, it was found that consistent with increased collagen deposition and vascular stiffening, aortas from c-myb<sup>h/h</sup> mice had higher maximal tensile strength (Fig. 2E).

**c-myb<sup>h/h</sup> resistance arteries do not manifest contractile defects**
Despite manifesting increased arterial collagen content and tensile strength, c-myb<sup>h/h</sup> mice were found to have decreased BP. To determine if a difference in resistance artery contractility was involved, perfusion myography was performed. Consistent with morphometry of carotid arteries, no differences in resting vessel diameter and wall thickness were observed in mesenteric arteries (Fig. 3A, B). When pressure was applied to arteries, it was found that the passive
diameter of $c$-$myb^{h/h}$ arteries was smaller than $c$-$myb^{WT}$ arteries at pressures $>$60 mmHg, suggesting that $c$-$myb^{h/h}$ arteries were stiffer (Fig. 3C), consistent with observations of increased vascular fibrosis observed in the CCA and increased tensile strength of thoracic aorta of $c$-$myb^{h/h}$ mice. Myogenic response (Fig. 3 D, E) and phenylephrine-induced vasoconstriction (Fig. 3F) of $c$-$myb^{h/h}$ arteries did not differ from $c$-$myb^{WT}$ vessels, demonstrating that $c$-$myb^{h/h}$ arteries do not manifest contractile defects. Interestingly, $c$-$myb^{h/h}$ arteries had defective acetylcholine-mediated vasodilation (Fig. 3G), which was overcome in the presence of sodium nitroprusside (SNP), a NO-donor (Fig. 3H), suggesting that endothelial NO-mediated vasodilation is defective in $c$-$myb^{h/h}$ arteries.

**$c$-$myb^{h/h}$ mice do not have defects in cardiac function**

Because vasoconstriction was not defective in $c$-$myb^{h/h}$ mice, 2D echocardiography was used to determine if defects in cardiac function were responsible for decreased blood pressure in $c$-$myb^{h/h}$ mice. No differences in cardiac output was detected in $c$-$myb^{h/h}$ mice (Table 1), suggesting that cardiac function was not responsible for differences in blood pressure in $c$-$myb^{h/h}$ mice.

**Plasma cytokine and biochemistry reveal systemic differences in $c$-$myb^{h/h}$ mice**

Because no differences in cardiac or vascular function that could explain the low blood pressure phenotype of $c$-$myb^{h/h}$ mice were detected, systemic/circulating factors that could underlie observed differences in blood pressure were examined. Several circulating factors have been shown to regulate vascular tone and blood pressure [376-378], with the ability to impact multiple organ systems that are involved in blood pressure homeostasis. When circulating plasma cytokines/chemokines/growth factors were examined, only keratinocyte chemoattractant (KC)
was found to be increased in the plasma of c-<i>myb</i><sup>h/h</sup> mice (c-<i>myb</i><sup>h/h</sup> 78.66 ± 8.21 pg/mL vs. c-<i>myb</i><sup>WT</sup> 51.71 ± 8.10 pg/mL; N = 7/group; P = 0.04; Table 2). Blood biochemistry panels on c-<i>myb</i><sup>WT</sup> and c-<i>myb</i><sup>h/h</sup> mice suggested differences in plasma potassium concentration (Supplemental Table 1), raising the possibility of altered kidney function in c-<i>myb</i><sup>h/h</sup> mice.

**KC does not regulate BP**

To validate plasma cytokine screen results, KC ELISA was performed on c-<i>myb</i><sup>WT</sup> and c-<i>myb</i><sup>h/h</sup> plasma (Supplemental Fig. 1A). To investigate if KC has any direct effects on BP, osmotic pumps were used to deliver KC (0, 100 and 1000 pg/kg/min). No differences in BP were detected 14 days after pump implantation, suggesting that KC does not have any direct effects on blood pressure homeostasis (Supplemental Fig. 1B). Additionally, changes in blood biochemistry consistent with c-<i>myb</i><sup>h/h</sup> mice were not found (Supplementary Table 2).

**c-<i>myb</i><sup>h/h</sup> mice have altered kidney function**

Having observed perturbations in plasma potassium concentrations in c-<i>myb</i><sup>h/h</sup> mice it was investigated if c-<i>myb</i><sup>h/h</sup> mice manifest defects in kidney function which may underlie the low blood pressure phenotype. To interrogate kidney function in c-<i>myb</i><sup>h/h</sup> mice, mice were placed in 24-hour metabolic cages to collect 24-hour urinary output. c-<i>myb</i><sup>h/h</sup> mice had higher 24-hr urine volume, greater creatinine clearance and fractional excretion of urea, suggesting perturbed kidney function (Table 3). Consistent with Hemavet plasma biochemistry results, Biochemistry Analyzer results validated that c-<i>myb</i><sup>h/h</sup> mice have decreased plasma potassium concentrations (Table 3).
**c-myb<sup>h/h</sup> bone marrow confers low blood pressure**

Mounting evidence of tissue-resident leukocyte populations regulating blood pressure homeostasis through the modulation of local tissue activity[358, 361, 362], suggest that changes in leukocyte populations or their function could underlie changes in blood pressure homeostasis. The c-myb<sup>h/h</sup> mouse manifests profound hematological abnormalities [15], hence, we next examined if BM-derived cell populations mediate the low blood pressure phenotype of c-myb<sup>h/h</sup> mice. At 8 wk after BMT, it was found that c-myb<sup>WT</sup> mice reconstituted with c-myb<sup>h/h</sup> BM (c-myb<sup>WT>h/h</sup>) had significantly reduced systolic and diastolic blood pressure compared to c-myb<sup>WT</sup> mice reconstituted with BM from c-myb<sup>WT</sup> (c-myb<sup>WT>WT</sup>) donors (c-myb<sup>WT>WT</sup> 121.2 ± 2.9 mmHg vs. c-myb<sup>h/h>WT</sup> 106.8 ± 1.1 mmHg; N= 8-9/group; P < 0.001). c-myb<sup>h/h>WT</sup> mice had blood pressures matching those of c-myb<sup>h/h</sup> mice reconstituted with c-myb<sup>h/h</sup> BM (c-myb<sup>h/h>h/h</sup>; c-myb<sup>h/h>WT</sup> 106.8 ± 1.1 mmHg vs. c-myb<sup>h/h>h/h</sup> 101.4 ± 2.9 mmHg; N= 9-10/group; P > 0.05; Fig. 4A). Conversely, c-myb<sup>h/h</sup> mice reconstituted with c-myb<sup>WT</sup> BM (c-myb<sup>WT>h/h</sup>) had increased blood pressure matching that of c-myb<sup>WT</sup> mice reconstituted with c-myb<sup>WT</sup> BM (c-myb<sup>WT>WT</sup>; c-myb<sup>WT>WT</sup> 121.2 ± 2.9 mmHg vs. c-myb<sup>WT>h/h</sup> 118.7 ± 1.5 mmHg; N= 8-9/group; P > 0.05; Fig. 4A). Left ventricular systolic BP data matched those of the aortic blood pressure readings (Fig. 4B). These reciprocal BMTs demonstrate that the low blood pressure phenotype is conferred by c-myb<sup>h/h</sup> BM.

**c-myb<sup>h/h</sup> bone marrow transplantation does not affect vascular contractile function**

BM-derived leukocytes have been shown to regulate vascular contractility, thus to confirm that vascular contractility was not affected by BM genotype, C57BL/6 (WT) mice were reconstituted with c-myb<sup>h/h</sup> (Hypo>WT) or c-myb<sup>WT</sup> (WT>WT) BM and vascular contractility was examined by
perfusion myography. There were no differences in myogenic response (Supplemental Fig. 2A) or phenylephrine-induced contraction (Supplemental Fig. 2B) of WT>WT and Hypo>WT vessels, demonstrating that BMT did not alter vascular contractile function in post-BMT c-myb<sup>h/h>WT</sup> mice.

**c-myb<sup>h/h</sup> have decreased renal B-cell numbers**

Having observed that c-myb<sup>h/h</sup> BMT conferred low blood pressure to c-myb<sup>WT</sup> mice, and that there were perturbations in renal function in c-myb<sup>h/h</sup> mice, we next investigated whether renal leukocyte populations were perturbed in c-myb<sup>h/h</sup> mice. Flow cytometry of renal leukocyte populations revealed decreased renal B220<sup>+</sup> B-cells in c-myb<sup>h/h</sup> kidneys (c-myb<sup>h/h</sup> 0.823 ± 0.183 x 10<sup>4</sup> cells/g vs. c-myb<sup>WT</sup> 2.707 ± 0.451 x 10<sup>4</sup> cells/g; N = 8-10/group; P = 0.0007; Fig. 5), with no differences in renal neutrophils, macrophages, T-cells or dendritic cells.

**c-myb<sup>h/h</sup> BMT confers abnormal kidney function**

Since kidney function and renal leukocyte populations were found to be abnormal in c-myb<sup>h/h</sup> mice, and c-myb<sup>h/h</sup> BMT conferred low blood pressure to c-myb<sup>WT</sup> mice, we also examined if c-myb<sup>h/h</sup> BMT also conferred abnormal kidney function. C57BL/6 (WT) mice were transplanted with c-myb<sup>WT</sup> or c-myb<sup>h/h</sup> BM, let recover for 8 weeks, and then assessed in 24-hour metabolic cages. Upon assessment of plasma and urinary biochemistry, several abnormalities were detected in c-myb<sup>h/h>WT</sup> mice (Table 4), suggesting that BMT of c-myb<sup>h/h</sup> BM also generates perturbations in kidney function.

**c-myb<sup>h/h</sup> mice are resistant to DOCA-salt hypertension**
While c-myb<sup>h/h</sup> mice have decreased BP at baseline, to determine if c-myb<sup>h/h</sup> mice are protected from experimental hypertension, mice underwent unilateral nephrectomy and were implanted with DOCA pellets. Compared to c-myb<sup>WT</sup> mice, when challenged with DOCA-salt hypertension, c-myb<sup>h/h</sup> mice had lower BP (c-myb<sup>WT</sup> 152.4 ± 5.5 mmHg vs. c-myb<sup>h/h</sup> 132.8 ± 6.5 mmHg; n=7/group; P < 0.05; Fig. 6), suggesting that diminished c-myb activity not only confers lower baseline blood pressure, it is also protective from experimentally induced hypertension.

**c-myb<sup>h/h</sup> mice on the FVB/N genetic background have no differences in blood pressure**

To determine if the phenotype observed in c-myb<sup>h/h</sup> mice on a C57BL/6 genetic background were unique to the C57BL/6 strain, the c-myb<sup>h/h</sup> mutation was backcrossed onto a FVB/N genetic background for 10 generations. When blood pressure was measured in FVB/N c-myb<sup>wt</sup> and FVB/N c-myb<sup>h/h</sup> mice, there were no differences in aortic or left ventricular systolic or diastolic blood pressure (Supplemental Fig. 3A, B). Comparison of complete blood counts on C57BL/6 and FVB/N strains of c-myb<sup>WT</sup> and c-myb<sup>h/h</sup> mice reveal differences in leukocyte populations reveal that RBC and platelet numbers are similar across strains, yet several differences in leukocyte populations exist (Supplemental Fig. 3C).

### 3.6 Discussion

Invasive hemodynamic assessment of blood pressure revealed that c-myb<sup>h/h</sup> mice had lower blood pressure than c-myb<sup>WT</sup> mice, despite having stiffer, more fibrotic arteries as determined histologically and by functional assessment of vessels. These results were confirmed in unrestrained, unanesthetized mice showing that c-myb<sup>h/h</sup> mice have lower blood pressure. These
results were the opposite of what would be expected of mice that display vascular fibrosis and stiffening, and blunted acetylcholine-mediated vasodilation of a resistance arterial bed, which are, by contrast, stereotypical of the vascular phenotype of hypertensive mice.

c-Myb has been shown to up-regulate collagen type-1 expression [137, 138, 200], therefore it was surprising to observe increased collagen staining and concomitant increases in tensile strength and decreases in vascular compliance in the arteries of c-myb<sup>h/h</sup> from various vascular beds. While we observe more collagen in vessels, it remains to be determined if there is also more fibrosis in other organs and tissues in the c-myb<sup>h/h</sup> mouse.

