Characterizing The Transcriptome of Sirt6-Deficient Aortic Smooth Muscle Cells

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

Several vascular diseases are marked by dysfunctional vascular smooth muscle cells (VSMCs). Our group has found that the knockout of the NAD$^+$-dependent histone deacetylase sirtuin 6 (Sirt6), specifically in VSMCs, increases oxidative stress-induced DNA damage, inflammation, and aortic aneurysms in mice. To study the molecular mechanisms that drive VSMC dysfunction in Sirt6-deficiency, I established a primary culture model of Sirt6 deletion in VSMCs with Cre-lox technology. Through RNA sequencing of Sirt6-deficient VSMCs, we have identified modest but coordinated upregulations in transcripts involved in nucleosome assembly, inflammation, cell death, and autoimmunity. Immunostaining in histological sections of VSMC-specific Sirt6-deficient mice exposed to increased oxidative stress via angiotensin II infusion revealed aberrant localization of proteins normally in the nucleus that could induce inflammation. We propose that aortic Sirt6 has an anti-inflammatory role in the vessel wall.

Keywords

Sirtuin 6, vascular smooth muscle cell, inflammation, RNA sequencing
Summary for Lay Audience

Cardiovascular diseases are the leading cause of death worldwide. Many of these diseases arise when a critical component of blood vessels called the vascular smooth muscle cell (VSMC) does not function properly. We and others have found that the absence of a protein within the body called sirtuin 6 (Sirt6) may lead to catastrophic dysfunction of blood vessels. Therefore, the purpose of this thesis was to examine how this process may work in mouse VSMCs.

I created a model of VSMCs that lack the gene for Sirt6. I then investigated the genes that are increased or decreased in the absence of Sirt6. In this study, I discovered a pathway that stimulates aggressive inflammation of blood vessels.

This knowledge could help in developing diagnostic techniques or therapies aimed at preventing the development of inflammatory vascular diseases.
Co-Authorship Statement

All chapters of this thesis were written by Ryan Wong and revised with recommendations from Dr. Hao Yin and Dr. J. Geoffrey Pickering. All schematics and artwork were created by Ryan Wong using BioRender.

The data presented in Chapter 2 is intended for adaptation and submission as an original research manuscript to a peer-reviewed journal. Ryan Wong was the primary contributor to all aspects of this study including study design and experimentation, data acquisition and quantification, analysis, and writing. Contributions to the work in this chapter include Dr. Jason Lee and Sharon Leung (execution of in vivo angiotensin II infusion experiment). Dr. Zengxuan Nong assisted with immunostaining. Dr. J. Geoffrey Pickering conceived and coordinated the study.
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To Dr. Hao Yin, your wealth of knowledge and enthusiasm has been invaluable to the completion of this degree. Thank you for teaching me all of the lab techniques I needed to complete this degree and lending a positive frame of mind even when experiments did not go to plan. I would also like to thank Dr. Jason Lee for guiding me through the beginning of this project. Thank you to Caroline O’Neil for keeping this lab together and making sure there were always reagents to use, and thank you to Dr. Zengxuan Nong for all your histology work. I would also like to thank my lab members Justin Ching-Johnson, Sabrina Staples, and Kevin Moore, for your friendship throughout this whole experience.

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<tr>
<td>AAA</td>
<td>Abdominal Aortic Aneurysm</td>
</tr>
<tr>
<td>Adeno-Cre</td>
<td>Adenovirus Containing Cre</td>
</tr>
<tr>
<td>Adeno-Null</td>
<td>Adenovirus Null Vector</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BER</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>CD45</td>
<td>Cluster of Differentiation 45 (Common Leukocyte Antigen)</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
</tr>
<tr>
<td>CHIP</td>
<td>C-Terminus of HSC70-Interacting Protein</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts per million</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C Motif Chemokine Ligand</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-Associated Molecular Patterns</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>ES</td>
<td>Enrichment Score</td>
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<tr>
<td>FDR</td>
<td>False Discovery Rate</td>
</tr>
<tr>
<td>FoxO1</td>
<td>Forkhead Homeobox 1</td>
</tr>
<tr>
<td>G6p</td>
<td>Glucose-6-Phosphatase</td>
</tr>
<tr>
<td>GCA</td>
<td>Giant Cell Aortitis</td>
</tr>
<tr>
<td>GCN5</td>
<td>General Control Non-Repressed 5</td>
</tr>
<tr>
<td>GH</td>
<td>Growth Hormone</td>
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<tr>
<td>GLUT1</td>
<td>Insulin Independent Glucose Transporter 1</td>
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<tr>
<td>GLUT4</td>
<td>Insulin Dependent Glucose Transporter 4</td>
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<tr>
<td>GOBP</td>
<td>Gene Ontology Biological Processes</td>
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<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>H3K9</td>
<td>Histone 3, Lysine 9</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia inducible factor 1α</td>
</tr>
<tr>
<td>IA</td>
<td>Infectious Aortitis</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopaedia of Genes and Genomes</td>
</tr>
<tr>
<td>KLF4</td>
<td>Krüppel-Like Factor 4</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
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LDH  Lactate Dehydrogenase
MIP-2  Macrophage Inflammatory Protein-2
miR  Micro RNA
MMP  Matrix Metalloproteinases
mRNA  Messenger RNA
mT/mG  tdTomato/eGFP
MYH11  Smooth Muscle Myosin Heavy Chain 11
MYOCD  Myocardin
NA  Non-Infectious Aortitis
NAD+  Nicotinamide Adenine Dinucleotide
NADPH  Nicotinamide Adenine Dinucleotide Phosphate-Hydrogen
NAM  Nicotinamide
NAMPT  Nicotinamide Phosphoribosyltransferase
NES  Normalized Enrichment Score
NET  Neutrophil Extracellular Trap
NF-κB  Nuclear Factor κB
NMN  Nicotinamide Mononucleotide
NMNAT  NMN Adenylyltransferase
OCT4  Octamer Binding Transcription Factor
PARP  Poly(ADP-Ribose) Polymerases
PC  Principal Component
PCA  Principal Component Analysis
PEPCK  Phosphoenolpyruvate Carboxykinase
PFK1  Phosphofructokinase
PGC-1α  Proliferator-Activated Receptor γ Coactivator-1α
qPCR  Real-Time Quantitative Polymerase Chain Reaction
RANTES  Regulated on Activation, Normal T Cell Expressed and Secreted
rDNA  Ribosomal DNA
RNA  Ribonucleic Acid
RNA-seq  Ribonucleic Acid Sequencing
ROS  Reactive Oxygen Species
RUNX2  Runt-Related Transcription Factor 2
SE  Standard Error
scRNAseq  Single Cell RNA Sequencing
Sir2  Silent Information Regulator 2
SIRT  Sirtuin
SIRT6KO  Sirtuin 6 Knockout
SIRT6WT  Sirtuin 6 Wildtype
SLE  Systemic Lupus Erythematosus
SMMHC  Smooth Muscle Myosin Heavy Chain
SM22α  Transgelin
SOX9   SRY-Box Transcription Factor 9
SRF    Serum Response Factor
TAA    Thoracic Aortic Aneurysm
TLR    Toll-Like Receptor
TNF-α  Tumour Necrosis Factor α
TPI    Triose Phosphate Isomerase
VSMC   Vascular Smooth Muscle Cell
WT     Wildtype
αSMA   Smooth Muscle α-Actin
λ      Wavelength

Sirtuin 6 Nomenclature
Sirt6   Murine Sirtuin 6 Protein
Sirt6   Murine Sirtuin 6 gene, messenger RNA, or DNA
SIRT6   Human Sirtuin 6 Protein
SIRT6   Human Sirtuin 6 gene, messenger RNA, or DNA
Chapter 1

1 General Introduction

1.1 Overview

Cardiovascular diseases are the leading cause of death worldwide, claiming 17.9 million lives annually, accounting for 32% of global deaths.\(^1\) A key determinant of vascular health is the condition of the vasculature and the health of the cells that comprise this vital system. One such cell type are the vascular smooth muscle cells (VSMC), which are the main constituent of the vascular media and a critical component to the integrity of arteries. VSMCs are responsible for supplying contractile tone, regulating blood pressure and maintaining the extracellular matrix.\(^2\)

Several vascular diseases can arise from the dysfunction of VSMCs, including atherosclerosis, restenosis, and aortic aneurysm.\(^3\)\(^-\)\(^5\) Unlike many adult somatic cells, VSMCs can dedifferentiate in response to injury, transitioning from a quiescent, contractile cell to a proliferative and migratory phenotype.\(^6\)\(^\)\(^7\) Through this phenotypic transition, VSMCs can exhibit characteristics of other cell types such as mesenchymal cells, macrophages, and osteoblasts, which are often seen in disease states.\(^8\)\(^\)\(^9\)
Understanding the complex regulation of the smooth muscle cell and its many phenotypes is therefore important to characterizing vascular disease.

Recently, our research group has found a novel link between the NAD\(^+\)-dependent histone deacetylase sirtuin 6 (SIRT6) and the development of aortic aneurysm, dissection, and aortitis.\(^10\) Sirtuin 6 is found in the nucleus, and has the ability to regulate processes that include inflammation, pluripotency, genomic stability, and longevity.\(^11\) Thus, SIRT6 may potentially modulate VSMCs in disease states.
The advent of next generation RNA sequencing (RNA-seq) has allowed the unbiased interrogation of transcriptional changes within a cell to uncover previously unrecognized pathways. The broad goal of my research was to understand the molecular regulation in an VSMC in the absence of Sirt6. To address this goal, I have pursued three specific objectives:

1) I generated an in vitro model of Sirt6 deficiency in mouse VSMCs using Cre-lox recombination.
2) I analyzed the transcriptome of Sirt6-deficient VSMCs using next generation RNA sequencing.
3) I validated select transcriptomic findings histologically on aortic sections from Sirt6-deficient mice.

1.2 Aortic structure and function

The aorta is the largest artery in the body, carrying oxygenated blood away from the left ventricle of the heart to all downstream blood vessels. It has two main functions: (1) to act as a large conduit for blood and (2) to provide elastic buffering for pulsatile stresses in response to ventricular contraction. The aorta can be divided into four anatomic sections beginning at the aortic root, which leads into the ascending thoracic aorta. The descending thoracic aorta begins just past the left subclavian artery and extends down to the diaphragm. The abdominal aorta begins just after the diaphragm down to the common iliac arteries (Figure 1.1A). The ascending thoracic portion bears the greatest hemodynamic stress, with the transmural pressure diminishing as blood flows down the aorta.
At a tissue level, the aortic wall consists of three distinct layers: the innermost layer, tunica intima, is comprised of an endothelial cell monolayer, supported by the basal lamina, and a subendothelial layer containing collagen, elastic fibrils, VSMCs, and fibroblasts. The middle layer, tunica media, has multiple layers of elastin and VSMCs. In humans, the tunica media is made up of 40 to 50 concentric rings of elongated VSMCs, collagen fibrils, and elastic lamellae, whereas, in mice, there are only 3 to 8 layers. The adventitia is the outermost layer and contains a heterogeneous collection of cells, including fibroblasts, progenitor cells, and immune cells such as macrophages, T-lymphocytes, and B-lymphocytes. (Figure 1.1B).

**Figure 1.1 Diagram of the aorta.**
A) Gross anatomy of the aorta. B) Layers that comprise the aortic wall.
1.3 Diseases of the aorta

The aorta is subjected to vast hemodynamic and oxidative stresses, and is thus central to several disorders impacting aortic structure and function. Ageing, environmental stresses, disease, and genetic predisposition can contribute to the weakening of the aorta and eventual failure. Below I describe three commonly manifested human aortic disorders.