In mice with deficient c-myb, it has been demonstrated that impaired wound healing is associated with decreased collagen synthesis in wounds [136]. In agreement with these data, we also demonstrated that c-myb<sup>h/h</sup> mice have decreased collagen mRNA expression compared to c-myb<sup>WT</sup> mice following vascular injury. The discrepancy of these data with the observation that at the steady-state, c-myb<sup>h/h</sup> mice have more vascular collagen suggest that c-Myb regulation of collagen synthesis may be divergent in vascular development and injury, and that the mechanisms that govern collagen synthesis in normal vascular development may not be the same as those involved in pathological processes. Additionally, we have found that after vessel injury, c-myb<sup>h/h</sup> vessels express lower levels of MMP-2 and MMP-9 [370]. If the expression of these collagenases is also decreased during vessel development, it is possible that there is altered or deficient collagen and ECM turnover in c-myb<sup>h/h</sup> mice, leading to buildup and excessive collagen in c-myb<sup>h/h</sup> vessel walls. Further investigation of vessel development in c-myb<sup>h/h</sup> mice could shed considerable mechanistic insights on why c-myb<sup>h/h</sup> mice have ‘paradoxically’ increased collagen deposition in the adult mouse.
It was hypothesized that \(c-myb\) deficiency would result in defective VSMC differentiation, as \(c-myb^+/c\)-embryonic stem cell-derived embryoid bodies as well as \(c-myb^{h/h}\)-derived adventitial vessel resident Sca1\(^+\) VSMC progenitor cells fail to efficiently differentiate into VSMCs [17, 370]. Decreased VSMC differentiation would lead to defective contractile function of vessels which might underlie the decreased blood pressure of \(c-myb^{h/h}\) mice. In addition, c-Myb regulation of intracellular calcium levels may also have a role in the regulation of vascular contractility. Perfusion myography data revealed, in agreement with histological and strength testing data, that arteries from \(c-myb^{h/h}\) mice were less compliant, as they distended less under the same pressure as vessels from \(c-myb^{WT}\) mice. Despite differences in vascular compliance, it was found that \(c-myb^{h/h}\) arteries had preserved myogenic response and preserved contractile response in response to phenylephrine stimulation, indicating that contractile function was not perturbed in \(c-myb^{h/h}\) mice. It is interesting to note that while \(c-myb\) has established roles in regulating intracellular calcium levels in VSMCs during cell cycle progression [222, 224], \textit{ex vivo} interrogation of contractile function from \(c-myb^{h/h}\) reveals preserved contractile function, suggesting that the \(c-myb\) regulation of intracellular calcium levels may be specific to cell cycle-related changes in calcium handling, and may not be relevant in mediating \textit{in vivo} contractile function. Surprisingly, it was observed that \(c-myb^{h/h}\) mice had a defective vasodilatory response to acetylcholine stimulation, which could be overcome using a nitric oxide donor. These data suggest that mechanisms regulating vasodilation are intact in \(c-myb^{h/h}\) VSMCs, reinforcing that \(c-myb\) deficiency in adult VSMCs causes no functional consequences to contractile function. However, these data are noteworthy in that they points to a previously unknown role for \(c-myb\) in the regulation of endothelial nitric oxide-mediated vasodilation.
myb is involved in intracellular calcium regulation in VSMCs [222, 224], thus it is possible that c-myb may regulate endothelial nitric oxide synthesis through a similar mechanism, although further experiments are required to establish such a relationship.

As no functional differences were found by perfusion myography or echocardiography in c-myb<sup>h/h</sup> mice, the possibility that differences in systemically circulating factors associated with changes in blood pressure regulation were explored as profound perturbations in leukocyte populations in c-myb<sup>h/h</sup> mice [15] may have altered cytokine plasma milieu of c-myb<sup>h/h</sup> mice. Examination of circulating cytokines and chemokines did not expose differences in factors previously associated with changes in blood pressure regulation such as IL-6, IL-17, RANTES, TNFα, and IFNγ, which have roles in blood pressure regulation in various organ systems. Only KC/CXCL1 was found to be increased in the plasma of c-myb<sup>h/h</sup> mice. However, exogenous KC infusion did not have an effect on blood pressure, suggesting that elevated plasma KC was not causative of low blood pressure in c-myb<sup>h/h</sup> mice. While most cytokines were found not to differ in plasma expression level, these results do not exclude the possibility that other cytokines or neurohormonal factors are affected in the c-myb<sup>h/h</sup> mouse. In addition, plasma expression levels do not exclude the possibility that local tissue-restricted expression of such factors is perturbed, precluding our ability to exclude cytokines from playing a role in this model. While KC does not appear to have a direct role in blood pressure regulation itself, it has been proposed as an early biomarker for acute kidney injury [379], suggesting that perturbations in kidney function may be present in c-myb<sup>h/h</sup> mice. In support of this data, a veterinary plasma biochemistry panel revealed decreases in plasma potassium ion concentrations in c-myb<sup>h/h</sup> mice. Elevated alkaline phosphatase was also observed in c-myb<sup>h/h</sup> mice. Elevated alkaline phosphatase is associated
with essential thrombocythaemia [380], one of the known hematological malignancies in the c-myb^{h/h} mouse [15]. Investigation of renal function in c-myb^{h/h} mice revealed that c-myb^{h/h} mice have increased 24-hour urinary output, greater creatinine clearance and fractional excretion of urea. Decreased plasma and urine potassium in c-myb^{h/h} mice was also observed, suggesting abnormal renal potassium handling.

While cardiac and vascular contractile function was normal in c-myb^{h/h} mice, other plausible mechanisms regulating blood pressure were explored. A growing body of evidence shows that various leukocyte subsets have profound roles in regulation of blood pressure homeostasis. Because c-myb^{h/h} mice have various hematological abnormalities, BMTs were performed to determine if low blood pressure was possibly regulated by BM-derived leukocytes. Interestingly, BMT of c-myb^{WT} BM into c-myb^{h/h} mice restored their blood pressures to c-myb^{WT} levels, and reciprocal BMT of c-myb^{h/h} BM into c-myb^{WT} recipients conferred low blood pressure to c-myb^{WT} animals, suggesting that the low blood pressure phenotype observed in c-myb^{h/h} mice was due to hematological abnormalities in c-myb^{h/h} mice. When the renal function of C57BL/6 (WT) mice reconstituted with c-myb^{h/h} BM was investigated, they also recapitulated many of the renal phenotypes of the c-myb^{h/h} mouse, suggesting that c-Myb function in BM-derived cells is crucial to the maintenance of renal function and blood pressure homeostasis.

Given the profound hematopoietic abnormalities in c-myb^{h/h} mice, it was likely that one or more renal leukocyte populations that are critical in the maintenance of normal homeostatic function may be perturbed. Investigation of renal leukocyte populations found that there were fewer B-cells in the kidneys of c-myb^{h/h} mice. While no differences in the number of other cell populations such as neutrophils, macrophages, dendritic cells and T-cells were found, it remains
possible that the function of these leukocyte populations is perturbed. A consistent phenotype of $c\text{-}myb^{h/h}$ and $c\text{-}myb^{h/h}/\text{WT}$ mice was decreased plasma potassium levels. Whether these leukocyte populations regulate the function of renal ion transporters known to regulate potassium levels such as KCNE1 [381], ENaC [382], ROMK1 [383], NCC [384] or NKCC2 [385] is of interest, as these may represent potential mechanisms of leukocyte-mediated BP homeostasis. While renal leukocyte populations were found to be perturbed, it remains possible that other tissue-resident leukocytes such as skin interstitial macrophages [358], vascular T-cells [362, 363, 386] are also perturbed in the $c\text{-}myb^{h/h}$ mice contributing to other mechanisms of leukocyte-mediated blood pressure homeostasis.

When $c\text{-}myb^{h/h}$ mice were bred from a C57/BL6 genetic background to an FVB/N background, it was found that the FVB/N mice did not manifest a low blood pressure. Interestingly, FVB/N mice had similar levels RBCs, suggesting that the low blood pressure were not due to the mild anemia observed in the C57BL6 $c\text{-}myb^{h/h}$ strain. Further characterization of leukocyte subsets between the C57BL/6 and FVB/N strain may provide useful insights into how differences in leukocyte number or functions may underlie the low blood pressure phenotype of the C57BL/6 $c\text{-}myb^{h/h}$ mouse.

3.7 Acknowledgements

3.8 Sources of Funding

This work was supported in part by Canadian Institutes of Health Research (CIHR) operating grant MOP-136850 (to M.H.). M.H. is a Career Investigator of the Heart and Stroke Foundation
of Ontario (CI5503). E.A.S was supported by a CIHR Small Health Organization Partnership Program Priority Area: Hypertension Doctoral Research Award, Ontario Graduate Scholarship and a Meredith & Malcolm Silver Scholarship in Cardiovascular Studies.

3.9 Disclosures

The authors declare no conflicts of interest.
3.10 Figure legends

Fig. 1: c-myb regulates blood pressure homeostasis

Assessment of blood pressure was performed on c-myb<sup>WT</sup> and c-myb<sup>h/h</sup> mice. Invasive hemodynamic assessment found decreased aortic systolic and diastolic blood pressure in c-myb<sup>h/h</sup> mice (A). Left ventricular systolic blood pressure and heart rate were also decreased in c-myb<sup>h/h</sup> mice (B). Radiotelemetric hemodynamic assessment of unanesthetized, unrestrained mice demonstrates c-myb<sup>h/h</sup> mice have decreased blood pressure assessed over a three-day period (C). Averaged hourly data of radioteneleetric data (D). Error bars represent SEM. (A, B) N=14 mice/genotype. (C, D) N = 10 mice/genotype. (A, B ) Student’s t-tests and (D) Two-way repeated measures ANOVA were performed. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001 vs c-myb<sup>WT</sup>. (C,D) Open and shaded bar below x-axis denotes photoperiod (open – day; shaded – night).

Fig. 2: c-myb<sup>h/h</sup> arteries have normal VSMC content and gene expression

Common carotid arteries from c-myb<sup>WT</sup> and c-myb<sup>h/h</sup> mice were stained with haematoxylin (H+E), Movat’s pentachrome (Movat), Massons trichrome (Masson) and picrosirius red (PSR) staining and imaged under brightfield or polarized light microscopy (A). Medial smooth muscle area (B) and cellularity (C) was quantified; no differences were found between c-myb<sup>WT</sup> and c-myb<sup>h/h</sup> mice. Expression of vascular smooth muscle contractile genes found no differences between c-myb<sup>WT</sup> and c-myb<sup>h/h</sup> arteries (D). c-myb<sup>h/h</sup> aortas had higher tensile strength than c-myb<sup>WT</sup> aortas (E). Error bars represent SEM. (B, C) N=14 mice/genotype. (D, E) N = 5-8 mice/genotype. (A) Two-way repeated measures ANOVA and (C, D, E) Student’s t-tests were performed. * P < 0.05 vs c-myb<sup>WT</sup>. 
Fig. 3: *c-myb*<sup>h/h</sup> resistance arteries do not have contractile defects.

No differences in resting vessel diameter and wall thickness were observed in mesenteric arteries (A, B). Passive diameter of *c-myb*<sup>h/h</sup> arteries was smaller than *c-myb*<sup>wt</sup> arteries at pressures >60 mmHg (C). Myogenic response (D, E) and phenylephrine-induced vasoconstriction (F) of *c-myb*<sup>h/h</sup> arteries did not differ from *c-myb*<sup>wt</sup> vessels. *c-myb*<sup>h/h</sup> arteries had defective acetylcholine-mediated vasodilation (G), which was overcome in the presence of sodium nitroprusside (SNP), a NO-donor (H). Error bars represent SEM. N=8-12 vessels from 4-6 mice /genotype. Two-way repeated measures ANOVA were performed. * P < 0.05 vs *c-myb*<sup>WT</sup>.

Fig. 4: *c-myb*<sup>h/h</sup> bone marrow confers low blood pressure

To determine if BM-derived cell populations mediate the low blood pressure phenotype of *c-myb*<sup>h/h</sup> mice, reciprocal BMTs were performed. After 8 weeks of reconstitution, it was found that *c-myb*<sup>wt</sup> mice reconstituted with *c-myb*<sup>h/h</sup> BM had significantly reduced systolic and diastolic blood pressure compared to *c-myb*<sup>WT</sup> mice reconstituted with BM from *c-myb*<sup>WT</sup> donors, matching that of *c-myb*<sup>h/h</sup> mice reconstituted with *c-myb*<sup>h/h</sup> BM (A). *c-myb*<sup>h/h</sup> mice reconstituted with *c-myb*<sup>WT</sup> BM had increased blood pressure matching that of *c-myb*<sup>WT</sup> mice reconstituted with *c-myb*<sup>WT</sup> BM (A). Left ventricular systolic BP data matched those of the aortic blood pressure readings (B). Error bars represent SEM. N=8-10 mice/group. Two-way repeated measures ANOVA were performed. * P < 0.05; ** P < 0.01; *** P < 0.001. *WT* represents *c-myb*<sup>WT</sup>; *h/h* represents *c-myb*<sup>h/h</sup>.

Fig. 5: *c-myb*<sup>h/h</sup> mice have decreased B-cell numbers in kidney
To determine if \(c-myb^{h/h}\) mice have alterations in renal leukocyte populations, flow cytometry of \(c-myb^{WT}\) and \(c-myb^{h/h}\) kidneys was performed. \(c-myb^{h/h}\) mice had decreased numbers of B220+ B-cells compared to \(c-myb^{WT}\) mice, while neutrophil, macrophage, dendritic cells and T-cell numbers were comparable to those of \(c-myb^{WT}\) mouse kidneys. Error bars represent SEM. \(N=8\)-10 mice/genotype. Student’s t-tests were performed. *** \(P < 0.001\) vs \(c-myb^{WT}\).

**Fig. 6: c-myb^{h/h} mice are resistant to DOCA-salt induced hypertension.**

9-10 week old \(c-myb^{wt}\) and \(c-myb^{h/h}\) mice underwent unilateral nephrectomy and were implanted with a 21 day slow release deoxycorticosterone acetate (DOCA) pellet (Innovative Research of America) and given 1% saline for drinking water or control (CTRL) pellet and normal drinking water. Blood pressure was determined 3 weeks post-nephrectomy and pellet implantation by invasive hemodynamic measurement. \(c-myb^{h/h}\) mice are refractory to hypertensive stimuli and do not develop as severe hypertension as \(c-myb^{WT}\) mice. Error bars represent SEM. \(N=7\)/group. 2-way repeated measures ANOVA was performed; * \(p < 0.05\), ** \(p < 0.01\)
3.11 Figures

Figure 3.1: *c-myb* regulates blood pressure homeostasis
Figure 3-2: *c-myb*<sup>h/h</sup> arteries have normal VSMC content and gene expression
Figure 3-3: *c-myb*<sup>h/h</sup> resistance arteries do not have contractile defects.
Figure 3-4: \(c-myb^{h/h}\) bone marrow confers low blood pressure
Figure 3-5: c-myb<sup>h/h</sup> mice have altered renal B-cell numbers
Figure 3-6: c-myb<sup>h/h</sup> are resistant to DOCA-salt induced hypertension.
3.12 Tables

3-1: Table 1: *c-myb*<sup>h/h</sup> mice do not have cardiac functional defects

<table>
<thead>
<tr>
<th></th>
<th><em>c-myb&lt;sup&gt;WT&lt;/sup&gt;</em></th>
<th><em>c-myb&lt;sup&gt;h/h&lt;/sup&gt;</em></th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVIDs (mm)</td>
<td>2.07±0.08</td>
<td>2.34±0.15</td>
<td><em>P</em> = 0.1119 (NS)</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.88±0.07</td>
<td>4.12±0.13</td>
<td><em>P</em> = 0.1276 (NS)</td>
</tr>
<tr>
<td>EF (%)</td>
<td>83.4±1.3</td>
<td>79.7±2.3</td>
<td><em>P</em> = 0.1649 (NS)</td>
</tr>
<tr>
<td>FS (%)</td>
<td>47.0±1.5</td>
<td>43.6±2.6</td>
<td><em>P</em> = 0.2114 (NS)</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>501.1±14.1</td>
<td>483.1±24.0</td>
<td><em>P</em> = 0.5171 (NS)</td>
</tr>
<tr>
<td>CO (L/min)</td>
<td>0.061±0.003</td>
<td>0.067±0.006</td>
<td><em>P</em> = 0.3874 (NS)</td>
</tr>
<tr>
<td>SV (mL/beat)</td>
<td>0.123±0.006</td>
<td>0.139±0.011</td>
<td><em>P</em> = 0.1822 (NS)</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.717±0.020</td>
<td>0.739±0.028</td>
<td><em>P</em> = 0.5281 (NS)</td>
</tr>
<tr>
<td>LVFWd (mm)</td>
<td>0.790±0.024</td>
<td>0.841±0.029</td>
<td><em>P</em> = 0.1899 (NS)</td>
</tr>
</tbody>
</table>

Echocardiography was performed on 10-12 week old *c-myb*<sup>WT</sup> and *c-myb*<sup>h/h</sup> mice under 1% isoflurane anesthesia. No differences in cardiac function were found by echocardiography.

Values are means ± SEM. N = 14 *c-myb*<sup>WT</sup>, 13 *c-myb*<sup>h/h</sup> mice. Stroke volume (SV) was calculated as SV = CO/HR. Student’s t-tests were performed. LVIDd – Left ventricular Internal Dimension at diastole; LVIDs – Left Ventricular Internal Dimension at systole; EF – Ejection Fraction; FS – Fractional Shortening; HR – Heart Rate; CO – Cardiac Output; IVSd – Interventricular Septum at diastole; LVFWd – Left Ventricular Free Wall at diastole; NS – Not significant.
3-2: Table 2: c-myb<sup>h/h</sup> mice do not have significantly altered plasma cytokine expression

<table>
<thead>
<tr>
<th>Cytokine (pg/ml)</th>
<th>c-myb&lt;sup&gt;WT&lt;/sup&gt;</th>
<th>c-myb&lt;sup&gt;h/h&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1a</td>
<td>4.840 ± 0.376</td>
<td>9.718± 2.877</td>
<td>P = 0.2516 (NS)</td>
</tr>
<tr>
<td>IL-1b</td>
<td>182.2 ± 28.6</td>
<td>188.5 ± 45.1</td>
<td>P = 0.9078 (NS)</td>
</tr>
<tr>
<td>IL-2</td>
<td>38.10 ± 6.04</td>
<td>37.68 ± 8.53</td>
<td>P = 0.9692 (NS)</td>
</tr>
<tr>
<td>IL-3</td>
<td>9.80 ± 1.45</td>
<td>13.74 ± 5.59</td>
<td>P = 0.5082 (NS)</td>
</tr>
<tr>
<td>IL-4</td>
<td>10.98 ± 1.09</td>
<td>9.66 ± 1.32</td>
<td>P = 0.4532 (NS)</td>
</tr>
<tr>
<td>IL-5</td>
<td>32.14 ± 4.12</td>
<td>29.14 ± 5.28</td>
<td>P = 0.6625 (NS)</td>
</tr>
<tr>
<td>IL-6</td>
<td>18.00 ± 2.3</td>
<td>18.70 ± 8.23</td>
<td>P = 0.9317 (NS)</td>
</tr>
<tr>
<td>IL-9</td>
<td>311.5 ± 83.48</td>
<td>303.6 ± 73.7</td>
<td>P = 0.9443 (NS)</td>
</tr>
<tr>
<td>IL-10</td>
<td>253.9 ± 45.68</td>
<td>229.1 ± 70.8</td>
<td>P = 0.7736 (NS)</td>
</tr>
<tr>
<td>IL-12(p40)</td>
<td>127.4 ± 6.6</td>
<td>131.3 ± 16.0</td>
<td>P = 0.8223 (NS)</td>
</tr>
<tr>
<td>IL-13</td>
<td>625.4 ± 114.4</td>
<td>580.8 ± 160.5</td>
<td>P = 0.8245 (NS)</td>
</tr>
<tr>
<td>IL-17</td>
<td>50.06 ± 7.80</td>
<td>45.76 ± 9.17</td>
<td>P = 0.7266 (NS)</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>49.14 ± 33.15</td>
<td>92.66 ± 80.5</td>
<td>P = 0.6434 (NS)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>57.90 ± 5.46</td>
<td>88.74 ±21.28</td>
<td>P = 0.1594 (NS)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>136.1 ± 21.9</td>
<td>151.4 ± 28.7</td>
<td>P = 0.6858 (NS)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>7.960 ± 2.137</td>
<td>8.23 ± 5.77</td>
<td>P = 0.9624 (NS)</td>
</tr>
<tr>
<td>KC</td>
<td>51.71 ± 8.10</td>
<td>78.66 ± 8.21 *</td>
<td>P = 0.0403 *</td>
</tr>
<tr>
<td>MCP-1</td>
<td>167.5 ± 26.0</td>
<td>169.6 ± 58.0</td>
<td>P = 0.9748 (NS)</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>11.46 ± 1.125</td>
<td>12.68 ± 1.824</td>
<td>P = 0.5774 (NS)</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>1516 ± 247</td>
<td>1870 ± 982</td>
<td>P = 0.7141 (NS)</td>
</tr>
<tr>
<td>RANTES</td>
<td>14.74 ± 1.95</td>
<td>19.11 ± 4.77</td>
<td>P = 0.4138 (NS)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1117 ± 134</td>
<td>1353 ± 485</td>
<td>P = 0.6486 (NS)</td>
</tr>
</tbody>
</table>

Blood was collected by cardiac puncture from 10-12 week old c-myb<sup>WT</sup> and c-myb<sup>h/h</sup> mice in 200 ul EDTA microvettles, and spun at 10<sup>5</sup>g for 10 minutes to collect plasma. BioRad Bio-Plex assay was run according to BioRd Mouse Cytokine/chemokine protocol. Values are means ± SEM. N= 7/group. Student’s t-test were performed; * p<0.05; NS – Not significant.
3-3: Table 3: $c$-myb$^{h/h}$ mice have altered kidney function.

<table>
<thead>
<tr>
<th></th>
<th>$c$-myb$^{WT}$</th>
<th>$c$-myb$^{h/h}$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma urea (mMol/L)</td>
<td>9.67 ± 0.38</td>
<td>8.96 ± 0.21</td>
<td>P = 0.1564 (NS)</td>
</tr>
<tr>
<td>Plasma creatinine (μMol/L)</td>
<td>16.50 ± 0.85</td>
<td>16.00 ± 1.58</td>
<td>P = 0.7679 (NS)</td>
</tr>
<tr>
<td>Plasma sodium (mMol/L)</td>
<td>145.2 ± 0.5</td>
<td>146.2 ± 0.7</td>
<td>P = 0.2538 (NS)</td>
</tr>
<tr>
<td>Plasma potassium (mMol/L)</td>
<td>7.22 ± 0.22</td>
<td>6.62 ± 0.25 *</td>
<td>P = 0.0388</td>
</tr>
<tr>
<td>Plasma chloride (mMol/L)</td>
<td>108.2 ± 0.9</td>
<td>106.8 ± 1.1</td>
<td>P = 0.3522 (NS)</td>
</tr>
<tr>
<td>Urine urea (mMol/L)</td>
<td>1039 ± 103</td>
<td>761 ± 32 *</td>
<td>P = 0.0276</td>
</tr>
<tr>
<td>Urine creatinine (μMol/L)</td>
<td>2517 ± 267</td>
<td>2088 ± 265</td>
<td>P = 0.2802 (NS)</td>
</tr>
<tr>
<td>Urine sodium (mMol/L)</td>
<td>93.50 ± 7.49</td>
<td>85.83 ± 6.24</td>
<td>P = 0.4496 (NS)</td>
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<tr>
<td>Urine potassium (mMol/L)</td>
<td>200.2 ± 14.1</td>
<td>161.7 ± 7.5 *</td>
<td>P = 0.0362</td>
</tr>
<tr>
<td>Urine chloride (mMol/L)</td>
<td>153.8 ± 13.6</td>
<td>127.2 ± 9.2</td>
<td>P = 0.1347 (NS)</td>
</tr>
<tr>
<td>Urine volume (ml)</td>
<td>1.97 ± 0.25</td>
<td>3.03 ± 0.30 *</td>
<td>P = 0.0215</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>0.198 ± 0.01</td>
<td>0.271 ± 0.02 *</td>
<td>P = 0.0107</td>
</tr>
<tr>
<td>Fractional excretion of urea</td>
<td>0.714 ± 0.04</td>
<td>0.611 ± 0.03 *</td>
<td>P = 0.0792</td>
</tr>
</tbody>
</table>

Blood was collected by cardiac puncture from 10-12 week old $c$-myb$^{WT}$ and $c$-myb$^{h/h}$ mice in 200 ul EDTA microvettes. Samples were analyzed on a Beckman AU480 Biochemistry Analyzer.

Values are means ± SEM. N = 5 $c$-myb$^{WT}$, 6 $c$-myb$^{h/h}$ mice. Student’s t-test were performed; * p<0.05; NS – Not significant.
3-4: Table 4: c-myb<sup>h/h</sup> bone marrow transplantation confers altered kidney function.

<table>
<thead>
<tr>
<th></th>
<th>c-myb&lt;sup&gt;WT&lt;/sup&gt; &gt; WT</th>
<th>c-myb&lt;sup&gt;h/h&lt;/sup&gt; &gt; WT</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma urea (mMol/L)</td>
<td>9.12 ± 0.11</td>
<td>9.28 ± 0.36</td>
<td>P = 0.6646 (NS)</td>
</tr>
<tr>
<td>Plasma creatinine (µMol/L)</td>
<td>20.67 ± 1.71</td>
<td>21.00 ± 1.27</td>
<td>P = 0.8784 (NS)</td>
</tr>
<tr>
<td>Plasma sodium (mMol/L)</td>
<td>151.3 ± 0.8</td>
<td>153.0 ± 1.2</td>
<td>P = 0.2781 (NS)</td>
</tr>
<tr>
<td>Plasma potassium (mMol/L)</td>
<td>7.73 ± 0.25</td>
<td>6.68 ± 0.20*</td>
<td>P = 0.0085</td>
</tr>
<tr>
<td>Plasma chloride (mMol/L)</td>
<td>111.7 ± 0.7</td>
<td>112.8 ± 1.5</td>
<td>P = 0.5020 (NS)</td>
</tr>
<tr>
<td>Urine urea (mMol/L)</td>
<td>617.7 ± 66.7</td>
<td>736.8 ± 24.9</td>
<td>P = 0.1252 (NS)</td>
</tr>
<tr>
<td>Urine creatinine (µMol/L)</td>
<td>1283 ± 132</td>
<td>1343 ± 62.2</td>
<td>P = 0.6883 (NS)</td>
</tr>
<tr>
<td>Urine sodium (mMol/L)</td>
<td>69.83 ± 3.42</td>
<td>78.67 ± 1.69*</td>
<td>P = 0.0430</td>
</tr>
<tr>
<td>Urine potassium (mMol/L)</td>
<td>128.5 ± 1.5</td>
<td>138.5 ± 3.2*</td>
<td>P = 0.0362</td>
</tr>
<tr>
<td>Urine chloride (mMol/L)</td>
<td>107.2 ± 4.8</td>
<td>119.8 ± 5.8</td>
<td>P = 0.0555 (NS)</td>
</tr>
<tr>
<td>Urine volume (ml)</td>
<td>3.13 ± 0.20</td>
<td>3.875 ± 0.35</td>
<td>P = 0.0942</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>0.274 ± 0.03</td>
<td>0.393 ± 0.05</td>
<td>P = 0.0559</td>
</tr>
<tr>
<td>Fractional excretion of urea</td>
<td>1.08 ± 0.07</td>
<td>1.27 ± 0.14</td>
<td>P = 0.2468 (NS)</td>
</tr>
</tbody>
</table>

C57BL/6 (WT) mice were transplanted with c-myb<sup>WT</sup> or c-myb<sup>h/h</sup> BM, let recover for 8 weeks 24-hour urinary output was collected using mice housed singly in metabolic cages. Blood was collected by cardiac puncture mice in 200 ul EDTA microvetties. Samples were analyzed on a Beckman AU480 Biochemistry Analyzer. Values are means ± SEM. N = 6/group. Student’s t-test were performed; * p<0.05; NS – Not significant.
3.13 Supplemental tables

3-5: Supplemental Table 1: $c$-$myb^{h/h}$ mice have differences in plasma biochemistry.