1.3.1 Aortitis

Aortitis is the all-encompassing term for the inflammation of the aorta and is characterized by the infiltration of lymphocytes, macrophages, and multinucleated giant cells into the aortic media and adventitia. There are two main forms of aortitis, the more common non-infectious aortitis (NIA), and the less common infectious aortitis (IA). NIA is usually caused by one of two conditions: Giant cell arteritis (GCA), which carries a prevalence of 220 per million in the UK, or a lifetime risk of and 1% in males and 0.5% in females in the US, or Takayasu arteritis (TA) which carry an incidence of 0.4 to 2.2 per million. There are few pathological features of NIA, including fibrosis, scarring of the aorta, also called “tree barking”, and granuloma formation. GCA is also associated with medial inflammation, and the formation of aortic aneurysms. TA is associated with intimal and adventitial fibrosis that may lead to luminal narrowing. Infectious aortitis occurs when there is an infiltration and colonization of pathogens in the aortic wall, either through pre-existing lesions or direct infiltration. In bacterial aortitis, atherosclerotic lesions or aneurysmal sacs are infiltrated with cells that mediate a chronic inflammatory response. Tuberculosis aortitis results from direct infiltration of the aorta from adjacent
infected tissues or blood.\textsuperscript{16} Moreover, Syphilitic aortitis is associated with thoracic aortic aneurysms.\textsuperscript{16}

1.3.2 Aortic aneurysm

Aortic aneurysms are defined by permanent, localized dilation of the aorta caused by pathogenic remodelling of the aortic wall.\textsuperscript{20} They are the second most common aortic disease after atherosclerosis.\textsuperscript{21} Aortic aneurysms can occur anywhere along the aorta, and are categorized by location. Aneurysms found above the diaphragm are classified as thoracic aortic aneurysms (TAA), and aneurysms found below the diaphragm are classified as abdominal aortic aneurysms (AAA).\textsuperscript{20} AAAs occur more commonly than TAAs, with a prevalence of 1.0-14.2\% in men, and 0.2-6.4\% in women, with the rate increasing with age.\textsuperscript{22} It is currently thought that AAAs are caused by chronic inflammation believed to lead to the destruction of the aortic media. Inflammation also leads to smooth muscle cell apoptosis, and the release of matrix metalloproteinases (MMP) and cytokines.\textsuperscript{23} Meanwhile, the incidence of TAAs ranges from 5 to 10 per 100,000 person-years.\textsuperscript{21} While TAAs are more frequent in men, women have poorer outcomes, being three times more likely to experience aortic dissection and rupture.\textsuperscript{21} TAAs can be brought on by genetic disorders of connective tissues such as Marfan’s syndrome, Ehlers–Danlos syndrome, Loeys–Dietz syndrome and Turner syndrome.\textsuperscript{24} However, non-syndromic causes of TAA may include bicuspid aortic valves, inflammatory conditions, familial thoracic aortic aneurysm and dissection, arteritis, syphilis, atherosclerosis, and trauma\textsuperscript{25–27} Aortic aneurysms have been described as “ticking time bombs” as these lesions are often asymptomatic but can progress to acute and deadly dissections and ruptures if left unmanaged.
1.3.3 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of medium and large arteries. Devastating effects of this disease occur when atherosclerotic plaques rupture or erode, creating thrombi that can cause the occlusion of arteries, leading to myocardial infarctions and stroke. Atherosclerotic plaques can be classified as unstable plaques versus stable plaques. Unstable plaques can easily rupture and are characterized by an accumulation of macrophages, a large necrotic core, and a thin fibrous cap comprised of phenotypically modulated VSMCs. Conversely, stable plaques have thick fibrous caps with a lower ratio of macrophages to VSMCs.

1.4 Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMCs) are the dominant cell type in the aortic media and are integral to the structural integrity of the aortic wall. During embryonic development, aortic VSMCs are derived from at least four different pools of stem cells. Vascular SMCs of the ascending aorta mostly derive from the second heart field, and cardiac neural crest, whereas those of the descending aorta mostly derive from the sclerotome, which includes cells from both the splenic mesoderm and somitic compartment.

Unlike many somatic cells, VSMCs retain characteristics of plasticity, being able to convert between two phenotypes that carry out their vital functions of (1) providing contractile tone, or (2) depositing, assembling, and maintaining the extracellular matrix (ECM). Ordinarily, VSMCs exist in a quiescent or “contractile” state where proliferation and synthesis of extracellular proteins proceed at a low rate. These mature, contractile
VSMCs can be identified by markers such as α-smooth muscle actin (αSMA), calponin, transgelin (SM22α), and smooth muscle myosin heavy chain (SMMHC), which are necessary for contraction. The plasticity of VSMCs also extends into the ability to transdifferentiate into cell types akin to immune cells and osteoblasts. This transdifferentiation is believed to drive vascular diseases such as atherosclerosis.

The phenotypic plasticity of VSMCs is mostly manifested in situations such as injury. Various stimuli such as growth factors, inflammatory signals, and mechanical forces can lead to VSMC dedifferentiation into a “synthetic” state whereby contractile proteins are downregulated. This phenotype modulation into a mesenchymal-like state allows VSMCs to migrate, proliferate, and synthesize ECM. A consequence of this plasticity is that aberrant signals from injury may cause a phenotypic switch when it is potentially unnecessary in the vasculature and contribute to vascular diseases such as atherosclerosis, aortic aneurysms, vascular inflammation, and surgical complications such as intimal hyperplasia.

The advent of single-cell sequencing (scRNAseq) has allowed the detailed characterization of “synthetic” VSMCs in disease states. For instance, VSMCs can take on properties of mesenchymal cells, fibroblasts, osteocytes, adipocytes, and macrophages. It is understood that the transcription factors krüppel-like factor 4 (KLF4), myocardin (MYOC), and octamer binding transcription factor (OCT4) have a key role in orchestrating these phenotypic changes.

KLF4 aids the transition of VSMCs into a pathogenic mesenchymal-like state by repressing the expression of the contractile genes SM22α, SMMHC, and αSMA. KLF4 acts via a cascade of events that include (1) binding to the G/C repressor region, (2)
recruiting histone deacetylases, (3) preventing the binding of serum response factor (SRF) to CArG elements and (4) antagonizing the binding of MYOC to SRF.\textsuperscript{44} The binding of MYOC to SRF, which exclusively occurs in cardiac and VSMCs, is required for the expression of contractile genes.\textsuperscript{44} Moreover, KLF4 can facilitate the transition to an osteogenic-like cell by activating runt-related transcription factor 2 (RUNX2) or SRY-box transcription factor 9 (SOX9)-induced transcriptional programming.\textsuperscript{45} KLF4 has also been associated with the transition of VSMCs to adipocyte-like, and fibroblast-like cells, though the mechanism has yet to be fully characterized.\textsuperscript{39}

Conversely, the expression of OCT4 may be protective against pathogenic dedifferentiation of VSMCs, with the expression of OCT4 being protective in mouse models of atherosclerosis by increasing the expression of migration-related genes.\textsuperscript{46} Moreover, OCT4 modulates VSMCs into a myofibroblast-like state to increase the expression of fibronectin, collagen-1 \(\alpha\)-1, and proteoglycans.\textsuperscript{44} Interestingly, OCT4 associates with promoters of genes that produce opposing phenotypes to KLF4 expressing VSMCs.\textsuperscript{47}

1.4.1 Primary culture of vascular smooth muscle cells

VSMCs, like many other cultured cells, will undergo many changes in phenotypes \textit{in vitro}. VSMCs in culture exhibit a shift to a ‘synthetic’ state, with a progressive downregulation of contractile markers \(\alpha\)SMA, calponin, SM22\(\alpha\) and SMMHC, and an associated upregulation in proliferation and migration. Morphologically, cells can lose their spindle-like shape and become a more rounded, rhomboid shape.\textsuperscript{7,35,48}

While there are many changes to the phenotype of VSMCs grown \textit{in vitro} compared with their \textit{in vivo} counterparts, there are many advantages to using cultured
cells to study disease states. Using cell culture allows better control of experimental variables such as growth conditions and genetic manipulation. Moreover, the homogeneity of cells removes the possibility of interfering environmental variables such as secretions from other cell types, which allows for data generation with a higher potential for reproducibility and consistency. Lastly, primary cell culture models allow for the amplification of biological material needed for genome sequencing.

1.5 Nicotinamide adenine dinucleotide and the NAMPT-mediated salvage pathway

Nicotinamide adenine dinucleotide (NAD\textsuperscript{+}) is an important dinucleotide in metabolic oxidation-reduction reactions. NAD\textsuperscript{+} can also act as a co-substrate in a diverse set of essential cellular processes, being consumed in the enzymatic reactions catalyzed by poly(ADP-ribose) polymerases (PARPs), cyclic ADP-ribose synthases, and sirtuins\textsuperscript{49}. These reactions produce a fluctuation in NAD\textsuperscript{+} levels that can influence how these enzymatic pathways function.\textsuperscript{50} Therefore, NAD\textsuperscript{+} levels must be maintained, and NAD\textsuperscript{+} is vital for cell function.

NAD\textsuperscript{+} can be synthesized de novo from dietary tryptophan, however, most NAD\textsuperscript{+} is produced in the salvage pathway.\textsuperscript{51} In this process, the NAD\textsuperscript{+} metabolite nicotinamide (NAM) is converted into nicotinamide mononucleotide (NMN) via the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT) and into NAD\textsuperscript{+} via NMN adenylyltransferase (NMNAT) (Figure 1.2).\textsuperscript{50,52} Interestingly, deletion of NAMPT causes developmental failure of mouse embryos and accelerates ageing-related mouse tissue dysfunction in the brain, skeletal muscle, and liver if ablated postnataally.\textsuperscript{53-56}
Figure 1.2 The NAD$^+$ salvage pathway associated with sirtuin cellular localization. NAMPT is the rate-limiting factor in the NAD$^+$ salvage pathway that converts NAM to NMN. Sirtuins are localized at different compartments of the cell. Some sirtuins are found in more than one cellular compartment. Sirtuins 3, 4, and 5 found in the mitochondria, sirtuins 1 and 2 are found in the cytoplasm, and sirtuins 1, 2, 5, 6, and 7 are found in the nucleus.
1.5.1 NAMPT knockout in VSMCs

Recently, our group found a role for NAMPT in aortic VSMC function.\textsuperscript{37,38} NAMPT was found to be reduced at both a transcript and protein level in the aortic media of patients with dilated ascending aortopathies. Moreover, there was an inverse relationship between VSMC NAMPT content and aortic diameter.

To understand the role of NAMPT in aortic health, an VSMC-specific Nampt knockout (Nampt-KO) was generated in mice. Nampt-KO alone created modest aortic dilation in ascending and descending thoracic regions. However, the addition of angiotensin II (AngII) infusion in Nampt-KO mice produced striking medial haemorrhage and dissection in 62.5\% of mice. Compared to control mice, Nampt-KO mice had a decrease in medial area in the descending thoracic aorta associated with a loss of VSMCs.

The loss of cells in the aortic media was driven by an increased susceptibility for Nampt-KO mice to induce premature VSMC senescence. Staining for oxidized nucleoside 8-oxo-2’-deoxyguanosine revealed a propensity to accumulate double-stranded DNA breaks, a driver of cell senescence.\textsuperscript{39} Moreover, culturing VSMCs with H$_2$O$_2$ increased single-stranded DNA fragments in Nampt-KO VSMCs via the suppression of PARP-1 activity. Notably, DNA strand breaks were also enriched in human aneurysmal ascending aortic VSMCs with low levels of NAMPT, highlighting a role for NAMPT in maintaining the structural integrity of the aorta.

1.6 Sir2 and sirtuins

Downstream of the NAD\textsuperscript{+} salvage pathway is an important conserved family of sirtuin proteins (SIRT1-7). Sirtuins are NAD\textsuperscript{+}-dependent, Class III histone/protein
deacetylases (HDAC). Mammalian sirtuins were originally described as homologues of the *Saccharomyces cerevisiae* transcriptional regulator silent information regulator 2 (*Sir2*), which silences mating-type locus genes. Sir2 and sirtuins gained prominence in the scientific community when Sir2 was shown to prolong the lifespan of *S. cerevisiae* by reducing the formation of deleterious rDNA circles thought to contribute to ageing in yeast.