<table>
<thead>
<tr>
<th></th>
<th>$c$-$myb^{WT}$</th>
<th>$c$-$myb^{h/h}$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (g/L)</td>
<td>33.0 ± 1.7</td>
<td>32.8 ± 2.1</td>
<td>$P = 0.9110$ (NS)</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>77.3 ± 3.2</td>
<td>111.5 ± 5.3 *</td>
<td>$P = 0.004$</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>156.7 ± 7.9</td>
<td>28.3 ± 0.9</td>
<td>$P = 0.1192$ (NS)</td>
</tr>
<tr>
<td>Amylase (U/L)</td>
<td>911.3 ± 92.3</td>
<td>827.0 ± 36.5</td>
<td>$P = 0.4888$ (NS)</td>
</tr>
<tr>
<td>Total Bilirubin (μMol/L)</td>
<td>4.7 ± 0.3</td>
<td>5.3 ± 0.5</td>
<td>$P = 0.3979$ (NS)</td>
</tr>
<tr>
<td>Urea nitrogen (mMol/L)</td>
<td>7.5 ± 0.4</td>
<td>8.8 ± 0.5</td>
<td>$P = 0.1162$ (NS)</td>
</tr>
<tr>
<td>Total Calcium (mMol/L)</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>$P = 0.4044$ (NS)</td>
</tr>
<tr>
<td>Phosphorus (mMol/L)</td>
<td>3.0 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>$P = 0.9082$ (NS)</td>
</tr>
<tr>
<td>Creatinine</td>
<td>&lt;18</td>
<td>&lt;18</td>
<td>$P = 0.9958$ (NS)</td>
</tr>
<tr>
<td>Glucose (mMol/L)</td>
<td>16.9 ± 1.1</td>
<td>16.9 ± 1.0</td>
<td>$P = 0.1360$ (NS)</td>
</tr>
<tr>
<td>Sodium (mMol/L)</td>
<td>155.0 ± 1.0</td>
<td>152.5 ± 1.7</td>
<td>$P = 0.0087$</td>
</tr>
<tr>
<td>Potassium (mMol/L)</td>
<td>8.3 ± 0.2</td>
<td>6.5 ± 0.3 *</td>
<td>$P = 0.5327$ (NS)</td>
</tr>
<tr>
<td>Total Protein (g/L)</td>
<td>52.0 ± 2.0</td>
<td>53.5 ± 1.3</td>
<td>$P = 0.3840$ (NS)</td>
</tr>
<tr>
<td>Globulin (g/L)</td>
<td>18.7 ± 0.7</td>
<td>20.5 ± 1.6</td>
<td>$P = 0.9110$ (NS)</td>
</tr>
</tbody>
</table>

Blood was collected by cardiac puncture from 10-12 week old $c$-$myb^{WT}$ and $c$-$myb^{h/h}$ mice in 200 ul EDTA microvetttes. Samples were run on a Hemavet complete diagnostic profile panel.

Values are means ± SEM. N = 3 $c$-$myb^{WT}$, 4 $c$-$myb^{h/h}$ mice. Student’s t-test were performed; * p<0.05; NS – Not significant.
3-6: Supplemental Table 2: Plasma biochemistry of KC treated mice.

<table>
<thead>
<tr>
<th></th>
<th>0 pg/kg/min</th>
<th>100 pg/kg/min</th>
<th>1000 pg/kg/min</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (g/L)</td>
<td>34.7 ± 0.7</td>
<td>37.7 ± 0.9</td>
<td>34.8 ± 1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>69.2 ± 3.7</td>
<td>84.7 ± 7.2</td>
<td>78.6 ± 2.0</td>
<td>NS</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/L)</td>
<td>120.8 ± 41.3</td>
<td>53.3 ± 6.9</td>
<td>119.7 ± 28.6</td>
<td>NS</td>
</tr>
<tr>
<td>Amylase (U/L)</td>
<td>757.8 ± 22.2</td>
<td>884.0 ± 29.8*</td>
<td>859.0 ± 24.5*</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Total Bilirubin (μMol/L)</td>
<td>4.2 ± 0.2</td>
<td>4.7 ± 0.3</td>
<td>3.8 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Urea nitrogen (mMol/L)</td>
<td>9.9 ± 0.5</td>
<td>8.5 ± 0.2</td>
<td>9.4 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Total Calcium (mMol/L)</td>
<td>2.5 ± 0.0</td>
<td>2.5 ± 0.0</td>
<td>2.5 ± 0.0</td>
<td>NS</td>
</tr>
<tr>
<td>Phosphorus (mMol/L)</td>
<td>2.3 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine</td>
<td>31.7 ± 4.2</td>
<td>20.0 ± 1.5</td>
<td>27.5 ± 4.4</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mMol/L)</td>
<td>14.7 ± 0.5</td>
<td>15.1 ± 0.6</td>
<td>14.2 ± 0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Sodium (mMol/L)</td>
<td>143.2 ± 0.9</td>
<td>141.7 ± 0.9</td>
<td>144.4 ± 1.0</td>
<td>NS</td>
</tr>
<tr>
<td>Potassium (mMol/L)</td>
<td>4.7 ± 0.2</td>
<td>3.6 ± 0.3</td>
<td>3.8 ± 0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Total Protein (g/L)</td>
<td>53.5 ± 0.7</td>
<td>53.3 ± 0.7</td>
<td>54.3 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Globulin (g/L)</td>
<td>18.8 ± 1.1</td>
<td>15.7 ± 0.3</td>
<td>19.5 ± 0.6</td>
<td>NS</td>
</tr>
</tbody>
</table>

Blood was collected by cardiac puncture from 12 week old C57BL/6 mice treated with KC (0, 100 or 1000 pg/kg/min) for 14 days by osmotic pump infusion. Samples were run on a Hemavet complete diagnostic profile panel. Changes in blood biochemistry consistent with c-myb<sup>−/−</sup> mice were not found. N = 3-8/group. Values are means ± SEM. One-way ANOVA were performed; * p<0.05 vs 0 pg/kg/min; NS – Not significant.
3.14 Supplemental Figure Legends

**Supplemental Fig. 1: KC does not regulate blood pressure.**

Cytokine array results were validated using KC ELISA. $c\text{-}myb^{h/h}$ mice have higher serum KC (A). To determine if KC directly regulates blood pressure, C57BL/6 mice were treated with 100 or 1000 ng/kg/min KC for 14 days. KC treatment did not affect blood pressure (B). Error bars represent SEM. (A) N=12/genotype; (B) 3-8/group. (B) Student’s t-test and (A) one-way ANOVA were performed; * P = 0.0051 versus $c\text{-}myb^{WT}$(WT).

**Supplemental Fig. 2: $c\text{-}myb^{h/h}$ BMT does not affect vascular function.**

Perfusion myography was performed on mesenteric arteries from C57BL/6 mice transplanted with either $c\text{-}myb^{wt}$(WT>WT) or $c\text{-}myb^{h/h}$(Hypo>WT) bone marrow. BMT from $c\text{-}myb^{wt}$ or $c\text{-}myb^{h/h}$ did not affect myogenic tone (A) or phenylephrine (PE)-induced contraction (B) post-BMT. Error bars represent SEM. N=6-11 arteries from 2-3 mice/group. (A) Two-way repeated measures ANOVA and (B) Student’s t-test were performed.

**Supplementary Fig. 3: FVB/N $c\text{-}myb^{h/h}$ mice have normal blood pressure.**

C57BL/6 $c\text{-}myb^{h/h}$ mice were backcrossed to a FVB/N background for 10 generations. Blood pressure of 12 week old FVB/N $c\text{-}myb^{WT}$ and FVB/N $c\text{-}myb^{h/h}$ with invasive hemodynamics as described previously. $c\text{-}myb^{h/h}$ on a FVB/N genetic background have normal aortic (A) and left ventricular (B) blood pressure. Error bars represent SEM. N = 17 FVB/N $c\text{-}myb^{WT}$(WT), 9 FVB/N $c\text{-}myb^{h/h}$(Hypo). (A, B) Student’s t-tests and (C) two-way ANOVA were performed; (A, B) ** P < 0.01 versus FVB/N $c\text{-}myb^{WT}$. (C) * P < 0.05; ** P < 0.01; **** P < 0.0001.
Figure 3-6:
Supplemental Fig. 1: KC does not regulate blood pressure.
Figure 3-7:
Supplemental Fig. 2: *c-myb*<sup>h/h</sup> BMT does not affect vascular function.
Figure 3-8:
Supplementary Fig. 3: FVB/N c-myb<sup>h/h</sup> mice have normal blood pressure.
CHAPTER 4:

4 c-Myb suppresses IgM+ B cell lymphopoiesis in atherosclerosis
c-Myb critically regulates B lymphocyte responses in atherosclerosis

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*,† authors contributed equally
4.1 Abstract:

Studies in mice and humans demonstrate a causal role for B cells in atherosclerosis. However, the potential design of B cell-targeted therapies may be complicated by observations that B cell subsets have opposing roles in atherogenesis; some subsets are atherogenic, while others appear to be protective. Our observation that bone marrow cells isolated from atherosclerotic mice express increased levels of c-Myb - a crucial regulator of hematopoiesis - led us to investigate the role of c-Myb in atherosclerosis progression. Using mice harboring a c-Myb hypomorphic allele, here we show that attenuated c-Myb activity dramatically decreases the severity of atherosclerosis by directly modulating atheroprotective IgM producing B lymphocytes. Protection in c-Myb-targeted animals was associated not only with decreased survival of B-cell subsets known to be atherogenic, but also increased production of athero-protective IgM antibodies. We also report for the first time that c-Myb has opposing effects on the survival of antibody producing plasma cells and their B cell predecessors. These data have implications in the therapeutic targeting of B-cells since it identifies a potential mechanistic pathway that circumvents the complexity of the B cell biology.
4.2 Introduction

In addition to inflammation in developing plaques, atherosclerosis develops through increased production of inflammatory cells in the bone marrow and spleen [235, 387-389]. The molecular mechanisms that control hematopoiesis in cardiovascular disease, however, remain incompletely understood. Several studies have indicated regulatory roles for B lymphocytes in the progression of atherosclerotic disease [294, 390], yet opposing roles of various B cell subsets in the development of atherosclerosis complicate the understanding of the overall role of B cells in atherosclerosis [315, 316, 391-393]. The two main subsets of B cells, B1 and B2 cells, are developmentally and functionally distinct. B1 cells produce the majority of circulating IgM [307], and are further subdivided into B1a and B1b B cells based on CD5 expression. CD5+ B1a cells produce natural IgM antibodies in response to non-antigenic stimuli, of which a substantial proportion recognize oxidation-specific epitopes, such as those found on oxidized low density lipoproteins, and reduce the development of atherosclerosis [308-310]. The primary mechanisms by which IgM is atheroprotective are via the prevention of phagocytosis of ox-LDL by macrophages by masking antigen epitopes, preventing the development of foam cells [394], and also by enhancing the phagocytosis of apoptotic foam cells, thereby reducing the necrotic core burden [391, 395, 396]. CD5- B1b cells also produce IgM in both antigen-independent and antigen-dependent manners and reduce the severity of atherosclerosis [314]. Marginal zone and follicular B2 cells, which represent the majority of B cells, have been primarily implicated as pro-atherogenic via the pathogenic activation of T cells [293, 315] and exacerbation of arterial
inflammation [316, 317]. IgM antibodies, particularly those directed toward modified lipoproteins, mediate atheroprotection [311, 312], while the role of IgG is less clear [313].

Among the pleiotropic roles the transcription factor c-Myb has in hematopoiesis, it has been shown to regulate several stages of B cell development [296, 304, 397, 398], including the migration of antibody secreting plasma cells from the spleen to the bone marrow [399]. However, it is currently unknown if c-myb regulates B lymphocytes in the pathogenesis of atherosclerosis. To determine whether c-Myb affects atherosclerosis severity, we studied mice harboring a c-myb hypomorphic allele (c-myb<sup>h/h</sup>: M303V) identified in an ENU-mutagenesis screen [15]. Having only 50% c-Myb activity, bypassing the lethality of c-myb<sup>−/−</sup> embryos [7, 31], c-myb<sup>h/h</sup> mice were crossed with <i>Ldlr</i><sup>−/−</sup> animals to create atherosclerosis-prone <i>Ldlr</i><sup>−/−</sup> c-myb<sup>h/h</sup> mice. Protection in c-Myb-targeted animals is due not only to decreased survival of atherogenic B2 cell subsets, but also increased production of athero-protective IgM antibodies.

### 4.3 Methods

**Animals.**

C57BL/6J (WT), low-density lipoprotein receptor–deficient (<i>Ldlr</i><sup>−/−</sup>; B6.129S7-Ldlr<sup>tm1Her</sup>/J), and mice homozygous for the Ighm<sup>tm1Cgn</sup> targeted mutation (μMT) (B6.129s2-Igh-6<sup>tm1Cgn</sup>/J) were purchased from Jackson Laboratories. c-myb<sup>h/h</sup> animals were derived as previously described [15], and subsequently backcrossed with <i>Ldlr</i><sup>−/−</sup> mice to generate <i>Ldlr</i><sup>−/−</sup> c-myb<sup>h/h</sup> mice. All mice used in the study were male. All protocols were approved by the Animal Resource Centre, University Health Network (AUP#1032, 1034, 1605, 2092).
Animal models and in vivo interventions.

High cholesterol diet consumption (HCD). At 6–8 weeks of age, Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice were placed on an HCD (20% fat by weight and 1.25% cholesterol; D12108C Research Diets Inc., New Brunswick, NJ) or remained on a chow diet for 12 weeks.

Bone marrow chimeras.

Ldlr<sup>−/−</sup> mice were lethally irradiated (950 cGy) and reconstituted with either wild type (wt) or c-myb<sup>h/h</sup> bone marrow. Briefly, bone marrow was harvested from femurs and tibias of donors; 2.0 x10<sup>6</sup> total cells, re-suspended in PBS, were injected intravenously via tail vein injection into recipients. Transplant recipients were allowed to recover for 8 weeks before starting HCD. For the generation of mixed chimeras, Ldlr<sup>−/−</sup> mice were lethally irradiated and reconstituted with a 50%:50% mix of bone marrow cells from c-myb<sup>h/h</sup> and μMT mice.

Cells.

Peripheral blood for flow cytometry-based analysis was collected by cardiac puncture using a 50 mM EDTA solution as an anticoagulant. Erythrocytes were lysed using BD FACS Lysing Solution (BD Biosciences). The total white blood cell count was determined by preparing a 1:10 dilution of (undiluted) peripheral blood obtained from the orbital sinus using heparin-coated capillary tubes in RBC Lysis Buffer (BioLegend). After organ harvest, single-cell suspensions were obtained as follows. For bone marrow, the femur and tibia of one leg were crushed with mortar and pestle in ice-cold 1x PBS and filtered through a 40-μm-nylon mesh. Spleens were homogenized through a 40-μm-nylon mesh, after which erythrocyte lysis was performed using RBC Lysis Buffer (BioLegend). Para-aortic (lumbar) lymph nodes were homogenized through a
40-μm-nylon mesh. Aortas were digested (from the root to the iliac bifurcation) according to a
method previously published [234]. This procedure involves perfusion of the aorta (with 20 ml
PBS) before digestion. Aortic tissue was cut in small pieces and subjected to enzymatic digestion
with 450 U ml\(^{-1}\) collagenase I, 125 U ml\(^{-1}\) collagenase XI, 60 U ml\(^{-1}\) DNase I and 60 U ml\(^{-1}\)
hyaluronidase (Sigma-Aldrich, St. Louis, MO) for 1 h at 37 °C while shaking. After digestion,
tissue samples were passed through a 40-μm-nylon mesh filter.

**RNA isolation and qRT-PCR analysis**

Single cell suspensions from spleen and bone marrow were prepared as above. To fractionate
B220 positive and B220 negative cells in the bone marrow, magnetic cell sorting (MACS) was
performed. Bone marrow cells were washed 3x in 1x PBS, then re-suspended in FACS buffer
(1x PBS, 0.5% BSA). Cells were labelled with anti-B220-FITC (BD Biosciences 553088) for 15
minutes on ice, washed in 1x PBS, and re-suspended in FACS buffer. Stained cells were then
incubated with anti-FITC micro-beads (Miltenyi biotec 130-042-701) for 30 minutes on ice,
washed in 1x PBS and re-suspended in FACS buffer. B220\(^+\) cells were isolated by positive
selection using LS columns (Miltenyi biotec 130-042-401). The B220\(^-\) fraction was collected
from flow through. RNA was extracted from cells using TRIzol reagent (Life Technologies
15596) according to manufacturer’s protocol. RNA concentration was obtained using a
Nanodrop ND100 spectrophotometer. cDNA was synthesized using qScript cDNA SuperMix
(Quanta BioSciences) according to manufacturer protocol. Transcripts were detected using
specific primers for c-myb (Forward: GCTGAAGAAGCTGGTGGAAC; Reverse:
CAACGCTTCGGACCATATTT) and Hprt (Forward: CTGGTGAAGGACCTCTCG;
Reverse: AACTTGCGCTCATCTTAGGC ) using SYBR Green Master (Roche) detected on a
LightCycler 480 (Roche). Hypoxanthine-guanine phosphoribosyltransferase (Hprt) was used as the housekeeping gene; values were compared using the $2^{-\Delta\Delta Ct}$ method. Samples were run in triplicate and averaged.

**Flow Cytometry**

For a complete list of antibodies used in this study, see Supplementary Table 1. Single cell preparations were stained for 30 min on ice unless otherwise noted before being fixed with Cytofix buffer (BD Biosciences). For cell viability/apoptosis analysis, Annexin V FITC apoptosis kit (BD Pharmingen 556547) was used according to manufacturer’s protocols. For cell cycle analysis, cells were fixed overnight in ice-cold ethanol (95%), followed by staining with FxCycle Violet (Life Technologies F10347). Intracellular IgM, IgG1, and IgG2a/b staining was performed as previously shown [400]. Data were acquired on a LSR II (BD Biosciences) and analyzed using FlowJo software (v.10; Tree Star). Positive and negative gating strategies were determined using isotype controls unless otherwise stated.

**Histology**

To determine aortic sinus lesion area, the aortic root and ~0.5mm of the ascending aorta was carefully dissected and fixed in 4% paraformaldehyde, cryoprotected in 20% sucrose in PBS before being embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek 4583), and flash frozen in isopentane on dry ice. Serial 5-μm transverse sections were made generating ~50-100 slices spanning the entirety of the aortic root. Sections were stained with haematoxylin and eosin (H&E) at even intervals to span the entire aortic root. Masson’s
trichrome staining was performed on sections that captured the maximum lesion area to visualize collagen content of lesions. Brightfield images were taken on a Leica DMLB microscope, captured with an Infinity2-2 CCD camera (Lumenera). To determine lipid content in atherosclerotic plaques, en-face oil red O (ORO) was performed on aortic arches isolated from animals on 12 wks HCD. Briefly, aortas were perfused with 10 ml ice-cold PBS, then fixed in 4%PFA over night. Aortas were stained for 30 min with a working solution of 3:2 ORO (0.5% w/v in isopropanol; Sigma O0625) stain:ddH2O, then washed in 70% isopropanol to remove non-specific staining. Aortas were then trimmed, cut open along the greater curvature, and pinned to siliconized plates to expose the luminal surface. Images of en-face stained aortas were taken on a Leica M165 FC stereomicroscope. Blinded experimenters performed morphometric analysis of lesion areas with ImageJ software (NIH).

**Total anti-IgG/IgM and anti-CuOxLDL IgG/IgM ELISAs.**

ELISAs were performed on flash-frozen serum samples. To determine CuOxLDL-specific IgG and IgM levels, MaxiSorp 96-well plates (Nunc 44-2404-21) were coated with 2 μg/well low density lipoprotein (Alfa Aesar J65591) in PBS overnight at 4 C°. Wells were blocked with 5% BSA in PBS for 1 h at room temperature (RT). Serum samples, serially diluted in reagent diluents (to a final concentration of 0.5% BSA), were incubated for 2 h at RT with low-density lipoprotein coated plates. Wells were washed 4x with 1x PBS and incubated with either goat anti-mouse IgM (1:1000; Invitrogen 626800) or goat anti-mouse IgG (1:1000; Invitrogen A10535) for 1 h at RT. Following a wash with 1x PBS, wells were incubated with polyclonal rabbit anti-goat immunoglobulin conjugated to biotin (Dako E0466) for 1 h at RT, then streptavidin conjugated to horseradish peroxidase (1:200; R&D Systems 890803) for 30 min at
RT. Wells were washed with 1x PBS and incubated with BioFX TMB chromogenic substrate solution (SurModics TMBH-1000-01) for 10-15 min at RT. H2SO4 was added to quench the reaction and plates were read at 450 nm on a microplate reader (Bio-Tek EL800). Total IgG and IgM levels were measured using IgG total Ready-SET-Go (eBioscience 88-50400-22) and IgM total Ready-SET-Go (eBioscience 88-50470-22) kits according to the manufacturer’s protocol.

**Enzyme-Linked Immunospot (ELISPOT).**
IgM producing cells were quantified using MultiScreen IP filter plates (Milipore MSIPS4510) coated with anti-mouse IgM (Jacksom ImmunoResearch 115-005-075) incubated overnight with 2.5x105 cells/well in B-cell culture media. IgM⁺ spots were detected using HRP conjugated anti-IgM (Sigma Aldrich A-8786) and AEC chromogen (Sigma Aldrich AEC101) according to manufacturer protocol. IgM⁺ spots were quantified using an ELISPOT plate reader (ImmunoSpot 5.0.2, CTL, Shaker Heights, OH, USA).

**Cell Culture.**
B220⁺ cells were isolated from spleens of wt and c-myb<sup>h/h</sup> mice by magnetic-activated cell sorting (MACS) and cultured for 4 days in the presence or absence of LPS. (Milentyi Biotec). Purified cells were cultured at 1 x 10⁵/ml and stimulated with LPS (20 μg/ml, Sigma) for 4 days. Supernatants were removed and secreted IgM was measured by ELISA.

**Statistics**
Results are expressed as either mean±SD or mean±SEM. Statistical tests included unpaired Student’s t-tests, 1- and 2-way ANOVA followed by Tukey or Bonferroni corrections for multiple comparisons. P ≤ 0.05 was considered to denote significance.

4.4 Results

c-Myb regulates atherosclerosis

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis of Ldlr−/− mice fed either normal chow or a diet high in cholesterol (HCD) revealed increased levels of c-myb (Chow 0.081 ± 0.009 vs. HCD 0.109 ± 0.008; N = 9/group; P = 0.0326) in leukocytes isolated from bone marrow of HCD mice (Fig. 1a). To determine whether c-Myb affects atherosclerosis severity, we examined atherogenesis in atherosclerosis-prone Ldlr−/−c-myb+/− mice. Loss of c-Myb activity did not alter plasma cholesterol and triglycerides of HCD-fed animals (Fig. 1b), yet yielded substantial reduction in atherosclerotic lesions in the aortic root (Fig. 1c) and arch (Fig. 1d, Supplementary Fig. 1a). Ldlr−/−c-myb+/− atherosclerotic lesions had decreased collagen content (Fig. 1e), and flow cytometry-based cell profiling on lesions revealed fewer inflammatory cells (Fig. 1f, Supplementary Fig. 1b).

Leukocyte c-Myb activity promotes atherosclerosis

Because HCD consumption increased c-myb expression in bone marrow leukocytes, we next investigated the role of hematopoietic cell-derived c-Myb on atherosclerosis progression. Lethally irradiated Ldlr−/− mice underwent bone marrow transplantation and were reconstituted with either wild type (c-myb+/−>Ldlr−/−) or c-myb+/− (c-myb+/−>Ldlr−/−) bone marrow. c-myb+/−>Ldlr−
mice on HCD and had less severe atherosclerosis than mice transplanted with $c$-$myb^{wt}$ cells (Fig. 1g), demonstrating that c-Myb activity in hematopoietic cells alone can regulate atherogenesis.

c-Myb is critical for B-cell lymphopoiesis in atherosclerosis

As atherosclerosis progresses, the number of blood monocytes rises, and leukocytosis predicts cardiovascular events in humans [401, 402]. Consistent with these observations and compared to chow-fed animals, $Ldlr^{-/-}$ mice on HCD had increased circulating monocytes, and T and B lymphocytes (Fig. 1h). Similar increases in blood monocytes and T cells were observed in $Ldlr^{-/-}c$-$myb^{h/h}$ HCD mice, however, $c$-$myb^{h/h}$ animals displayed profoundly diminished B cell responses, regardless of diet (Fig. 1h, Supplementary Fig. 1c). Decreased numbers of B cells were also observed in the spleen, bone marrow, and para-aortic lymph nodes, but not peritoneal cavities of $Ldlr^{-/-}c$-$myb^{h/h}$ HCD mice (Fig. 2a-d, Supplementary Fig. 2a). Flow cytometry indicated decreased numbers of B cell progenitors in the bone marrow of $Ldlr^{-/-}c$-$myb^{h/h}$ HCD mice including early Fraction A cells, pro- and pre-B cells, and immature Fraction E cells (Fig. 2a, c), consistent with known roles for c-Myb in B cell development [15, 203, 206, 304, 397]. Decreased progenitor cell numbers was associated with diminished numbers of transitional as well as mature B2 B cell subsets in the bone marrow and spleen (Fig. 2a-d). Yet, c-Myb had no effect on splenic marginal zone (MZ) cells, and increased B1b B cell populations (Fig. 2b, d). c-Myb effects were B lineage-restricted since we did not observe decreased numbers of non-committed hematopoietic stem and progenitor cells (Supplementary Fig. 2b, c). Moreover, these effects of c-Myb did not depend on altered cell proliferation (Fig. 2e). Rather, c-Myb
promotes B cell survival as assessed by annexin V staining (Fig. 2f) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Supplementary Fig. 2d) [206].

**Atherogenic potential of c-Myb depends on direct effects on B cells**

Sorted B220\(^+\) and B220\(^-\) cells from bone marrow of atherosclerotic mice showed that B220\(^+\) B cells express ~4.5-fold elevated levels of c-\(myb\) (B200\(^+\) 0.290 ± 0.031 vs. B220\(^-\) 0.064 ± 0.008; N=5/group; P < 0.0001) as shown by qRT-PCR (Fig. 2g). To determine whether the atherogenic potential of c-Myb depends on direct effects on B cells, mixed chimeras were generated by reconstituting lethally irradiated \(Ldlr^/-\) mice with a mixture of c-\(myb^h/h\) and \(\mu\)MT bone marrow cells. In these mice (c-\(myb^h/h\):\(\mu\)MT), \(\mu\)MT marrow contributed c-\(myb^{wt/wt}\) cells to all leukocyte subsets except B cells, whereas the c-\(myb^h/h\) marrow contributed only c-\(myb^h/h\) cells. Therefore, of the cell populations in the reconstituted mice, only B cells lacked c-Myb activity. Comparison of blood leukocytes from these animals with c-\(myb^{h/h}:wt\) controls (ie. in which wild type marrow contributed c-\(myb^{wt/wt}\) cells to all leukocyte subsets) revealed differences between experimental groups in only the number of circulating B cells (Fig. 2h). Decreased lesion development in c-\(myb^{h/h}:\mu\)MT chimeras further demonstrated that the atherogenic potential of c-Myb depends on direct effects on B cells (Fig. 2i).