Sirtuins are expressed ubiquitously but localized in different cellular compartments of the cell. In their respective compartments, sirtuins primarily exert protein deacetylation of both histone and nonhistone substrates. By far, SIRT1 is the most studied sirtuin. It is normally localized in the nucleus but can translocate to the plasma membrane and cytoplasm under stress and disease states (Figure 1.2). SIRT2 has isoforms in both the nucleus and cytoplasm. SIRT6 is a nuclear sirtuin, and SIRT7 is localized to the nucleolus (Figure 1.2). SIRT3 and SIRT4 are localized to the mitochondria. And SIRT5 has isoforms in the mitochondria and the nucleus (Figure 1.2).

In the nucleus, sirtuins primarily act as regulators of gene expression by deacetylating histones on lysine residues or protein substrates such as the acetyltransferase general control non-repressed 5 (GCN5). The deacetylation of histones creates a closed conformation of chromatin, preventing the binding of RNA polymerase II to DNA, thus inhibiting gene transcription. In the mitochondria, sirtuins regulate energy metabolism and the mitochondrial stress response. All sirtuins have mono-ADP-ribosyl transferase activity except for SIRT5. Importantly, sirtuins can be stimulated by an increase in NAD⁺ concentration, and conversely, a decrease in NAD⁺
can suppress their activity. SIRT6 can uniquely bind NAD$^+$ in the absence of an acetylated substrate. Hence it has been proposed that sirtuins act as biosensors for metabolism.\textsuperscript{70,71}

1.7 Sirtuin 6

The nuclear sirtuin, SIRT6, has emerged as a particularly interesting sirtuin due to the ability to regulate a wide range of vital processes including lifespan (Figure 1.3). SIRT6 has been found to regulate DNA repair, telomere maintenance, and cellular metabolism, including glycolysis and gluconeogenesis, lipogenesis and lipolysis, inflammation, tumour suppression, and pluripotency. Through these functions, SIRT6 contributes to the overall longevity and health of mammals.\textsuperscript{72,11}

Biochemically, SIRT6 is primarily recognized as an NAD$^+$-dependent HDAC, with mild mono-ADP-ribosyltransferase activity. It carries out its HDAC activity on acetyl groups on lysine 9 (H3K9ac), lysine 18 (H3K18ac) and lysine 56 (H3K56ac) residues of histone H3, regulating chromatin silencing.\textsuperscript{73} SIRT6 can also directly deacetylate non-histone proteins such as FOXO1, GCN5, PKM2 and NAMPT\textsuperscript{72}. SIRT6 also exhibits lysine deacylation activity, including for TNF-\textalpha, which results in activation\textsuperscript{74}. Interestingly, the deacylation activity of SIRT6 is 300 times higher than its deacetylation activity, further illustrating the multifaceted role of SIRT6 in the regulation of several biological processes.\textsuperscript{75}
Figure 1.3 The multifaceted roles of sirtuin 6.
The main function of SIRT6 is as a transcriptional regulator of several genes through histone deacetylation. SIRT6 can also directly deacetylate, ADP-ribosylate, and deacetylate proteins. Figure adapted with permission from Kugel and Mostoslavsky, 2014.
1.7.1 Regulation of sirtuin 6 expression and stability

Murine Sirt6 is expressed highest in the muscle, brain, and heart. However, several factors change how and when Sirt6 is expressed. For instance, Sirt6 levels will decline with age. Regulation of Sirt6 expression happens at both a protein and mRNA level. The expression of two microRNAs, miR-34a and -122, have been correlated with decreases in Sirt6. miR-34a has been shown to be inversely correlated with Sirt6 in squamous cell carcinomas. Similarly, miR-33a, -33b, -122, and -766 have been shown to bind directly to the 3’UTR of Sirt6 to interfere with translation into a protein. In intestinal epithelial cells, interferon-γ inhibits Sirt6 gene expression by inducing expression of miR-92b, which also binds to the 3’UTR of Sirt6 to interfere with its translation.

Sirt6 can also be regulated transcriptionally. The transcription factor, c-FOS, binds to an AP-1 binding site at the Sirt6 promoter, activating Sirt6 gene expression. Conversely, Sirt6 is negatively regulated by PARP-1. Also, E2F1 directly binds the Sirt promoter and suppresses gene expression activity under both normoxic and hypoxic culture conditions.

Post-translationally, the stability of Sirt6 depends on a ubiquitin ligase called c-terminus of HSC70-interacting protein (CHIP), which protects it from proteasomal degradation. The free fatty acid palmitate reduces Sirt6 by downregulating the expression of CHIP mRNA. Moreover, a ubiquitin-specific peptidase USP10 can also protect Sirt6 from proteasomal degradation. Metabolic status is also a potent regulator of Sirt6 expression. Sirt6 levels increase in mice after fasting. Calorie restriction (low glucose) in culture has also been shown to increase Sirt6 expression in fibroblasts.
1.7.2 Sirtuin 6 knockout and transgenic animals

A seminal study of global Sirt6 knockout (SIRT6KO) in mice by Mostoslavsky et al. found induction of lymphopenia, loss of subcutaneous fat, lordokyphosis, osteopenia, and eventual death from severe hypoglycemia in mice by postnatal day 28. At the cellular level, knockout of Sirt6 in MEFs decreased proliferation and increased genomic instability and sensitivity to DNA damaging agents. Thus, SIRT6KO induced deficiencies in base excision repair (BER).

A separate study sought to understand the mechanisms that produced the severe metabolic phenotype in SIRT6KO mice. Interestingly, the hypoglycemic phenotype was driven by an upregulation in the insulin-independent glucose transporter 1 (GLUT1) and insulin-dependent glucose transporter 4 (GLUT4). Moreover, early lethality in these mice was rescued by feeding mice with glucose-containing water. Notably, mice that survived beyond four weeks of age went on to develop chronic liver inflammation at two months of age.

SIRT6 is also critical in humans as its loss in embryonic development results in perinatal lethality. Conversely, overexpression of SIRT6 has been shown to be beneficial, protecting against premature senescence in human VSMCs and nucleus pulposus cells. Another study investigating Sirt6 overexpression in mice also found that overexpression led to increased glucose tolerance, reduced adipose inflammation, and a more youthful metabolic profile. Moreover, overexpression of Sirt6 in mice leads to an increase in lifespan from a decrease in insulin-like growth factor-1 (IGF-1), and decreased IGF-1 signalling.
The effect of SIRT6KO is tissue dependent. As such, tissue-specific knockouts of Sirt6 have been generated in the brain, fat, liver, and heart. Notably, neural ablation of Sirt6 does not lead to fatal hypoglycemia but instead retards the growth of mice at four weeks of age. However, mice will reach their normal weight at 6-8 months of age, albeit with increased adiposity. This phenotype was likely brought on by a decrease in signalling from growth hormone (GH) and IGF-1. Fat-specific ablation of Sirt6 led to increased adiposity and adipose tissue inflammation. Liver-specific ablation of Sirt6 led to increased glycolysis, triglyceride synthesis, and the formation of a fatty liver. Lastly, heart-specific SIRT6KO mice developed cardiac hypertrophy from hyperactivated IGF signalling.

Therefore, Sirt6 is an essential protein in maintaining growth, metabolism, and genomic integrity. Because many of these pathways are implicated in disease states, characterizing Sirt6 functions in different tissues is important.

1.7.3 Sirtuin 6 in glucose metabolism

During normoxic conditions, cells convert glucose into pyruvate, which is then transported into the mitochondria to produce ATP via the electron transport chain in aerobic respiration. However, under hypoxic conditions, cells will shift into anaerobic respiration, where cells use glycolysis to produce ATP. Since the discovery of hypoglycemia in SIRT6KO mice, there has been substantial investigation on the link between Sirt6 and the regulation of glucose metabolism. A study into the mechanisms that produced the hypoglycemic phenotype discovered that Sirt6 regulates glycolytic genes controlled by hypoxia inducible factor-1 (HIF-1α) through the deacetylation of H3K9 at target gene promoters. Specifically, there was an upregulation of GLUT1,
lactate dehydrogenase (LDH), triose phosphate isomerase (TPI), aldolase, and phosphofructokinase (PFK1).\textsuperscript{100}

Not only can Sirt6 drive glycolysis, but it can also increase the supply of circulating glucose through gluconeogenesis. Dominy et al. suggested a compensatory mechanism in response to the hypoglycemia seen in SIRT6KO mice. Peroxisome proliferator-activated receptor \(\gamma\) coactivator-1\(\alpha\) (PGC-1\(\alpha\)) is a key transcriptional regulator for gluconeogenesis which controls the expression of glucose-6-phosphatase (G6p) and phosphoenolpyruvate carboxykinase (PEPCK).\textsuperscript{101} Sirt6 directly deacetylates GCN5, a factor which represses PGC-1\(\alpha\) through acetylation. With the knockout of Sirt6, GCN5 activity is decreased, leading to the activation of PGC-1\(\alpha\) and downstream genes. Hence, the knockout of Sirt6 increases gluconeogenesis.

Another important transcription factor in gluconeogenesis regulated by Sirt6 is forkhead homeobox 1 (FoxO1). FoxO1 activates gluconeogenesis by binding directly to the promoters G6P and PEPCK, the rate-limiting factors in gluconeogenesis.\textsuperscript{102} Sirt6 can directly deacetylate FoxO1 and exclude the import of FoxO1 into the nucleus, thus reducing gluconeogenesis.

The ability of Sirt6 to orchestrate glucose metabolism is especially important in the context of the Warburg effect, which is the phenomenon that describes an increased use of glycolysis for ATP production.\textsuperscript{103,104} Immune cells are dependent on glycolysis for growth, survival and secretory functions.\textsuperscript{105} Therefore, Sirt6 regulation of glucose metabolism is important in the context of immunity and immune reactions.
1.7.4 Sirtuin 6 in inflammation

It has been proposed that Sirt6 is a master regulator in inflammation based on its control over glucose metabolism and the expression of inflammatory cytokines.\textsuperscript{103,106} Sirt6 regulates immune response pathways mainly through epigenetic changes carried out by its deacetylase activity and deacylation of proteins.

A critical component of the inflammatory response is a shift in metabolic programming from mitochondrial oxidative phosphorylation to aerobic glycolysis.\textsuperscript{105,107} Much like in cancer cells, where this process was first discovered and termed the Warburg effect, the switch to aerobic glycolysis supports the growth, differentiation, and effector functions of immune cells.\textsuperscript{105} Moreover, throughout the process of inflammation, inflammatory cells must switch between three key metabolic pathways: glycolysis, glutaminolysis, and oxidative phosphorylation.\textsuperscript{104}

SIRT6 is highly expressed in organs and cells that regulate the immune system, such as the thymus, appendix, spleen, lymph node, tonsil, bone marrow, and white blood cells.\textsuperscript{103} Thus, SIRT6 may facilitate the shifts in metabolic pathways needed for immune cell action.\textsuperscript{103} For instance, SIRT6 can antagonize the HIF-1$\alpha$ pathway by deacetylating H3K9 at target gene promoters.\textsuperscript{100} A key pathway regulating the Warburg effect and glycolysis is the serine-threonine kinase PI3/Akt, which upregulates the glucose transporter GLUT1 and activates PFK1.\textsuperscript{108} Akt-mediated phosphorylation also inhibits the translocation of FoxO1, which inhibits glycolytic gene expression.\textsuperscript{109} SIRT6-mediated fatty acid deacylation prevents R-Ras2 association with Akt, thus inhibiting the activation of Akt.\textsuperscript{110} Sirt6 also regulates Akt at a transcriptional level by physically interacting with
c-JUN, a transcription factor for Akt, where it is recruited to chromatin to deacetylate H3K9, halting transcription.

The nuclear factor kappa-B (NF-κB) family of transcription factors are comprised of five structurally similar proteins that can form homo- or heterodimers to modulate gene transcription. A common heterodimer is made up of p65/RelA, and p50, which is normally repressed by IκB.\textsuperscript{111} SIRT6 can bind to the NF-κB subunit RelA and deacetylate H3K9 at the promoters of downstream NF-κB genes which results in the downregulation of NF-κB-mediated inflammatory genes. Indeed, the knockdown of SIRT6 in human umbilical vein endothelial cells lead to an increase of pro-inflammatory cytokines IL-1β, IL-6, IL-8, and ECM remodelling enzymes MMP2 and MMP9.\textsuperscript{103,106,112} Moreover, SIRT6 can also upregulate the NF-κB inhibitor IκB which causes NF-κB to translocate into the cytoplasm.\textsuperscript{113} SIRT6 can also modulate Myc, a key transcription factor with roles in the metabolic shift to glycolysis through its control over glutaminolysis, and clonal expansion of immune cells.\textsuperscript{114} SIRT6 physically interacts with Myc and deacetylates H3K56 at the promoters of Myc-regulated ribosomal protein genes, thus reducing the translation of proteins.\textsuperscript{115} Moreover, through Myc, SIRT6 also decreases glutaminase expression, thereby regulating glutaminolysis.\textsuperscript{115}

SIRT6 can also regulate inflammation by increasing the secretion of tumour necrosis factor-α (TNF-α), a key inflammatory cytokine. SIRT6 can enhance the secretion of TNF-α by deacylating at lysine residues 19 and 20.\textsuperscript{74} Moreover, when overexpressed, SIRT6 increases the translational efficiency of TNF-α.\textsuperscript{116}
1.8 Sirtuin 6 in vascular disorders

Cardiovascular diseases (CVD) are currently the leading cause of death in humans, with nearly 18 million people dying each year. While the precise mechanisms that drive CVD are complex and have yet to be fully uncovered, SIRT6 has emerged as a potentially targetable modulator of many of the pathways seen in CVD.

1.8.1 Sirtuin 6 and atherosclerosis

Recently, Grootaert et al. found a protective role of SIRT6 against the development of atherosclerosis by preventing telomere-induced cellular senescence. Moreover, atherosclerotic plaques from human and mouse samples were deficient in SIRT6, and VSMCs grown from these samples were prone to senescence. The deficiency in SIRT6 was attributed to a decrease in CHIP, a protein that stabilizes SIRT6 and prevented proteasomal degradation. Overexpression of SIRT6 in mice prevented the development of atherosclerosis.

Another vascular complication of atherosclerosis is vascular calcification. In a study investigating calcification in chronic kidney disease, Sirt6 was found to suppress osteogenic transdifferentiation in VSMCs in mice. Sirt6 directly deacetylated the osteogenic transcription factor Runx2 and promoted its degradation. Thus, Sirt6 deficiency is emerging as an important factor in the process of atherosclerosis.

1.8.2 Sirtuin 6 and aortic disease, data from our lab

Oxidative stress threatens the integrity of the vascular wall. The dysregulated generation of reactive oxygen species (ROS) by nicotinamide adenine dinucleotide
phosphate-hydrogen (NADPH) oxidase is a key route to oxidative stress that has been implicated in the development of vascular pathologies. AngII can induce a marked increase in ROS by activating NADPH oxidase. Our group has found that the knockout of the NAMPT, the rate limiting enzyme in the NAD\(^+\) salvage/recycling pathway, results in a drastic increase in oxidative DNA damage, senescence, and loss of aortic integrity. This was in part due to an impaired action of the NAD\(^+\)-dependent DNA repair enzyme PARP-1. Thus, our group undertook a study to understand if the protein, SIRT6, which is also downstream of the NAD\(^+\) salvage pathway, is involved in the management of oxidative stress in the aorta.

We generated mice with a tamoxifen-inducible, VSMC-specific Sirt6 knockout by crossing male C57BL/6J mice carrying an allele for SMMHC-Cre-ER\(^{2}\) with a female FVB/6J mutant mice with loxP sites flanking exons 2-3 of Sirt6. To induce a knockout, 8 week-old mice were subjected to 5 days of tamoxifen injections. Compared with wild-type mice treated with corn oil, tamoxifen-induced SIRT6KO at alone did not alter the morphology of the aorta. The aortic sections from four regions of the aorta, the aortic arch, ascending thoracic, descending thoracic, and abdominal aorta, appeared healthy.

To understand how Sirt6 may impact prolonged oxidative stress, mice were then infused with 28 days of AngII via an implanted osmotic pump. This resulted in a pronounced and abnormal aortic phenotype. SIRT6KO mice treated with AngII had a marked increase in petechial haemorrhage as indicated by the presence of hemosiderin in the outer layers of the media and adventitia. Moreover, SIRT6KO mice also had increased aortic aneurysms marked by permanent dilation and loss of VSMCs in the media of the aorta. These effects were most pronounced in the ascending thoracic aorta.
Notably, 100% of SIRT6KO mice (n=6) had a proliferative inflammatory cell infiltration into the media and adventitia as indicated by positive leukocyte common antigen (CD45) and Ki-67 staining. These results showed that SIRT6KO, in conjunction with AngII, induce an aortitic phenotype.\textsuperscript{10}

This \textit{in vivo} work laid the foundation for this current study. While an inflammatory phenotype was seen \textit{in vivo}, we were curious to understand the altered pathways within VSMCs to produce this drastic phenotype. Moreover, overt aortitis is quite uncommon in mouse models.\textsuperscript{123} Therefore, I undertook an \textit{in vitro} approach to study how SIRT6KO alters the transcriptome of VSMCs.

### 1.9 Histones and inflammation

The genetic code within the nucleus is organized as strands of DNA tightly wound around proteins, collectively called chromatin (Figure 1.4A). The basic unit of chromatin is called the nucleosome core particle, consisting of 147bp of DNA wrapped approximately twice around an octamer of positively charged core histones. Each octamer is made up of two copies of each core histone: H2A, H2B, H3, and H4.\textsuperscript{124} Connecting each nucleosome is a strand of 10-60bp of DNA protected by histone H1.\textsuperscript{125}

Histones can be modified to change gene expression in two main ways, acetylation and methylation (Figure 1.4A). In the former, acetyl groups on lysine residues of histones weaken the positive charge on histones and thus the electrostatic interactions with DNA. This loosening of DNA wrapping around the histone octamer allows DNA to be accessible to RNA polymerase II for gene expression.\textsuperscript{126} Methylation, however, can
both silence or activate transcription, depending on the pattern and residue on which it is found.\textsuperscript{127}

However, an interesting case arises when histones are found outside of the nucleosome. Extracellular histones can behave as danger-associated molecular patterns (DAMPs) to activate the immune response and the release of inflammatory cytokines to induce sterile inflammation (Figure 1.4B).\textsuperscript{128} For example, circulating histones commonly found in sepsis can activate the nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome.\textsuperscript{129} The NLRP3 inflammasome is a cytosolic protein that coordinates the process of sterile inflammation by mediating the activation of caspase-1, and the secretion of IL-1\(\beta\)/IL-18 in response to microbial infection and cellular damage.\textsuperscript{130} Therefore, histones may activate sterile inflammation. Moreover, externalized histones from neutrophils can also induce lytic cell death in VSMCs by forming pores in the membranes of VSMCs.\textsuperscript{131}

Histones need not be released into the extracellular space to induce inflammation. Histones may traffic from the nucleus to the cytoplasm and to membrane-bound locations to induce inflammatory signalling.\textsuperscript{132} For example, the histone H3 has been found in the cytoplasm and on the plasma membrane of apoptotic microglia, which is thought to contribute to the creation of autoreactive nuclear antigens in autoimmune disease.\textsuperscript{132} The possibility of Sirt6 control in the expression and localization of histones has not yet been explored.
Figure 1.4 The role of histones in the cell
A) The organization of the DNA into histones, nucleosomes, and chromatin. Histones may be epigenetically modified with acetylation (Ac) or methylation (Me) marks. B) The role of extracellular histones in the process of inflammation.
1.10 Aims of the thesis

Cardiovascular disease remains the leading cause of death worldwide. Understanding the molecular mechanisms that drive these conditions will be paramount to developing diagnostic techniques and therapies. Our group and others have identified a key NAD+-dependent HDAC, Sirt6, as having a range of controls over processes that underlie cardiovascular disease. Importantly, knockout of Sirt6 in VSMCs produced an inflammatory phenotype which emulates aortitis.

The objective of my work was to characterize the transcriptome of Sirt6-deficient murine aortic VSMCs.

I hypothesize that the knockout of Sirt6 in VSMCs will uncover previously unknown Sirt6 regulated inflammatory pathways in this critical cell type of the vascular wall.

In chapter 2, my main objective was to uncover potential VSMC driven mechanisms that may contribute to inflammation, aneurysm, and VSMC loss in cardiovascular disease. I developed an in vitro system of Sirt6 deficiency from cultured murine aortic VSMCs. I then characterized the transcriptomes of these cells with next-generation RNA-seq. These data were then validated with independent cell culture experiments and histology from samples of Sirt6 deficiency in vivo.

The extended discussion in chapter 3 summarizes the thesis results, outlines the strengths and limitations of our findings, and describes future work that will aid in validating our novel pathway of VSMC-driven aortic inflammation.
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Chapter 2

2 Characterizing the transcriptome of Sirt6-deficient aortic smooth muscle cells

2.1 Introduction

Aortitis is the term that describes the devastating condition of an inflamed aortic wall. During aortitis, the inflammatory process is often aggressive and can result in the loss of vascular smooth muscle cells (VSMCs) and extracellular matrix (ECM). In this weakened state, the aorta can lose integrity, become aneurysmal, or dissect. The latter is a catastrophic and lethal event.

Broadly, there are two forms of aortitis: the more common non-infectious aortitis (NA) and less common infectious aortitis (IA). In the United States, NA is predominantly caused by giant cell arteritis (GCA), which carries a lifetime risk of 1% in women and 0.5% in men. In NA, the aortic media and adventitia are infiltrated with lymphocytes, macrophages, and multinucleated giant cells. NA is thought to be an autoimmune condition. However, specific antigenic stimuli have yet to be identified. Moreover, autoimmune disorders such as systemic lupus erythematosus (SLE) can lead to aortitis. IA most commonly occurs when lesions from atherosclerosis and aneurysmal disease become colonized by microorganisms such as bacteria. IA can also occur by direct infiltration of the aorta from surrounding infections of tissue or blood. Clinical diagnosis of aortitis is difficult since there are no biomarkers for aortitis, and symptoms often present in a non-specific manner. Currently, diagnosis of aortitis is suspected by echocardiography, and confirmed with computed tomography or magnetic resonance imaging.
imaging and positron emission tomography. The cellular mechanisms that drive aortitis are poorly characterized.

Sirtuin 6 (SIRT6) is a member of the sirtuin family of NAD+-dependent, class III histone deacetylase enzymes. Located in the nucleus, SIRT6 mainly remolds chromatin, deacetylating histone H3 at lysine residues (H3K) -9, -18 and -56, to regulate the transcription of several genes and pathways. For instance, SIRT6 can modulate the process of inflammation. NF-κB is a key transcription factor that regulates several inflammatory cytokines such as IL-1, IL-6, IL-8, IL-12, TNF-α and chemokines such as CP1, IL-18, RANTES, MIP-2, CXCL1, and CXCL10. SIRT6 deacetylates H3K9 at the promoters of NF-κB-regulated genes. As such, SIRT6 activity could potentially suppress the expression of several critical inflammatory cytokines released during the immune response. Another key process in the immune response is the upregulation of glycolysis in immune cells, termed the Warburg effect, through the expression of genes regulated by HIF-1α. SIRT6 may regulate inflammation through its deacetylation of H3K9 at glycolytic gene promoters downstream of HIF-1α, such as lactate dehydrogenase (LDH), triose phosphate isomersase (TPI), aldolase, and phosphofructokinase (PFK1). Moreover, the Warburg effect is also present in proliferating VSMCs in atherosclerosis. SIRT6 can also directly deacylate the inflammatory cytokine TNFα to modulate secretion. Importantly, all these pro-inflammatory cascades have been reported in cells or tissues outside the vasculature.