**c-Myb restricts the development of IgM antibody secreting cells**

B cells have numerous functions including the production of cytokines and immunoglobulins (reviewed in [403, 404]). IgM antibodies, particularly those directed toward modified lipoproteins, mediate atheroprotection [311, 312], while the role of IgG is less clear [313]. Regardless of diet, \(Ldlr^/-c-myb^{h/h}\) mice exhibited increased levels of serum IgM (\(Ldlr^/-\))
0.548 ± 0.111 mg/ml vs. Ldlr<sup>-/-</sup> c-myb<sup>h/h</sup> 1.338 ± 0.252 mg/mL; N=5-9/group; P < 0.05), but not IgG compared to Ldlr<sup>-/-</sup> animals (Fig. 3a and Supplementary Fig. 3a). Measurement of antibodies directed specifically at copper-oxidized low density lipoprotein (CuOxLDL) similarly revealed increases in anti-CuOxLDL IgM (Fig. 3b), indicating an intrinsic capacity of these animals to mount immunoglobulin responses to modified lipoproteins. Analysis of intracellular immunoglobulin stores by flow cytometry (Fig. 3c) and immunofluorescence microscopy (Fig. 3d) revealed increased numbers of IgM-producing B cells in spleens of Ldlr<sup>-/-</sup> c-myb<sup>h/h</sup> HCD mice compared to control animals despite drastically reduced total B cell number in Ldlr<sup>-/-</sup> c-myb<sup>h/h</sup> HCD mice. This was confirmed by enzyme-linked immunospot assay (Fig. 3e, Supplementary Fig. 3b). B cells from Ldlr<sup>-/-</sup> c-myb<sup>wt</sup> and Ldlr<sup>-/-</sup> c-myb<sup>h/h</sup> mice generated similar size spots, suggesting c-myb had no effect on IgM production on a per-cell basis (Supplementary Fig. 3c). Consistent with this observation, B220<sup>+</sup> cells from wt and c-myb<sup>h/h</sup> mice produced similar amounts of IgM in vitro following exposure to lipopolysaccharide (LPS) (Fig. 3f). Phenotypic characterization of IgM producers revealed them to be B220<sup>+</sup>IgM<sup>+</sup>CD23<sup>lo</sup>CD5<sup>-</sup>CD1d<sup>-</sup> B1b cells [391] (Fig. 3g).

4.5 Discussion

The progression of atherosclerosis is mediated by continual inflammation, with different tissues and cell subsets having considerably varying roles in the pathogenesis of atherosclerosis. In particular, specific B cell subsets have been shown to have both pro- and anti-atherogenic effects, however the transcriptional control of the generation of different subsets in atherosclerosis remains poorly understood. We show that a mouse harboring a point mutation in
the hematopoietic transcription factor c-Myb results in decreased atherosclerosis. Consistent with established roles of circulating IgM as being atheroprotective, \textit{Ldlr}^{-/-} c-myb^{h/h} mice had increased plasma IgM titers, as well as increased numbers of IgM secreting and intracellular IgM\(^+\) B cells. Additionally, \textit{Ldlr}^{-/-} c-myb^{h/h} mice had decreased pro-atherogenic marginal zone B cells and increased anti-atherogenic B1b B cell subsets.

While the data clearly show that \textit{c-myb} regulates B cell lymphopoiesis in atherosclerosis, the mechanisms by which c-Myb is involved are still unclear. Firstly, it is unclear why there are increases in anti-atherogenic B1b cells in \textit{Ldlr}^{-/-} c-myb^{h/h} mice. Consistent with plasma cell generation observed in \textit{CD19}^{CRE} c-myb^{loxp/loxp} in response to T-dependent immunization [399], in \textit{Ldlr}^{-/-} c-myb^{h/h} mice on HCD we observed similar numbers of IgM and IgG ASCs in the bone marrow as compared to \textit{Ldlr}^{-/-} c-myb^{WT} mice, presumably due to defective migration of mature plasma cells to the bone marrow. However, it is unclear if the increased number of splenic IgM producing cells in \textit{Ldlr}^{-/-} c-myb^{h/h} mice is due to defective migration of mature plasma cells. The mechanism underlying excess generation of IgM\(^+\) ASCs in the spleen of \textit{Ldlr}^{-/-} c-myb^{h/h} mice could be driven in part by the defective migration of cells generated in the spleen to the BM, to excess proliferation of plasmablasts, or the failure to of immature short-lived plasmablasts to die. Upon stimulation, B1 cells undergo immense proliferation and differentiation, become IgM-secreting plasmablasts that form extra-follicular foci within the spleen [319]. These foci are relative long lived, and remain as a collection of plasmablasts that do not fully mature to plasma cells. The origin of these immature plasmablasts has been determined to be from B1b cells [319], despite the relative initial rarity of B1b cells in the spleen. Normally, the populations of these immature plasmablasts is regulated by apoptosis [320], but is remains to be determined if
increased B1b cells in \textit{Ldlr^{-/-} c-myb^{h/h}} mice is mediated by defects in the involution of immature B1b-derived plasmablasts.

The regulation of plasma cell differentiation at the molecular level is primarily achieved by the coordinate down-regulation of Pax5, a transcription factor expressed throughout B cell lineages, maintaining mature B cell identity and function [321, 322], and expression is absent from plasma cells [324]. Pax5 maintains B cell identity through the activation of genes critical for B cell function [321, 323], and represses the expression of genes necessary for plasma cell development and function [325-328]. Opposite to Pax5, Blimp1 (Prdm1) expression is required for plasma cell differentiation and the up-regulation of genes required for plasma cell function [329-332]. Blimp1 silences B cell genes and promotes the exit from the cell cycle [333], and also represses Pax5 [334, 335], resulting in the de-repression of Pax5 target genes. c-Myb and Pax5 are known to interact and regulate the Rag2 promoter in immature B cells [336, 337], however, it is unknown if c-Myb and Pax5 also interact in regulating genes related to plasma cell differentiation. While there is no evidence that c-Myb and Blimp1 directly interact in the differentiation or function of plasma cells, several factors known to regulate Blimp1 are also known to have interactions with c-Myb. ETS1 is known to interfere with plasma cell maturation via Blimp1; c-Myb and ETS1 are known to interact in the differentiation of myeloid cells [338, 339], thus c-Myb and ETS1 may coordinately work to inhibit Blimp1-mediated plasma cell maturation. Additionally, transcriptional repression by Blimp1 also involves the recruitment of chromatin modifying histone deacetylases [340]. Histone deacetylases also recruit other transcription factors to either activate or repress transcription of target genes. In particular, the transcriptional co-repressors mSin3a and NCoR are two factors known to bind histone
deacetylase [405] and also c-Myb [406]. Hence, intact function of c-Myb may be required for Blimp1-mediated maturation of plasma cells. Interestingly, interfering with Blimp1 via a transgenic truncated form of Blimp1, causes an increase in proliferation and survival of short-lived, IgM secreting plasma cells, resulting in elevated serum IgM [407]. Whether any of these proposed mechanisms are operant in \(Ldlr^{+/c-myb^{hh}}\) mice remains to be determined.

A recent network-driven integrative analysis of whole blood gene expression profiles from Framingham Heart Study participants suggested a causal role for B cells in cardiovascular disease [408]. Genes critically involved in B cell receptor signaling, differentiation, and activation emerged as key drivers of disease. The design of B cell targeted therapies may prove complicated as B cell subsets appear to have opposing roles in atherogenesis, exerting either protective [294, 391] or pathogenic functions [315, 316]. Our results demonstrate that in addition to decreasing atherosclerosis severity, targeted reduction of c-Myb activity circumvents this complexity by depleting potentially harmful B2 cells while boosting production of protective IgM.

4.6 Acknowledgments

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Studies. S.E. was supported by an Ontario Graduate Scholarship. The authors declare no conflicts of interest.

4.7 Author Contributions

E.A.S. and S.E. conceived the project, designed and performed experiments, and analyzed and interpreted data. A.L., N.D., R.B., C.C.J.Z., D.T., and D.S. performed experiments and helped interpret the data. H.S. performed histology. C.M.T.B., I.H., P.L., F.K.S., M.R.S., M.C., and B.B.R. provided materials, intellectual input and edited the manuscript. M.H. conceived the project, provided materials and intellectual input. C.S.R. conceived the project, designed and performed experiments, supervised the study, and wrote the manuscript.

4.8 Figure Legends

Figure 1. c-Myb promotes atherosclerosis. (a) RT-PCR detection of c-Myb expression conducted on leukocytes from bone marrow of Ldlr<sup>−/−</sup> mice fed either normal chow or a diet high in cholesterol (HCD) for 12 weeks (mean ± SEM, n = 9 per group). * P < 0.05. (b) Total cholesterol and triglycerides in plasma from Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice (mean ± SEM, n = 7-8). (c) Quantification of lesion size in aortic roots of Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice (mean ± SEM, n = 14-15). * P < 0.05. (d) en face lipid Oil Red O staining of aortic arches from Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice (mean ± SEM, n = 7-8). * P < 0.05. (e) Masson trichrome staining of aortic root sections of Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice (mean ± SEM, n = 11-12). * P < 0.05. (f) Flow cytometry analysis revealed fewer inflammatory cells including macrophages and B cells in atherosclerotic aortas from Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice (mean ± SEM, n = 8-10). * P < 0.05. (g) Quantification of aortic root lesion size in bone marrow chimeras generated by
reconstituting lethally irradiated \( Ldlr^{-/-} \) mice with either wild type (wt) or \( c-myb^{h/h} \) bone marrow cells. (mean \( \pm \) SEM, \( n = 9 \)). * \( P < 0.05 \). (h) Peripheral blood monocytes, neutrophils, and T and B lymphocytes in \( Ldlr^{-/-}c-myb^{wt} \) and \( Ldlr^{-/-}c-myb^{h/h} \) mice fed either chow or HCD (mean \( \pm \) SEM, \( n = 13-19 \)). * \( P < 0.05 \).

**Figure 2. Atherogenic potential of c-Myb depends on direct effects on B cells.** (a) Flow cytometry-based profiling of B lineage restricted progenitors and mature B cells from bone marrow of \( Ldlr^{-/-} \) and \( Ldlr^{-/-}c-myb^{h/h} \) mice. Fr ‘A’, Fraction A; Fr ‘E’, Fraction E (mean \( \pm \) SEM, \( n = 7-8 \)). * \( P < 0.05 \). (b) Phenotype of B cell subsets in the spleen of same animals as (a). MZ, marginal zone; FO, follicular; T1/T2/T3, transitional (mean \( \pm \) SEM, \( n = 7-8 \)). * \( P < 0.05 \). (c) Enumeration of B cell progenitors in (a) (mean \( \pm \) SEM, \( n = 7-8 \)). * \( P < 0.05 \). (d) Enumeration of B cells in (b) (mean \( \pm \) SEM, \( n = 7-8 \)). * \( P < 0.05 \). (e) Cell cycle analysis of spleen and bone marrow B cells from \( Ldlr^{-/-} \) HCD and \( Ldlr^{-/-}c-myb^{h/h} \) HCD mice (mean \( \pm \) SEM, \( n = 7-8 \)). * \( P < 0.05 \). (f) Flow cytometry analysis of Annexin V staining of B cells from spleen and bone marrow of \( Ldlr^{-/-} \) HCD and \( Ldlr^{-/-}c-myb^{h/h} \) HCD mice (mean \( \pm \) SEM, \( n = 7-8 \)). * \( P < 0.05 \). (g) \( c-myb \) mRNA expression in B220+ and B220− cells isolated from the bone marrow of \( Ldlr^{-/-} \) mice fed either chow or HCD (mean \( \pm \) SEM, \( n = 5 \)). * \( P < 0.05 \). (h) Leukocyte profile of peripheral blood from mixed chimeras generated by reconstituting irradiated \( Ldlr^{-/-} \) mice with a mixture of either \( c-myb^{h/h} \) and \( \mu MT \) bone marrow cells (\( c-myb^{h/h};\mu MT \) chimeras) or \( c-myb^{h/h} \) and wild type (wt) bone marrow cells (\( c-myb^{h/h};\text{wt} \) chimeras) (mean \( \pm \) SEM, \( n = 6 \)). * \( P < 0.05 \). (i) Quantification of aortic root lesion size in mice from (f).
Figure 3. c-Myb attenuates IgM responses in atherosclerosis. (a) Total serum IgM (mean ± SEM, n = 5-9). * P < 0.05. (b) Serum anti-copper-oxidized oxLDL (CuOxLDL) IgM levels assessed by enzyme-linked immunosorbant assay (ELISA) (mean ± SEM, n = 5-9). * P < 0.05. (c) IgM antibody secreting cells (ASCs) in the spleen and bone marrow of Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice assessed by ELISPOT (mean ± SEM, n = 3-5). * P < 0.05. (d) Total IgM in culture supernatants assessed by ELISA. B220<sup>+</sup> cells were isolated from spleens of wt and c-myb<sup>h/h</sup> mice by magnetic-activated cell sorting (MACS) and cultured for 4 days in the presence or absence of LPS. Data show 3 individual experiments pooled. (e) Enumeration of IgM-producing cells in spleens of Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice by flow cytometry (mean ± SEM, n = 3-5). * P < 0.05. Data show one of two representative experiments. (f) Immunofluorescence showing IgM staining in the spleen of Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice. Shown is a representative image from one of 3 animals examined. (g) Representative dot plots show intracellular immunoglobulin M (IgM) stores of B220<sup>+</sup>IgM<sup>+</sup>CD23<sup>lo-neg</sup>CD1d<sup>−</sup> cells in the spleen of Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>c-myb<sup>h/h</sup> mice.
Figure 4-1: c-Myb promotes atherosclerosis
Figure 4-2: Atherogenic potential of c-Myb depends on direct effects on B cells.
Figure 4-3: c-Myb attenuates IgM responses in atherosclerosis.
4.10 Supplemental tables

4-1: Supplementary Table 1. Antibodies used in flow cytometry analysis

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4.11 Supplementary Figure Legends

**Supplementary Figure 1.** (a) en face lipid Oil Red O staining of aortic arches from \( \textit{Ldlr}^{-/-} \) HCD and \( \textit{Ldlr}^{-/-}c\text{-myb}^{h/h} \) HCD mice. Data show % of the aortic area that stained positive for ORO (left panel) and the total area of the aortic section analysed (right panel) (mean ± SEM, n = 7-8). * \( P < 0.05 \). (b) Gating strategy for flow cytometry-based analysis of immune cells in aortae. Data show representative plots. (c) Peripheral blood B lymphocyte percentage in \( \textit{Ldlr}^{-/-}c\text{-myb}^{wt} \) and \( \textit{Ldlr}^{-/-}c\text{-myb}^{h/h} \) mice fed either chow or HCD (mean ± SEM, n = 13-19). * \( P < 0.05 \).

**Supplementary Figure 2.** (a) Numbers of B lymphocytes in spleen, bone marrow, para-aortic lymph nodes, and peritoneal cavity of \( \textit{Ldlr}^{-/-} \) and \( \textit{Ldlr}^{-/-}c\text{-myb}^{h/h} \) mice (mean ± SEM, spleen, bone marrow, para-aortic lymph nodes n = 7-8, peritoneal cavity n = 4). * \( P < 0.05 \). (b) Flow cytometry-based analysis of non-committed hematopoietic stem and progenitor cells in the bone marrow. Data show representative plots. LT-HSC, long term-hematopoietic stem cell; MPP, multi-potent progenitors; HPC, hematopoietic progenitor cells. (c) Enumeration of hematopoietic stem and progenitor cell populations in \( \textit{Ldlr}^{-/-} \) and \( \textit{Ldlr}^{-/-}c\text{-myb}^{h/h} \) mice (mean ± SEM, n = 9-10). (d) Intracellular TUNEL staining in B220\(^{+}\)IgD\(^{+}\) B lymphocytes from spleens of \( \textit{Ldlr}^{-/-} \) HCD and \( \textit{Ldlr}^{-/-}c\text{-myb}^{h/h} \) HCD mice (mean ± SD, n = 5). * \( P < 0.05 \). FMO, Fluorescence minus one.

**Supplementary Figure 3.** (a) Total serum IgG (mean ± SEM, n = 5-9). * \( P < 0.05 \). (b) IgM antibody secreting cells (ASCs) in the spleen and bone marrow of wt and \( c\text{-myb}^{h/h} \) mice assessed by ELISPOT (mean ± SEM, n = 5). * \( P < 0.05 \). (c) Quantification of spot size in (b) (mean ± SEM, n = 5).
Figure 4-4: Supplemental Figure 1
Figure 4-5: Supplemental Figure 2
Figure 4-6: Supplemental Figure 1
CHAPTER 5:

5 Concluding Discussion and Future Directions
While the insights gained in this dissertation add significantly to understanding the multitude of areas in which c-myb has a regulatory role, there are still significant new areas to be explored given the novel insights brought to light in the three preceding chapters.

5.1 Lineage tracing Sca1⁺ adventitial progenitor cells: embryological origins and pathological involvement

While the embryological origins of VSMCs have been largely elucidated and even shown to have distinct roles in the development of cardiovascular pathology, the embryological origins of the adventitia remains unresolved. While adventitia would presumably originate from the same tissues as the medial SMC that underlie the adventitia, in lineage tracing studies of medial SMCs, adventitial cells do not share the same embryological origins. Lineage tracing studies by several groups have identified and ruled out potential embryological origins for the adventitia, yet the origins remain unknown. Passman et al. found that while the medial SMCs of the aortic arches and carotid arteries were neural crest derived (Wnt1-cre⁺), they failed to identify any adventitial cells that were also Wnt1-cre⁺ suggesting that the adventitia overlying the SMCs was of a different embryological origin [265]. In a study examining optic muscle, it was observed that Myf5-cre marked the adventitia of some of the arteries around the eye, suggesting that adventitia in this area may arise from a somitic mesodermal lineage. CD45⁺Sca1⁺ cells that localize to the vasculature of muscle have also been found to express Myf5, possibly suggesting that Myf5-dependent lineage tagging in these mice may be a later event than occurs in embryogenesis [409, 410]. Late-expression of Myf5 might suggest these cells may not be somitic in origin, but a result re-expression of Myf5.

While SMCs of the aorta have been shown to arise from different embryological origins with differing susceptibility to disease, generation of chimeric mice using tagged c-myb⁻/⁻ (β-gal⁺)
ES cells demonstrated paucity of β-gal+ (c-myb+/−) cells throughout SMC-rich tissues like the thoracic aorta, intestines, as well as the root of the aorta [17], all of which arise from separate embryonic origins. This suggests that while SMC heterogeneity exists based on embryological origins, the regulation of SMC differentiation may be c-myb-responsive regardless of the embryonic origins of the affected SMC precursors. Determination of the origins of Sca1+ adventitial cells may provide insights into the origins of the adventitia, but it remains possible that c-myb-dependent processes in Sca1+ VSMC progenitor cells may not vary as a function of embryological origin, but are rather universal regulators of SMC differentiation.

Given the diverse cell types present in the adventitia, the adventitia may not be composed of cells of a single origin, but rather recruited from a variety of embryological tissues. In particular to Sca1+ adventitial cells, a complicating factor into elucidating their origins, as well as their contributions to disease may be that there is significant heterogeneity within the Sca1+ population alone. With both hematopoietic and vessel-resident cells constituting this population, it would not be inconceivable that multiple tissues give rise to Sca1+ cells of the adventitia. Future experiments to delineate the origin of adventitia may provide valuable insights into a tissue that has become appreciated as having a regulatory role in vessel biology.

It was found that Sca1+ cells proliferate in a c-myb dependent fashion in response to vessel injury, however it remains to be shown conclusively what the role of Sca1+ cells are in the injury response. While lineage tagging studies using a Sca1-GFP or other Sca1-reporter would be helpful in identifying cells originating from Sca1+ cells (which subsequently lose Sca1 expression), several additional considerations would have to be made when attempting these studies. As previously mentioned, Sca1+ cells originate from both BM- and vessel-resident cells, thus the need to differentiate these population would be required. This may be overcome by
BMTs to replace BM-derived Sca1\(^+\) cells with non-marked cells, but does not address the possibility that BM-derived vessel resident cells may be radio-insensitive, and thus not ablated fully by BMT. It may be possible to overcome this using a vessel-graft model into a non-Sca1-reporter line; however differences in vessel-injury models may make it difficult to generalize these results to other injury models.

A further difficulty of lineage tracing cells of Sca1\(^+\) origin is the considerable heterogeneity within the population. In this dissertation it was identified that cKit\(^+\)Sca1\(^+\) cells had the greatest proliferative potential, while other marker expression did not correlate to a highly proliferative population. Precisely defining a single population of Sca1\(^+\) cells to identify will necessitate the creation of bi- or multi-transgenic lineage tracing animals. While we identified that cKit\(^+\)Sca1\(^+\) cells were highly proliferative, it is not known if these cells represent a more primitive progenitor, the activated state of the Sca1\(^+\) VSMC progenitor cell, or if they represent an entirely separate population from other marker-expressing populations. Future experiments could aim to isolate these specific sub-populations to determine their relative capacity for proliferation as well as determine if there is differential differentiation capacity within separate populations. While it was found that the expression of CD34 and Flk1 decreased during injury, it remains possible that these are markers of quiescent, non-activated Sca1\(^+\) progenitor cells, thus further studies could determine if these markers are expressed in activated vs. non-active Sca1\(^+\) progenitor cells.

5.2 \(c\)-Myb-mediated proliferation and differentiation: further mechanisms and lineages
Interestingly, in the \(c\)-\(myb\)\(^{+/+}\) mouse, development of VSMCs as well as Sca1\(^+\) VSMC progenitor cells appears unperturbed. This suggests that while \(c\)-\(myb\) regulates the proliferation of mature VSCMs as well as the differentiation of vessel-resident SMC progenitor cells, partial loss of \(c\)-
myb function does not result in the complete absence of mature VSMCs. In chimeric animals generated with LacZ\textsuperscript{+}c-myb\textsuperscript{−/−} ESC displayed significant decreases in chimerism in SMC-rich tissues [17], however c-myb\textsuperscript{−/−} EBs were still capable of generating SMCs. This suggests that some SMCs may overcome the deficiency in c-myb, or that ablation of c-myb is sufficient to significantly reduce SMC differentiation, but not sufficient to completely eliminate it. These observations suggest that there may be alternative pathways or some redundant mechanisms ensuring SMC development can occur in the absence of c-myb. Determination of these pathways will add further insight into the mechanisms of differentiation of SMCs.

In our study we show that the mechanism by which c-myb appears to regulate VSMC differentiation is the transcriptional regulation of myocardin, a master regulator of SMC differentiation. However, it is worthwhile to note that hypomorphic c-myb still resulted in some level of SMC differentiation, suggesting that c-Myb with DNA binding capacity is sufficient for some level of SMC gene induction, whereas total ablation of c-Myb results in very little SMC gene expression[17]. The diminished capacity of hypomorphic c-Myb suggests that the recruitment of transcriptional co-activators by c-Myb to specific sites in the myocardin promoter during the induction of SMC differentiation remains important. P300, an HDAC, has been found to be important in the induction of myocardin expression, and c-Myb is a known binding partner of p300[15, 411, 412]. Future studies on the mechanism by which c-Myb regulates SMC differentiation could also look at the transcriptional binding partners of c-Myb which regulate SMC-gene expression. Future studies could employ techniques such as ChIP-seq to identify, in an unbiased fashion, transcriptional targets of c-Myb relevant to SMC differentiation. Similarly, a proteomic examination of c-Myb binding partners during SMC differentiation could also yield
similarly important refinements of the processes c-Myb is involved in during SMC
differentiation.

A further area of investigation to come from the current study is whether or not c-myb
regulates the differentiation of Sca1+ progenitor cells into any other lineages present in the vessel
wall. Given the role of c-myb in myeloid differentiation, c-myb might also regulate
differentiation of Sca1+ cells into vessel-resident myeloid populations. In addition, regulation of
EC and pericyte differentiation by c-myb has never been reported in any stem/progenitor
population and remains an area that requires examination. While there was no contractile
difference in c-myb/h/h mice, they manifested an impaired vasodilatory response to acetylcholine
stimulation, which was overcome by a nitric oxide donor, SNP. Nitric oxide has been implicated
as having a role in the differentiation and function of ESC-derived endothelial cells [413], so it is
plausible that c-myb may have a role in endothelial cell differentiation. This is the first
demonstration that c-myb may regulate the function of endothelial cells. While it is uncertain
what aspect of nitric oxide synthesis is defective in c-myb/h/h mice, nonetheless, these results offer
an exciting new insight into possible additional roles in vascular-resident cells and provide a new
direct for further research.

In addition to regulating differentiation of VSMC progenitor cells, it was also found that c-
myb regulated the proliferation of Sca1+ progenitor cells both in vitro and in vivo. What remains
to be determined however is the mechanism by which c-myb regulates the proliferation of these
cells. c-myb has been shown to regulate proliferation through the regulation of cyclin E
expression in colonic crypt cells[13, 414], yet it has also been shown to regulate cell cycle
progression in VSMCs via a IP3R-dependent Ca2+-mediated pathway[221-224]. Elucidation of
the relevant pathways regulated by \textit{c-myb} could lead to targeted therapies designed to disrupt the proliferation of Sca1$^+$ VSMC progenitor cells that may be involved in vascular remodelling.

5.3 Challenging the dogma: insights into VSMC de-differentiation and progenitor cell involvement in neointimal remodeling.

While the current dogma of VSMC biology centers on the ability of fully differentiated mature VSMCs to de-differentiate, down-regulate expression of mature VSMC markers and become highly proliferative cells in response to mitogenic stimuli, mounting evidence suggests there may be alternative pathways of generating VSMCs. Indeed, it has been shown that there may be some phenotypic heterogeneity within VSMC populations, with some VSMCs poised to become highly proliferating cells, while others remain quiescent. Further evidence challenging this dogma is the presence of small populations of vessel-resident VSMC progenitor cells that can differentiate into cells that express markers of fully differentiated VSMCs. In the current study we show that a small population of Sca1$^+$ cells in the adventitia can differentiate to VSMCs in a c-Myb-dependent manner, further demonstrating a role for c-Myb in VSMC biology.

In the vessel injury data, it is notable that despite lower VSMC gene expression in injured vessels of \textit{c-myb}$^{+/-}$ mice versus \textit{c-myb}$^{+/+}$ mice, which would presumably suggest readily de-differentiated cells poised to proliferate, there was reduced neointimal remodeling. This could suggest that changes in gene expression in a small population of cells such as vessel-resident progenitor cells are responsible for the decrease in neointimal remodeling, and that gene expression changes in large numbers of VSMCs obscures the detection of changes in these small populations of progenitor cells. Alternatively, it could suggest that de-differentiation alone is insufficient for phenotypic switching to occur; that c-Myb is absolutely necessary for VSMC
proliferation regardless of the state of mature VSMC marker expression in VSMCs. Again, specific lineage-tagging experiments could allow for conclusive evidence showing progenitor cell involvement in generating neointimal VSMCs in injury. It would also allow for the examination of gene expression changes in the small populations of VSMC-generating cells that might otherwise be obscured by other signals from more populous cells. While these experiments will challenge the existing dogma of phenotypic modulation, it is likely that both populations of proliferation-poised mature VSMCs and vessel-resident VSMC progenitor cells contribute in some form to neointimal remodeling.