Recently, our group has found that the NAD+-dependent protein Sirt6 maintains the integrity of the aorta. We generated an VSMC-specific SIRT6 knockout mouse and subjected them to 28 days of AngII infusion. Compared with wildtype mice, SIRT6KO
mice exhibited increased dilation, rupture, haemorrhage, and a remarkable inflammatory infiltrate in the adventitia and into the media. These features were indicative of aortitis. However, the mechanistic roles of VSMC Sirt6 in the pathogenesis of aortitis are unknown.

In this study, I have undertaken an in vitro approach to study the effect of Sirt6 deficiency in VSMCs. I developed an VSMC culture system to effectively delete the Sirt6 gene in mouse VSMCs using Cre-lox technology. The transcriptomes of Sirt6-deficient, and Sirt6 wildtype VSMCs were then ascertained with next-generation RNA sequencing. Because the upregulation of histone expression was a profound finding, I also immunostained Sirt6 wildtype and knockout aortas to explore for extracellular localization. Overall, these findings uncover a previously unrecognized regulation of histones by Sirt6 and provide insights into the role of VSMCs in the progression of aortitis.

2.2 Methods

2.2.1 Animals

Experiments were conducted per the University of Western Ontario Animal Care Committee, which follows the policies set out by the Canadian Council on Animal Care. Male C57BL/6J mice carrying an SMMHC-Cre-ER² allele and Rosa26-mT/mG allele were crossed with female FVB/6J floxed mutant mice with loxP sites flanking exons 2-3 of Sirt6 (The Jackson Laboratory, Sirt6tm1.1Cxd/J, 017334) to produce male offspring heterozygous for floxed Sirt6 (Sirt6<sup>flox/wt</sup>) with Cre and mT/mG expression. These offspring were backcrossed for > 10 generations to produce Myh11-CreERT2, Rosa26-
mt/mG, Sirt6\textsuperscript{flox/wt} mice of C57BL/6J genetic background. These heterozygous offspring were then mated together to yield homozygous floxed mice (Figure 2.1. To verify the genotype of these mice, animals were anaesthetized with isofluorane, and tail samples were collected at 21 days of age. SIRT6 WT and mutant alleles were genotyped using primers P1: 5’ AGT GAG GGG CTA ATG GGA AC 3’ and P2: 5’ AAC CCA CCT CTC TCC CCT AA 3’ (Figure 2.2B). All currently known isoforms of murine Sirt6 contain exons 2-3, thus a removal of these sites would yield a non-functional transcript (Gene ID: 50721, updated on 23-Jun-2022).\textsuperscript{20-22}
Figure 2.1 Breeding strategy to produce C57BL/6J mice with **Sirt6**^flx/flx^, SMMHC-CreER^{12}, Rosa26-mT/mG mice.

1) FVB mice with Sirt6^flx/flx^ alleles were crossed with C57BL/6J mice carrying an SMMHC-CreER^{12}, Rosa26-mT/mG to create mice with Sirt6^flx/wt^ alleles. 2) Mice were backcrossed to a C57BL/6J for 10 generations. 3) C57BL/6J mice with Sirt6^flx/wt^ alleles were inbred to create C57BL/6J mice with SMMHC-CreER^{12}, Rosa26-mT/mG, and Sirt6^flx/flx^ alleles.
2.2.2 Isolation and culture of murine aortic smooth muscle cell

Cells were isolated per the protocol established by Ray et al. to generate VSMCs from a single murine aorta. Briefly, 6–8-week-old male mice were euthanized with isofluorane overdose. The murine abdominal cavity was opened down the midline of the animal. The aorta spanning from the ascending to suprarenal sections was removed from the abdominal cavity intact with the heart. Under a dissecting microscope (Olympus), the aorta was separated from the heart at the aortic root. Perivascular adipose tissue and adventitia was removed from the aorta with fine forceps. The aorta was splayed open and endothelial cells were scraped from the intimal side. The aorta was sectioned into 1-2 mm² fragments and enzymatically digested by collagenase type II (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C for 2-3 hours.

Digested cells were then pelleted, resuspended, and grown on 0.1% fibronectin-coated polystyrene cell culture dishes (Thermo Fisher Scientific, Waltham, MA, USA). VSMCs were initially grown with smooth muscle growth media-2 (SmGM-2, Lonza) to selectively promote the growth of VSMCs. Subsequent cell passages were performed by trypsinizing cells, pelleting and then seeding on fibronectin-coated polystyrene cell culture dishes with DMEM with 10% FBS. Cells were trypsinized and passaged every 2-3 days when cells reached confluence. Cells at passage 2 were used for adenoviral transduction.

2.2.3 Adenoviral transduction of Cre recombinase into VSMCs

To generate Sirt6 knockout (SIRT6KO) VSMCs, cells derived from mice with SMMHC-CreER², Rosa26-mT/mG, and Sirt6lox/lox alleles were transduced with
adenovirus containing Cre-recombinase (adeno-Cre) (Vector Biolabs, Malvern, PA, Cat #1045). To generate Sirt6 wildtype (SIRT6WT) cultures, cells were transduced with adenovirus without exogenous gene expression (adeno Null) (Vector Biolabs, Malvern, PA, Cat #1240) (Figure 2.2A). Cells were grown with 0.5% Poly-L-Lysine supplemented media during transduction to reduce electrostatic interactions with the adenovirus protein coat and to enhance transduction efficiency. Adenovirus was administered at a multiplicity of infection of 200. Following transduction for 24 hours, culture media was changed and reverted to DMEM supplemented with 10% FBS.
**Figure 2.2 Schematic of adenoviral transduction of VSMCs with Cre-recombinase.**

A) Schematic of cell culture and method to produce wildtype and Sirt6-deficient murine VSMCs. B) Adenovirus expressing Cre recombinase was used to induce cleavage at loxP sites flanking exons 2-3 of the Sirt6 gene. Simultaneously, Cre recombinase also cleaved Rosa26-mT/mG at loxP sites flanking tdTomato and the stop codon. The resulting expression was a truncated, non-functional Sirt6 gene and eGFP expression.
2.2.4 RNA harvesting and qPCR

Control and Sirt6-deficient cells were harvested for RNA at passage 4, 10 days after adenoviral transduction at Passage 2 to create wildtype and Sirt6-deficient VSMC. Qiazol® (Qiagen) and chloroform were added to cell samples and centrifuged for 15 min. The upper aqueous phase was mixed with 100% ethanol before being transferred to an miRNeasy column. From this point on, the miRNeasy Plus Mini Kit protocol (Qiagen) was used to complete RNA extraction. RNA was eluted with 20μl of RNase-free water. Total RNA samples were quantified using NanoDrop (Thermo Fisher Scientific, Waltham, MA). Three (3) samples of Sirt6-deficient and 3 samples of Sirt6-WT RNA were used for next generation sequencing. In a subsequent cell culture experiment, another 3 samples of Sirt6-deficient and 3 samples of Sirt6-WT RNA were harvested for qPCR.

RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) on a Mastercycler epGradient S thermocycler (Eppendorf AG). Using quantitative PCR (ViiA7, Applied Biosystems), abundances of mRNA for Sirt6 and select genes were compared to Beta-2-Microglobulin, a housekeeping gene. Each 10ul reaction contained 1 ng of cDNA/well, and samples were run in triplicate. Mouse Sirt6 transcript readings were normalized to B2M levels and analyzed using the ΔΔCT method on a ViiA7 PCR system (Life Technologies).

2.2.5 Immunocytochemistry

Aortic VSMCs grown on glass coverslips were fixed in 4% paraformaldehyde for 20 minutes and immunolabeled for Sirt6 (1:100, D8D12, Cell Signalling). Smooth
muscle α-actin was stained with Acti-stain™ 488 (1:150, PHDG1, Cytoskeleton Inc) or Acti-stain™ 555 (1:150, PHDH1, Cytoskeleton Inc). To detect Sirt6, a donkey anti-rabbit secondary antibody was used with an excitation wavelength of 488 nm (1:100, Cat# 711-545-152, Jackson ImmunoResearch). A DAPI-containing mounting media was used to counterstain for nuclei (Invitrogen, P36931). Cells were imaged with a Nikon AR-1 confocal microscope (Nikon). Positive nuclear signals as indicated by DAPI staining were counted to represent the total number of VSMCs. Cells with both DAPI and Sirt6 staining were counted and expressed as a fraction of total VSMCs to derive Sirt6 knockdown efficiency in SIRT6WT and SIRT6KO cells.

2.2.6 Histology

Sirt6-deficient and WT aortas from 12-week-old male mice were used for histology. WT and Sirt6-deficient mice were anaesthetized with isoflurane and perfusion-fixed at physiological pressure with 4% paraformaldehyde. Tissue was kept in 4% paraformaldehyde overnight and immersed in 70% ethanol the next day for storage. The heart connected to the aorta was dissected, and the surrounding fat and tissue were removed. For macroscopic examination, the heart and aorta were imaged with a dissecting microscope (Olympus). Aortic segments were embedded in paraffin, and 5 μm-thick-transverse sections were stained with hematoxylin and eosin (H&E).

Sectioned and stained slides were scanned on a Leica Aperio AT2 slide scanner (Leica GmbH). The resulting images were evaluated on QuPath version 0.2.3 (https://qupath.github.io).25
2.2.7 Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded 5 μm-thick aortic sections were immunolabeled with primary antibodies overnight at 4°C: smooth muscle α-actin (1:200, M085, Agilent), and Histone H3 (1:200, Sigma H0164). To detect smooth muscle α-actin, a fluorescent anti-mouse secondary antibody was used with an excitation wavelength of 488nm (Alexa Fluor 488, 1:100, Cat# 715-545-150, Jackson Immunoresearch). To detect histone H3, a secondary biotinylated donkey anti-rabbit secondary antibody was used (1:100, Cat# 711-065-152, Jackson Immunoresearch), a fluorophore-conjugated streptavidin reagent was then used to detect biotin with an excitation wavelength of 549nm (DyLight 549, 1:100, SA-5549-1, Vector Biolabs). Nuclei were stained with DRAQ5 (1:1000, ab108410, Abcam). Images were acquired using a Nikon AR-1 confocal microscope (Nikon). Sirt6 positive VSMCs were counted in 3 fields of view in WT and KO VSMCs and expressed as a fraction of total cells in each field of view.

2.2.8 RNA sequencing

RNA sequencing for Sirt6-deficient (n=3) and Sirt6-WT VSMCs (n=3) was performed at the London Regional Genomics Centre (Robarts Research Institute, London, Ontario, Canada) using the Illumina NextSeq 500 (Illumina Inc., San Diego, CA).

Total RNA was processed using Vazyme VAHTS Total RNA-seq (H/M/R) Library Prep Kit for Illumina (Vazyme, Nanjing, China). Samples were depleted for rRNA, and fragmented. Then, cDNA was synthesized with sample-specific tags and
amplified by PCR. cDNA was then purified with magnetic beads. Libraries were then pooled into a single library, and size distribution was assessed on an Agilent High Sensitivity DNA Bioanalyzer chip and quantitated using the Qubit 2.0 Fluorimeter (Thermo Fisher Scientific, Waltham, MA).

The library was sequenced on an Illumina NextSeq 500 as a 76 bp single-end run, using a single High Output v2 kit (75 cycles). Fastq data files were analyzed using Partek Flow (St. Louis, MO). After importation, data were aligned to the Mus musculus mm10 genome using STAR 2.7.3a and annotated using Ensembl Transcripts release 102. Features with more than 15 reads were normalized as Counts Per Million (CPM).

2.2.9 Gene Set Enrichment Analysis

The annotated gene list with associated CPM was imported into the Gene Set Enrichment Analysis (GSEA) software version 4.1.0 (Broad Institute, San Diego, CA). Genes were ranked using the signal2noise ranking metric. The resulting gene lists were queried for coordinate expression within a priori-defined groups of genes by Gene Ontology Biological Processes (GOBP), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome. Gene sets with more than 500 genes and less than 4 were not queried. Results were expressed as normalized enrichment scores (NES) with q-values (false discovery rate; FDR)\textsuperscript{26}

2.2.10 Statistical analysis

All data were expressed as mean ± standard error (SE). A p < 0.05 was considered statistically different. Normality tests were performed using the D’Agostino-Pearson omnibus normality test. Ratio paired t-tests were performed for the knockout of Sirt6
based on fold-change of gene expression (qPCR), cell counts of mT to mG conversion, and cell counts of Sirt6 positive nuclear staining. Unpaired t-tests were performed for counts of extranuclear localization of histone H3. Two-way ANOVA was performed for fold-changes of select histone transcripts in the independent validation cohort of VSMCs. Statistical analysis was determined using Graphpad Prism software version 9.0.