5.4 Role of leukocyte c-Myb in neointimal remodelling.

In addition to the vessel-intrinsic injury phenotype, we also showed that transplantation of c-myb<sup>h/h</sup> BM into c-myb<sup>WT</sup> mice was able to confer protection from neointimal remodelling. Whether this is due to changing the leukocyte populations recruited to the vessel wall during injury remains to be determined. While leukocyte recruitment was not nominally different in c-myb<sup>h/h</sup> mice, it was not determined if there are differences in the populations of leukocytes recruited. In addition, we show that BP differences also segregate with BMT; hence it is not known how much of a protective benefit is conferred through direct leukocyte effects on vessel injury, and how much of the phenotype is dependent on BP differences. Using the FVB/N strain of c-myb<sup>h/h</sup> mice could help overcome this confounding factor, as the FVB/N c-myb<sup>h/h</sup> mice do not manifest BP differences. However, it is unknown if the C57Bl/6 and FVB/N c-myb<sup>h/h</sup> also manifest differences in hematopoietic differentiation or function, which may further complicate these studies.

5.5 Paradoxical ECM regulation: fibrosis, vascular stiffening and defective collagen synthesis
c-Myb has been shown to up-regulate collagen type-1 expression [137, 138, 200], therefore it was surprising to observe increased collagen staining and concomitant increases in tensile strength and decreases in vascular compliance in the arteries of $c$-$myb^{h/h}$ from various vascular beds. While we observe more collagen in vessels, it remains to be determined if there is also more fibrosis in other organs and tissues in the $c$-$myb^{h/h}$ mouse.

In mice with deficient $c$-$myb$, it has been demonstrated that impaired wound healing is associated with decreased collagen synthesis in wounds [136]. In agreement with these data, we also demonstrated that $c$-$myb^{h/h}$ mice have decreased collagen mRNA expression compared to $c$-$myb^{WT}$ mice following vascular injury. The discrepancy of these data with the observation that at the steady-state $c$-$myb^{h/h}$ mice have more vascular collagen, suggest that c-Myb regulation of collagen synthesis may be divergent in vascular development and injury, and that the mechanisms that govern collagen synthesis in normal vascular development may not be the same as those involved in pathological processes. It is interesting to note that in $c$-$myb^−/−$:WT chimeric animals, there is an almost complete absence of $c$-$myb^−/−$-derived SMCs, and indeed, very little staining of $c$-$myb^−/−$-derived cells in the aorta at all, however in the $c$-$myb^{h/h}$ mouse we observe normal numbers of both VSMCs as well as adventitial Sca1$^+$ cells. This could suggest that developmentally, only partial function (DNA binding) is sufficient to generate normal VSMC compartment in the adult mouse; perhaps collagen synthesis during development is DNA binding-dependent. However this alone would not explain why there is increased collagen staining. Our current investigation did not examine the expression of all collagen types in the adult aorta, thus determination of all other collagen types could reveal increased expression of other collagens. Similarly, we have not examined the spatiotemporal expression of collagen genes during vascular development.
While c-Myb is known to regulate collagen gene expression, a finding from the injury study could possibly explain why there is more collagen expression in the vessels of $c\text{-}myb^{h/h}$ mice. Consistent with previous studies, we show that collagen gene expression is decreased in injured $c\text{-}myb^{h/h}$ mice; however, post-injury mRNA expression data of matrix metalloproteinase-2 (MMP-2) and MMP-9 (also known as collagenase A and B), show that $c\text{-}myb^{h/h}$ vessels express decreased MMP-2 and MMP-9 following injury. It is possible that developmental expression of these MMPs is also hindered, thus developing vessels may not as efficiently degrade or remodel collagen, leading to aberrant collagen deposition in $c\text{-}myb^{h/h}$ arteries. Determining MMP expression and activity in developing vasculature of $c\text{-}myb^{h/h}$ mice could help to shed light on the seemingly paradoxical collagen deposition and functional consequences observed in $c\text{-}myb^{h/h}$ vessels.

5.6 c-Myb in blood pressure regulation

In the examination of cardiovascular function in $c\text{-}myb^{h/h}$ mice, it was surprising to find that they did not manifest any differences in cardiac or vascular function. While we found decreased blood pressure in $c\text{-}myb^{h/h}$ mice, it was interesting that there are no differences in SMC formation, nor are there differences in SMC function. These results suggest that while $c\text{-}myb$ may regulate differentiation of VSMCs, it does not affect the development of normal contractile SMC, nor does $c\text{-}myb$ regulate the contractile function of SMCs as was originally hypothesized. While literature exists on the role of $c\text{-}myb$ in the proliferation of VSCMs, our study is the first to examine a role in the contractile function of $c\text{-}myb$ in mature SMCs. It is interesting to note that despite regulating VSMC cell cycle via a Ca$^{2+}$-dependent mechanism, $c\text{-}myb$ does not appear to regulate SMC contractile function, which is also dependent on Ca$^{2+}$. Cell-cycle dependent regulation of PMCA1 expression by c-Myb was shown to function during G$_1$/S-phase, whereas
the regulation of PMCA1 expression did not occur during G\textsubscript{0} \textit{in vitro} \cite{222}. The reason for this could be that \textit{c-myb} has differential effects during cell cycle regulation in proliferating versus contractile VSMCs.

In attempting to assay factors that may be responsible for systemic changes in blood pressure regulation, the cytokine array identified KC as the only factor to be differentially expressed in the serum of \textit{c-myb}\textsuperscript{h/h} mice. However, we found that KC most likely was not causally associated with blood pressure regulation. While systemic differences in these factors may not be present in \textit{c-myb}\textsuperscript{h/h} mice, it remains possible that local expression of these factors have mechanistic roles in blood pressure regulation in \textit{c-myb}\textsuperscript{h/h} mice. It is also possible that cytokines, mitogens and neurohormonal factors (such as rennin, angiotensin, aldosterone and others) not assayed in the plasma cytokine array are perturbed in the \textit{c-myb}\textsuperscript{h/h} mice. Hence, investigation of other systemic factors may yield substantial mechanistic insights into \textit{c-myb}-mediated regulation of blood pressure regulation.

While we were able to show a link between BM and renal function, the mechanisms remain unclear. Future investigations to determine any differences in renal leukocyte populations will begin to uncover the mechanism of leukocyte-mediated changes in renal function. While we have identified that B-cells are decreased in \textit{c-myb}\textsuperscript{h/h} kidneys, it remains to be determined if and how these leukocyte populations modulate renal function. While we did not find differences in the number of other renal leukocyte populations, it remains possible that despite similar numbers, their functions are perturbed. Similarly, while we find that renal B-cells are decreased in number, this does not exclude the possibility that B-cells may regulate the function of other renal leukocyte and non-leukocyte cell populations. Finally, while immunophenotyping was performed on renal leukocyte populations, it remains possible that
further refinements in identification of sub-types of these populations (macrophages, dendritic cells, T-cells, and B-cells) could yield further insight into the regulation of renal function by leukocytes and how \textit{c-myb} regulates these populations.

Given the profound hematopoietic abnormalities in \textit{c-myb}^{h/h} mice, it is likely that one or more renal leukocyte populations that are critical in the maintenance of normal homeostatic function may be perturbed. When comparing the data of \textit{c-myb}^{h/h} mice and \textit{c-myb}^{h/h}\textgreater\textit{WT} mice, both of which had low blood pressure and altered renal function, it was found that the parameters commonly affected in both these mice were plasma potassium level, urinary output and creatinine clearance suggesting that these parameters were regulated by BM-derived cell populations. However, how this is achieved remains unknown. Whether leukocyte populations regulate the function of renal ion transporters known to regulate potassium levels such as KCNE1[381], ENaC[382], ROMK1[383], NCC[384] or NKCC2[385] could be potential contributing mechanisms of leukocyte-mediated BP homeostasis.

In order to develop a causal relationship of hypomorphic B-cells and changes in renal function, an experimental paradigm employed in the atherosclerosis study could also be used. By generating chimeric animals that are reconstituted with either \textit{c-myb}^{h/h}:\textit{WT} or \textit{c-myb}^{h/h}:\textmu\textit{MT} (\textmu\textit{MT} mice lack B-cells, but all other lineages are normal) BM, it could be assessed if restoration of WT B-cells can restore normal renal function in chimeric animals. In addition, comparative immunophenotyping of renal leukocyte populations in low blood pressure C57BL/6 \textit{c-myb}^{h/h} mice and normal blood pressure FVB/N \textit{c-myb}^{h/h} mice might also yield insights into renal leukocyte populations responsible for blood pressure homeostasis.
Inflammation in hypertension is commonly reported, and a well studied field and the causative mechanisms of leukocyte-mediate regulation of blood pressure are just beginning to become understood [376, 377, 415]. Persistent changes in plasma potassium in both c-myb\textsuperscript{h/h} mice and c-myb\textsuperscript{h/h>WT} mice suggest that leukocytes may also be mediating the function of specific ion transporters, thus determination of expression levels of various ion transporter may also be investigated in c-myb\textsuperscript{h/h} mouse kidneys. Both innate and adaptive immune mechanisms have been implicated in the pathogenesis of hypertension, thus the potential immunomodulatory mechanisms that may be active in c-myb\textsuperscript{h/h} mice are numerous in nature. Neural, renal and vascular function in the kidney are all affected by leukocyte populations, thus it is also likely that more than one leukocyte population moderates blood pressure in the c-myb\textsuperscript{h/h} mouse. Elucidation of these mechanisms may yield exciting new therapeutic targets, and thus represent a worthwhile area for further investigation.

5.7 c-Myb in atherogenesis

While our results show that B cell subsets seem to be predominantly affected in the \textit{Ldlr}\textsuperscript{-/-} c-myb\textsuperscript{h/h} mouse, and that the phenotype is dependent on c-myb activity in BM-derived cells, there are most likely other uncharacterized defects in hematopoietic cell subsets in the \textit{Ldlr}\textsuperscript{-/-} c-myb\textsuperscript{h/h} mouse. While we observed that there were fewer macrophages in \textit{Ldlr}\textsuperscript{-/-} c-myb\textsuperscript{h/h} mice after HCD, it remains to be determined if that is a result of defective recruitment of monocytes, defects in \textit{in situ} proliferation of lesional macrophages [234], or mediated by other mechanisms such as differences in circulating systemic factors.

Although we found that there were no significant differences in plasma cytokines in c-myb\textsuperscript{h/h} mice, it remains to be determined if this persists following antigen-stimulation or injury. Also, while we identified that most cytokines assayed in the cytokine array were not different in
the plasma of c-myb<sup>h/h</sup> mice, nonetheless, it remains possible that the local tissue-restricted expression of these same cytokines may be perturbed. Additionally, while we showed that protection from atherosclerosis occurred with BMT of c-myb<sup>h/h</sup> BM into Ldlr<sup>-/-</sup> c-myb<sup>WT</sup> mouse, it remains to be determined if c-myb also has a role in the contribution of vessel-resident cells in the pathogenesis of atherosclerosis. As c-myb has functions in VSMCs, adventitial progenitor cells, and likely endothelial cells, it is likely that vascular-resident cells will also have altered function in Ldlr<sup>-/-</sup> c-myb<sup>h/h</sup> mouse. In the preceding chapter, we demonstrated that c-myb has roles both in vessel-intrinsic as well as BM-mediated protection from vessel remodeling using reciprocal BMTs[370]. Thus, it is likely that defective c-myb activity in vessel-resident cells would also confer some level of protection during the pathogenesis of atherosclerosis. Thus, studies involving Ldlr<sup>-/-</sup> c-myb<sup>h/h</sup> mice transplanted with c-myb<sup>WT</sup> BM could clarify if c-myb also has roles in vessel resident cells in the pathogenesis of atherosclerosis.

It is interesting to note that in the injury and blood pressure studies, we see defective collagen expression and then increased collagen deposition. These seemingly paradoxical results may be in part due to changes in MMP expression in c-myb<sup>h/h</sup> vessels affecting ECM turnover and degradation. While decreased expression of collagens in atheroma might suggest that plaques are less structurally stable, concomitant decreases in MMPs might mitigate any weaknesses in atheroma, however plaques would presumably still be exposed to hemodynamic forces which may cause plaque rupture. Future studies could examine more advanced lesions to see if lesions of Ldlr<sup>-/-</sup> c-myb<sup>h/h</sup> are more susceptible to plaque rupture. Additionally, because c-myb has been shown to regulate collagen and other ECM components[136] and we observe increased collagen deposition basally in c-myb<sup>h/h</sup> mice, it would be interesting to examine what role c-myb has in the pathogenesis of aortic aneurysm.
The results of the studies of *c-myb* function in vessel-resident Sca1*+* VSMC progenitor cells, the role of *c-myb* in the pathogenesis of atherosclerosis and the role of *c-myb* in regulating blood pressure homeostasis add significantly to the understanding of the role of *c-myb* in cardiovascular physiology. Through multiple in vitro and in vivo models of cardiovascular diseases, this dissertation has expanded significantly on the role of *c-myb* in cardiovascular physiology. Significant new insights have also created further areas of investigation that will hopefully shed further insight into processes that can be targeted in cardiovascular disease.
6 References


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