2.3 Result

2.3.1 Confirmation of sirtuin 6 knockdown in aortic medial VSMC, evidence from mRNA, protein, and novel reporter mouse strain

To determine the consequences of Sirt6 deficiency in VSMCs, we first developed a novel mT/mG Cre-reporter mouse with VSMC-specific inducible SIRT6KO. Mice homozygous for the Sirt6 transcript with loxP flanking exons 2-3 were bred with mice expressing a Rosa26-mT/mG Cre-Reporter. Aortic VSMCs from these mice were then cultured. Knockout of Sirt6 was achieved by transducing VSMCs with adenovirus containing Cre-recombinase (adeno-Cre). VSMCs transduced with adenovirus containing no DNA (adeno-Null) were used as controls. In the absence of Cre-recombinase, VSMCs can be expected to express tdTomato and fluoresce red. However, when Cre-recombinase is introduced into the cells, VSMCs can be expected to switch to eGFP expression yielding green fluorescence. VSMCs were fixed and imaged using fluorescence microscopy at 488nm or 549nm excitation wavelengths. This revealed that 88% (p=0.0068) VSMCs subjected to adeno-Cre converted to green fluorescence, whereas adeno-Null treated cells retained red fluorescence (Figure 2.3). Next, qPCR was used to detect Sirt6 mRNA levels in aortic VSMCs from adeno-Cre, and adeno-Null treated
mice. In VSMCs subjected to adeno-Cre Sirt6, mRNA abundance was reduced to 6% of that of VSMCs subjected to the adeno-Null vector (n=5, p=0.0086, Figure 2.4C). Lastly, we stained for Sirt6 fixed cells (Figure 2.4A). This revealed a 97% reduction in Sirt6-positive cells compared with WT controls (p=0.0044, Figure 2.4B).
A

tdTomato Detection
(λ = 549nm excitation)

Wildtype

Sirt6 KO

eGFP Detection
(λ = 488nm excitation)

B

p=0.0068

% eGFP+ expression

Sirt6 WT

Sirt6 KO
Figure 2.3 Knockout of Sirt6 from aortic VSMCs

A) Images of cells shifting in fluorescence from tdTomato (mT) to eGFP (mG) overlaid with DAPI nuclear stain. VSMCs treated with adeno-null (top) retained mT signal under excitation at $\lambda = 549\text{nm}$ and did not fluoresce green with excitation at $\lambda = 488\text{nm}$. VSMCs treated with adeno-Cre (bottom) lost mT fluorescence under excitation at $\lambda = 549\text{nm}$ and gained mG fluorescence with excitation at $\lambda = 488\text{nm}$. B) Nuclei associated with a green cytoplasm were counted as a fraction of the total number of VSMC in 4 fields of view determine knockdown efficiency. A ratio paired t-test was performed based on cell counts. In total, 88% of VSMCs treated with adeno-Cre converted from mT to mG ($p=0.0068$).
A

SIRT6 Detection
(647nm excitation)

Nuclear localization of SIRT6
(405nm + 647nm excitation)

SIRT6 WT

SIRT6 KO

B

% SIRT6 positive

\[ p = 0.0044 \]

C

Relative Sirt6 mRNA abundance

\[ p = 0.0086 \]
Figure 2.4 Confirmation of Sirt6 knockout in murine VSMCs.
A) WT and KO VSMCs were immunostained for Sirt6 (left) and were overlaid with DAPI (right) to show nuclear localization of SIRT6. B) Sirt6 positive VSMCs were counted in 3 fields of view in WT and KO VSMCs and expressed as a fraction of total cells in each field of view. P-values were derived using ratio paired t-tests. Cell counts showed a 97% reduction of Sirt6 protein (p=0.0044) in Sirt6-deficient VSMCs compared with Sirt6-WT VSMCs. C) Relative mRNA abundance of Sirt6 (n=5) was calculated using the ΔΔCT method relative to β₂ macroglobulin. Ratio-paired t-tests were used to derive p-values. Cells treated with adeno-Cre shows 94% knockdown of Sirt6 mRNA compared with WT (p=0.0086).
2.3.2 Transcriptomes of sirtuin 6 deficient aortic VSMCs

Having established a highly efficient system for depleting Sirt6 in aortic VSMCs, I next characterized the transcriptome of Sirt6-deficient VSMCs using next generation RNA-seq. RNA was extracted from cultures of murine aortic VSMCs and sequenced. Differential expression was assessed by comparing read counts of genes expressed in Sirt6-deficient versus control VSMCs. Transcriptome-wide, 18499 mRNA transcripts were identified. A gene was considered present in the transcriptome if there were greater than 15 counts per million (CPM) for each gene in six independent cell cultures. In total, 1.5% of transcripts were identified as differentially expressed genes (DEG). A gene was considered a DEG if it had an up- or downregulation associated with a p<0.05. In total, 139 genes were upregulated, and 146 genes downregulated in SIRT6KO VSMCs vs Control (p< 0.05, Figure 2.5). We performed an unsupervised principal component analysis, which showed separation between Sirt6-deficient and Sirt6-WT VSMCs for two groups. However, there were two points that overlapped, indicating outliers from one group of VSMCs (Figure 2.6).
Figure 2.5 Volcano plot of all genes sequenced.

Each point represents a single gene. In total, 18499 genes were sequenced from 6 independent cell cultures (3 KO and 3 WT). 139 genes were significantly upregulated (red, p<0.05) and 146 genes were significantly downregulated (blue, p<0.05).
Figure 2.6 Principal component analysis (PCA) of all cohorts of VSMCs sequenced. PCA showing the 3-dimensional representation of the first 3 principal components (PC1, PC2, and PC3 with percentages in parentheses referring to % variance by individual PC) with maximal separation of the data points. Each red dot represented a dataset from a Sirt6-knockout VSMCs, and each blue dot represented a dataset from Sirt6-WT VSMCs. There was visual separation on the PCA plot except two outliers from one batch of cells (Sirt6 KO 1 and Sirt6 WT 1).
2.3.3 Pathway enrichment analysis

To interpret the potential biological relevance of the gene expression data, I used gene set enrichment analysis (GSEA) to perform an unbiased assessment of gene sets found within the genes sequenced from Sirt6-deficient and Sirt6-WT VSMCs. These gene sets were assembled and defined by 3 separate genome-based organizations namely KEGG, GOBP, and Reactome.\textsuperscript{27-32} First, GSEA ranked all 18499 sequenced genes from most positively differentially expressed to most negatively differentially expressed. Next, \textit{a priori} gene sets curated by each organization were queried within the expression data to determine an enrichment score (ES). Clustering of genes at the top of the expression data would yield a positive ES of high magnitude; clustering at the bottom would yield a negative ES of high magnitude; and random distribution of genes would yield a negative ES of low magnitude.

2.3.3.1 The top enriched KEGG pathway is “Systemic Lupus Erythematosus”

I queried the KEGG database for pathways enriched in the sequencing data (Figure 2.7A). Within the top 10 pathways identified were pathways that are related to 3 functional clusters: Inflammation (“systemic lupus erythematosus”, and “leishmania infection”), cellular replication (“ribosome”, “cell cycle”, “pentose phosphate pathway”, and “DNA replication”), and energy metabolism (“PPAR signalling pathway”, “galactose metabolism”, “valine, leucine, and isoleusine degradation”, and “fructose and mannose metabolism”).
“Systemic Lupus Erythematosus (SLE)” pathway was the top enriched pathway (NES=2.31, q<0.001) (Figure 2.8A). Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of autoreactive T and B cells that produce autoantibodies that damage organs.\textsuperscript{33} KEGG defined SLE with a set of 139 genes, of which 81 are histones. 59% of SLE-contributing genes were represented in the sequencing data. Within the genes contributing to the enrichment score of the SLE pathway, also known as the leading edge, were several histone genes: 13 of 47 histone H2 variants, 4 of 19 H3 variants, and 2 of 15 H4 variants. Lastly, of the top 10 genes contributing to the leading edge, 5 are histones (H3C4, H2AC19, H2BC5, H3C7 and \textsuperscript{36}Figure 2.8B).

2.3.3.2 The top enriched Gene Ontology Biological Process pathway is “Nucleosome Assembly”

Next, I used the Gene Ontology Biological Processes (GOBP) pathway database to identify other potentially biologically relevant pathways in the sequencing data (Figure 2.7B). GOBP similarly identified pathways related to cellular replication (“nucleosome assembly”, “chromosome condensation”, “DNA replication independent chromatin organization”, “nucleosome organization” and “chromatin assembly or disassembly”) and inflammation (“cellular response to oxygen radical”, interleukin 27-mediated signaling pathway” and “cellular oxidant detoxification”).

Among the GOBP pathways queried, “Nucleosome Assembly” was the top enriched pathway (NES=2.18, q=0.015) (Figure 2.9A). Genes in this pathway relate to the compaction of DNA around histones within the nucleus.\textsuperscript{34} GOBP defined the
nucleosome assembly pathway with a set of 125 genes, of which 37 are histones. 55% of nucleosome assembly-contributing genes were represented in our expression data.

Contributing to the leading edge of this pathway also were several histone genes: 7 of 10 H1 variants, 7 of 29 H2, 4 of 16 H3 variants, and 3 of 15 H4 variants. Lastly, of the top 10 genes contributing to the leading edge, 6 are histones (H1-0, H4C12, H2BC5, H1-1, H1-5, H4C14, and H3C7) (Figure 2.9B).

2.3.3.3 The top Reactome pathway is “DNA Replication Pre-Initiation”

I also used gene sets defined by the Reactome pathway database to query the sequencing data (Figure 2.7C). Reactome is one of the newest pathway databases.32 Like in KEGG and GOBP queries of the sequencing data, a cluster of pathways related to cellular replication: “DNA replication pre-initiation”, “ERC6 CSB and EHMT2 G9A positively regulates rRNA expression”, “DNA replication”, “condensation of prophase chromosomes”, and “assembly of the ORC complex at the origin of replication”) was also identified using Reactome. Additionally, pathways related to epigenetic modifications (“DNA methylation”, “PRC2 methylates histones and DNA”, and “HDMS demethylates histones”), and senescence (“DNA damage telomere stress induced senescence”) were also identified.

Among the Reactome pathways queried, “DNA Replication Pre-Initiation” was identified as the top enriched pathway (NES=2.47, q=0.0000423) (Figure 2.10A). The genes in this pathway encode for proteins the form pre-replicative complex at the origin of replication. Genes in this pathway must be expressed in the G1 phase for the cell cycle to proceed.35 Reactome has defined DNA replication pre-initiation with a set of 146
genes, of which 80 are histones. 58% of the DNA replication pre-initiation-contributing genes were represented in our expression data. Contributing to the leading edge of this pathway were again several histone genes: 11 of 30 H2 variants, 4 of 15 H3 variants, and 3 of 14 H4 variants. Of the top 10 genes contributing to the leading edge, 6 are histones (H4C12, H2BC5, H3C7, H2BC13, and H2AC20) (Figure 2.10B).

In aggregate, histones are enriched in three biologically relevant pathways with 12 histone genes overlapping in each of KEGG Systemic Lupus Erythematosus, GOBP Nucleosome Assembly, and Reactome DNA Replication Pre-Initiation. These findings indicate a previously unrecognized regulatory link between Sirt6 and histone genes (Table 1).
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<th>GOBP: Nucleosome Assembly</th>
<th>Reactome: DNA Replication Pre-Initiation</th>
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Table 1: Overlap of histone genes amongst the most enriched gene sets from each of three databases.
Figure 2.7 Bubble plots of top 10 enriched pathways of KEGG, GOBP, and Reactome.

Each bubble represents a pathway identified by each database. The area of each bubble represents the proportion of genes within a given pathway that were identified in the RNA-seq data generated in this study. A colorimetric scale represents the -log q-value of each identified pathway. The top 10 pathways identified with each pathway database were ranked from the highest NES (bottom) to the lowest (top). A) Systemic lupus erythematosus is the top enriched KEGG pathway. B) Nucleosome assembly is the top enriched GOBP pathway. C) DNA replication pre-initiation is the top enriched Reactome pathway.
Figure 2.8 Gene set enrichment analysis plots for KEGG Systemic Lupus Erythematosus.

A) Enrichment plot for KEGG Systemic Lupus Erythematosus illustrating a peak enrichment score of 0.60, and an NES of 2.31 (q<0.001)  
B) Heatmap of the 15 genes that contributed to the leading edge. Red indicates an upregulation of transcripts, whereas blue represents a downregulation in transcripts.
Figure 2.9 Gene set enrichment analysis plots for GOBP Nucleosome Assembly

A) Enrichment plot for GOBP Nucleosome Assembly illustrating a peak enrichment score of 0.55, and an NES of 2.18 (q=0.015) B) Heatmap of the 15 genes that contributed to the leading edge. Red indicates an upregulation of transcripts, whereas blue represents a downregulation in transcripts.
Figure 2.10 Gene set enrichment analysis plots for Reactome DNA Replication Pre-Initiation

A) Enrichment plot for Reactome DNA Replication Pre-Initiation illustrating a peak enrichment score of 0.58, and an NES of 2.47 (q=0.0000423). B) Heatmap of the 15 genes that contributed to the leading edge. Red indicates an upregulation of transcripts, whereas blue represents a downregulation in transcripts.
2.3.4 Validation of histone upregulation

I next sought to ascertain if the upregulation of histones could be reproduced in an independent cell culture experiment. Mouse aortic VSMCs were cultured from another cohort of mice of the same genotype. Select histone genes that were seen to be upregulated in RNA-seq analysis were chosen to be validated by qPCR. RNA-seq revealed the upregulations in H3C4 (1.3-fold), H2BC5 (1.3-fold), H2AC19 (1.3-fold), H3C7 (1.5-fold), and H2AC20 (1.3-fold). qPCR of independent cell culture of Sirt6-deficient VSMCs also showed upregulations in H3C4 (2.7-fold), H2BC5 (1.6-fold), H2AC19 (1.5-fold), H3C7 (2.6-fold), and H2AC20 (1.6-fold). In aggregate, these histone transcripts were significantly upregulated in Sirt6-deficient VSMCs (p=0.0083).
Figure 2.11 Assessment of histone gene expression using independent aortic cell culture.
Independent cell cultures were conducted to create *Sirt6*-WT and deficient VSMCs. qPCR was used to measure the relative abundance of select histone mRNA transcripts. These qPCR results were expressed as fold changes of histone mRNA in *Sirt6*-deficient VSMCs vs WT VSMCs (red bars). Fold changes from the independent cell culture was compared with fold changes of the same transcripts from RNA-Seq results. Independent cell cultures confirmed a consistent upregulation in these histone transcripts.
2.3.5 Assessment of histone H3 in aortic VSMCs in vivo: Evidence of cytoplasmic localization

Histones are ubiquitously expressed nuclear proteins. Given the modest, but consistent upregulation seen in cultured VSMCs deficient in Sirt6, I next asked if there were any identifiable histone changes at the protein level in vivo. Histological sections were cut from aortas of Sirt6-deficient and Sirt6-wildtype mice that had undergone 28 days of AngII infusion. Two different strains were used, C57BL/6 and FVB mice. Sections were immunostained for Histone H3, and counterstained with DAPI.

In WT aortas, histones are predominantly localized to the nucleus of VSMCs (Figure 2.12C), with 4.5% of VSMCs presenting with extranuclear histone H3 (Figure 2.11). However, in Sirt6 knockout VSMCs (Figure 2.12G), I observed a 2.7-fold increase VSMCs with extranuclear localization of histone H3 (p=0.0152), with 12.4% of VSMCs presenting with extranuclear histone H3 (Figure 2.11).
Figure 2.12 Counts of medial VSMCs with extranuclear histone H3.

Extranuclear histones were quantified by counting VSMCs with histone H3 signal extending beyond the bounds of the stained nuclei. Three fields of view were used for each group of cells. P-values were derived using unpaired t-tests. Overall, Sirt6-deficient VSMCs have a 2.7-fold increase in extranuclear histone H3 (p=0.0123).
Figure 2.13 Evidence of cytoplasmic histones in a Sirt6-deficient mouse aorta.
Aortas from 12-week-old Sirt6-deficient and WT male mice were fixed in paraformaldehyde and stained for DRAQ5 (blue), histone H3 (red), and αSMA (green). 
A) DRAQ5 stained nuclei (blue) in Sirt6-WT vSMCs. B) Overlay of DRAQ5, and histone H3 C) Nuclei are contoured in yellow showing colocalization of histone H3 and nuclei. 
D) Overlay of DRAQ5 (blue), histone H3 (red), and αSMA (green) illustrating that identified cells are VSMCs. E) DRAQ5 stained nuclei (blue) in Sirt6-deficient VSMCs. F) Nuclei are contoured in yellow, showing extranuclear localization of histone H3 (red) evidenced by histone H3 signal (red) outside the nuclei 
H) Overlay of DRAQ5 (blue), histone H3 (red), and αSMA (green) illustrating that identified cells are VSMCs.
2.4 Discussion

Recently, our group has shown that the VSMC-specific knockout of Sirt6 in mice induces aortic ruptures and remarkable aortic inflammation.\textsuperscript{19} In this study, I have developed an \textit{in vitro} system to study Sirt6 deficiency in VSMCs and undertaken a transcriptomic analysis. I identified a modest and unexpected increase in the expression of several histones. Moreover, \textit{in vivo} preliminary experiments, I found evidence that in VSMC-specific, Sirt6-deficient aortas, histones may reside outside the VSMC nucleus.

I first sought to characterize the transcriptomic changes in Sirt6-deficient VSMCs with next-generation RNA sequencing. Sequencing yielded a total of 18499 genes sequenced, with 146 genes downregulated, and 139 genes upregulated. Relative to other studies, we have identified comparatively few differentially expressed genes. For instance, Zhang et al. used RNA-seq to interrogate Sirt6-deficient thymic epithelial cells and found 920 downregulated genes and 1217 upregulated.\textsuperscript{36} Moreover, another study aggregated publicly available microarray data of Sirt6-deficient and Sirt6 wildtype mice to identify genes involved in the phenotypic switching in VSMCs in aortic aneurysms.\textsuperscript{37} In the study by Guan et al., 377 genes were significantly downregulated, and 298 genes were significantly upregulated with a fold change of at least 2.\textsuperscript{37} Considering that Sirt6 can regulate several transcription factors through its deacetylation activity,\textsuperscript{38} our findings of few differentially expressed genes is especially surprising. This finding may be due to an outlier group (Figure 2.6) that may have skewed sequencing results. Moreover, it is conceivable that there are yet to be identified compensatory mechanisms for genetic dysregulation in Sirt6-deficient VSMCs.

I used GSEA to query the sequencing data for pathways identified by three different genome databases (KEGG, GOBP, and Reactome). Pathways related to cellular
replication were upregulated in Sirt6-deficient VSMCs. It has been previously shown that the loss of SIRT6 increases the proliferation of cancer cells, leading to tumour formation.\textsuperscript{39} Moreover, SIRT6 deficiency has been shown to increase the expression of ribosomal genes which allow for the synthesis of proteins in the proliferation of cancer cells.\textsuperscript{40} While this study presents some results which are consistent with the literature, it is the first to show this process in aortic VSMCs. Furthermore, pathways related to energy metabolism were found in the top 10 pathways identified by KEGG. SIRT6-deficiency leads to abnormal glucose and lipid metabolism.\textsuperscript{41} Cultured myoblasts from SIRT6-deficient mice have increased glucose uptake due to increased membrane expression of GLUT1 and GLUT4.\textsuperscript{42} Moreover, SIRT6 deficiency increases glycolysis through the upregulation of glycolytic genes.\textsuperscript{41} In this study, neither glucose transporter, nor glycolytic genes to be were not found significantly changed. However, pathways related to glucose metabolism were identified by KEGG, namely “galactose metabolism”, and “fructose and mannose metabolism”. SIRT6 deficiency also increases transcription factor PPARγ on the promoters of critical fatty acid transporters, thereby increasing the expression of fatty acid transporters.\textsuperscript{43} Consistent with the literature, KEGG identified an enrichment of the PPAR signalling pathway in the genomes of Sirt6-deficient cells in this study. It has also been recently shown that SIRT6 knockdown via AAV-delivered siRNA results in decreases in the contractile markers α-SMA, and SM22α.\textsuperscript{44} While these contractile markers were identified in the sequencing results of this study, they were not significantly downregulated.

It is notable that while the magnitudes of upregulation in histone transcripts found in this study were small, several isoforms of all five families of histones were
consistently upregulated in Sirt6-deficient VSMCs. Typically, the expression of histones is dictated by the cell cycle, with mRNA being only upregulated during cell cycle transitions between the G1 and S-phase.\textsuperscript{44} Moreover, the accumulation of non-chromatin bound histone proteins is toxic to the cell. Thus the expression of histone mRNA must be strictly regulated.\textsuperscript{45} SIRT6 is a multifunctional protein that can modulate the expression of several genes through its histone deacetylase activity.\textsuperscript{38,46} It is known that SIRT6 can repress gene expression by deacetylating lysine residues 9, 18 and 56 of histone H3 at promoter regions of target genes.\textsuperscript{11} Deacetylation of histones prevents that region of DNA from being accessed by transcriptional machinery, which silences gene expression. Interestingly, it has been shown that the upregulation of histones H3 and H4 during the G1/S-phase transition in the cell cycle is associated with increased acetylation at H3K9.\textsuperscript{47,48} Thus, it is conceivable that the upregulation of histones in this study may have taken place in a Sirt6-dependent manner through a similar mechanism. With decreased levels of Sirt6 in the cell, there may have been an increase in acetylation at the promoters of histone genes, leading to increased histone transcription.

Through immunostaining for histone H3 in histological sections from VSMC-specific Sirt6-deficient mice, we also showed preliminary evidence for extranuclear localization of histone H3 in VSMCs. Ordinarily, the role of histones is to organize the genetic code into a tightly wound chromatin.\textsuperscript{49} However, when found outside the nucleus, histones can behave as an immune-activating signal called a danger-associated molecular pattern (DAMPs).\textsuperscript{50,51} It is known that histones can traffic from the nucleus to the cytoplasm and cell membrane to induce inflammatory signalling.\textsuperscript{52} For instance, apoptotic microglia can traffic histone H3 to cell membranes which are thought to
enhance the apoptotic response.\textsuperscript{53} Indeed, apoptotic signalling has also been shown to induce the release of histones from Jurkat and HeLa cells.\textsuperscript{54} Necrosis can also lead to a release of histones through its uncontrolled rupture of the plasma membrane.\textsuperscript{55} Externalized histones have been well documented in a special form of neutrophils called neutrophil extracellular traps (NETs).\textsuperscript{56} Moreover, it has been shown that externalized Histone H4 from NETs can induce lytic cell death in VSMCs.\textsuperscript{52} While it is possible that Sirt6-deficient VSMCs were exhibiting histone translocation seen in apoptosis, there was no indication morphologically that the cells were undergoing apoptosis. The mechanism of how these cells exhibited extranuclear Histone H3 remains an open question and warrants further study.

Our group previously identified an aortitic phenotype in mice with VSMC-specific SIRT6 deficiency. A common cause of aortitis is giant cell arteritis (GCA). In the first step in the pathophysiology of GCA, toll-like receptors (TLRs) on adventitial dendritic cells are activated to induce the secretion of chemokines such as CCL-19 and -21.\textsuperscript{57} In the second step of GCA progression, secreted cytokines recruit T-cells, monocytes, and macrophages. Finally, the aorta will undergo vascular remodelling.\textsuperscript{58} Notably, extracellular histones can activate TLR2, TLR4, and TLR9.\textsuperscript{59,60} Together with our findings of extranuclear histones in SIRT6-deficient VSMCs, it is possible that histones drive inflammation seen in VSMC-specific SIRT6-deficient mice.

2.5 Limitations

There are limitations to the generalizability of this study. First, the mice used in this study carried the Myh11-Cre allele only on the Y-chromosome. Therefore we were
only able to harvest VSMCs from male mice.\textsuperscript{61,62} It will be important to investigate VSMCs from female mice to test for sex differences in the inflammatory response in future studies. Moreover, exclusively young, and otherwise healthy mice were used for experimentation.

It is understood the use of Cre-recombinase may produce random, off target recombination at cryptic loxP sites found within the mammalian genome.\textsuperscript{63} It will therefore be important to study the effect of Cre administered in wild-type control lacking loxP sites flanking \textit{Sirt6} to control for this phenomenon.

The RNA-seq entailed 3 biological replicates in each arm. This relatively small sample size is a limitation. Moreover, principal component analysis determined there was one outlier group in the sequencing results. Therefore, it will be important to increase replicate numbers for more statistical power in future studies.

2.6 Conclusion

I have developed an \textit{in vitro} model of \textit{Sirt6} deficiency to study the transcriptomic changes resulting from its loss. I quantified the changes in the transcriptome through next-generation RNA sequencing. This study yielded an unexpected increase in histone transcripts upon \textit{Sirt6}-deficiency. I also showed in preliminary data that this was associated with extranuclear histones in the inflamed aortic media of VSMC-specific \textit{Sirt6}-deficient mice. These findings offer potentially novel insights into the role of Sirt6 in vascular biology and raises the possibility of a Sirt6-histone axis in supressing inflammation in the stressed aorta.
2.7 References for Chapter 2


Chapter 3

3 Summary and conclusion

The findings in this thesis provide new insights into the role of Sirt6 in VSMC biology. I established a valuable primary culture system of murine VSMCs with inducible Sirt6 deficiency. Using this model, I discovered a set of differentially expressed genes upon Sirt6 deletion. This included a remarkably coordinated upregulation of multiple histone transcripts following the knockout of Sirt6. Moreover, I have uncovered preliminary evidence of increased extranuclear histone H3 in aortic histological sections from angiotensin II-infused mice with postnatal inactivation of Sirt6 in VSMCs. Histones found outside the nucleus can function as damage-associated molecular patterns to elicit inflammation. Moreover, cytoplasmic and plasma-membrane bound histones have been associated with the development of autoantibodies in autoimmune disease. Therefore, these findings of this study suggest a previously unrecognized anti-inflammatory role of Sirt6 in smooth muscle cells via repressing the expression and cytoplasmic shuttling of histones (Figure 3.1).
A

SIRT6 Replete SMC

cell membrane

cytoplasm

nucleus

Deacetylation of H3K9 at promoters of Histone

Histone mRNA Transcription Inhibited
Figure 3.1 Proposed mechanism of Sirt6 mediated histone upregulation.

A) In Sirt6 replete VSMCs, H3K9ac is deacetylated at the promoters of histone genes. This closes chromatin to transcription by RNA polymerase II. B) In Sirt6 depleted VSMCs, H3K9ac is potentially hyperacetylated at the promoters of histone genes. This allows increased transcription of histone genes, accounting for the upregulation in histone transcripts seen in this study. Increased transcripts will then be translated into histone proteins. Excess histone proteins may traffic to the plasma membranes of VSMCs to activate the NLRP3 inflammasome and recruit inflammatory cells to the aortic media. Transcripts may also traffic to the cytoplasm or nucleus where they may sensitize VSMCs to genotoxic stresses like ROS. Sirt6 depletion may also exacerbate the release of NF-κB-controlled genes.
3.1 Establishment of an *in vitro* model of sirtuin 6 deficiency

I first established an *in vitro* model of Sirt6 deficiency by culturing cells from a C57BL/6 mouse carrying *SMMHC-CreER*², Rosa26-푚T/mG, and Sirt6<sub>flox/flox</sub> alleles. These cells were then transduced with adeno-Cre to induce genetic recombination at loxP sites to delete *Sirt6* and shift fluorescent reporter expression from tdTomato (푚T) to eGFP (mG). The efficiency of Sirt6 knockout after 10 days in culture was validated in 3 ways, conversion of 푚T/mG fluorescent reporter, *Sirt6* mRNA expression quantified via qPCR, and Sirt6 protein expression quantified via immunofluorescence. Compared with wild-type mice, knockout cells had an adenoviral transduction efficiency of 88% (p=0.0068) based on fluorescent shifts from mT to mG. *Sirt6* mRNA was downregulated by 94% (p=0.0086); protein by 97% (p=0.0044).

My study is the first to genetically delete *Sirt6* in cultured VSMCs. There are 3 previous reports on the functional role of Sirt6 in VSMCs.³⁻⁵ These studies have relied on RNA interference to downregulate *Sirt6*. For instance, Yao et al. used siRNA to silence *Sirt6* in murine VSMCs but this only resulted in a knockdown of approximately 57%.³ Li et al. also used siRNA against *Sirt6* in murine VSMCs and reported a knockdown of approximately 90%.⁵ Grootaert et al. used two different shRNA species to knockdown *SIRT6* in human VSMCs to achieve knockdown efficiencies of 62.5 to 90%. The genetic KO model I undertook offers advantages over RNAi or shRNA approaches. First, there is evidently a higher knockout efficiency of 94%. Second, the integrated 푚T/mG Cre reporter allows the ability to monitor the knockout efficiencies in live cells. Third, examining for red and green fluorescence even after fixation provides an additional
indicator of gene knockout together with assessments of Sirt6 transcript and protein abundance. Fourth, this strategy circumvents questions regarding non-specific effects of siRNA or shRNA transcripts. It is recognized that non-specific effects of the adenovirus could arise. However, by design, such effects would be controlled for as the control VSMCs were also subjected to adenovirus infection.

3.2 Histones were enriched in sirtuin 6-deficient VSMCs

I characterized the transcriptomic changes in Sirt6-deficient VSMCs using RNA-seq. A total of 18499 genes were sequenced, with 146 genes found to be significantly downregulated and 139 genes upregulated.

Using a GSEA-based bioinformatics pipeline, I observed a concerted upregulation of multiple histone genes, which included isoforms of all five families of histone (H1, H2A, H2B, H3, and H4). Although the magnitude of upregulations was modest, the profile became evident based on the top-ranked pathways in 3 gene set databases, “systemic lupus erythematous” (KEGG), “nucleosome assembly” (GO biological process), and “DNA replication pre-initiation” (Reactome).

Histones are usually located in the nucleus of cells, where they are core organizers that pack DNA into nucleosomes. Moreover, histones are generally only transcriptionally upregulated during the cell cycle transition between G1 and S to accommodate the increased synthesis of DNA. It is therefore surprising to see an upregulation of histone transcripts in VSMCs following the knockout of Sirt6. Interestingly, Singh et al. found that excess histones were deleterious, sensitizing yeast to
DNA damage by genotoxic agents.\(^8\) We previously saw increased oxidative stress-induced DNA damage \textit{in vivo} in \textit{Sirt}6-deficient mice treated with angiotensin II. Thus, it is possible that increased histones may also sensitize VSMCs to DNA damage.

Assessment of histological sections of aortas from mice with VSMC-specific \textit{Sirt}6 deficiency and treated with AngII proved to be exciting. I found evidence of increased extranuclear histone H3 in \textit{Sirt}6-deficient samples compared with wild-type controls. Interestingly, in microglia, it has been shown that histones located outside of the nucleus can function as DAMPs to activate the immune response.\(^2,9,10\) Moreover, extracellular histones in hepatocytes can activate toll-like receptors to induce local secretion of cytokines to attract immune cells.\(^11,12\) This line of evidence, in combination with our findings in this study, seems relevant to the aortitis phenotype observed in the mouse model of \textit{Sirt}6 deficiency. In mice with VSMC-specific \textit{Sirt}6 KO and AngII infusion, we found CD45+ leukocyte infiltrate across the full thickness of aortic media.\(^13,14\) Therefore, it is possible that the \textit{Sirt}6 deficiency in VSMCs transcriptionally increases histone expression, and those excess histones retained in an extranuclear space may serve as pro-inflammatory signals to exacerbate aortic inflammation.

### 3.3 Future directions

The findings of 1) increased histone transcripts and 2) evidence of extranuclear histones warrant further investigation. These phenomena are potential new mechanisms to understand vascular inflammation and describe the poorly characterized cellular mechanisms of aortitis.
To fully characterize the involvement of Sirt6 in the expression of histones, it will be essential to show direct involvement of the SIRT6 protein with histone genes or gene products. While my results have shown an upregulation of histone transcripts and evidence of extranuclear histones, the mechanisms by which this happens in a Sirt6-dependent manner are unknown. I suspect that the regulation may occur via an H3K9 hyperacetylation event following Sirt6 deficiency. To begin to test this hypothesis, it will be essential to quantify the acetylation status of H3K9. This can be done by immunoprecipitation or immunoblotting of cultured VSMCs or by immunostaining for H3K9ac either in histological sections or cultured cells. To test for the direct involvement of SIRT6 in the deacetylation of H3K9ac at regions related to histone genes, chromatin immunoprecipitation with RNA sequencing (ChIP-seq) may be used.

Culture studies will also be necessary to understand the localization of histone proteins, and if histones are truly externalized in VSMCs. Externalization of histones is one way that cells can initiate an inflammatory response via activation of TLRs on immune cells. To characterize the cellular localization of histones, cultured VSMCs could be subjected to cellular fractionation with subsequent purification of histone proteins from each cellular subfraction. To understand if Sirt6-deficient VSMCs release histones into the extracellular space, an enzyme-linked immunosorbent assay (ELISA) may be used to probe culture media for histone proteins.

It is also important to note that in vivo model of Sirt6-deficiency required the use of AngII to elicit an inflammatory response and extranuclear localization of histones. AngII, in addition to increasing blood pressure in vivo, also increases oxidative stress in the vasculature. Thus, there may need to be extra inflammatory stimuli supplemented
to cultured VSMCs to induce histone release. This can be done by culturing cells with inflammatory cytokines such as TNFα, interleukins, or hydrogen peroxide to characterize the conditions needed to release histones.

3.4 Conclusions

In conclusion, I have developed a novel in vitro VSMC model with inducible Sirt6 deficiency. This model was used to interrogate the Sirt6-regulated transcriptomes in VSMCs. I discovered that the knockout of Sirt6 in VSMCs induces an increase in the abundance of multiple histone transcripts. I also showed evidence that Sirt6-deficiency in VSMCs might cause the extranuclear localization of histone H3. These findings provide new insights into a potential mechanism of how Sirt6 may initiate or exacerbate inflammatory conditions of the aorta.
3.5 References for Chapter 3


inflammatory liver injury through Toll-like receptor 9 in mice. *Hepatology*.
2011;54(3):999–1008.


Appendices

Appendix A: Ethics Approval for Animal Use

Appendix A:
Ethics Approval for Animal Use

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2018-161 has been approved by the Animal Care Committee (ACC)
and will be approved through to the above review date.

Please at this time review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:
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   b) University Council on Animal Care Policies and related Animal Care Committee procedures
      http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.html
2) As per UCAC’s Animal Use Protocols Policy,
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   b) external approvals associated with this AUP, including permits and scientific/departamental peer approvals, are complete and accurate;
   c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
   d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
      http://uwo.ca/research/services/animalethics/animal_use_protocols.html
3) As per MAPS 7.10 all individuals listed within this AUP as having hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training (training@uwo.ca); and
   c) be overseen by me to ensure appropriate care and use of animals.
4) As per MAPS 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to AVS Veterinarians and ACC Leaders;
   c) UCAC policies and related ACC procedures will be followed, including but not limited to:
      i) Research Animal Procurement
      ii) Animal Care and Use Records
      iii) Sick Animal Response
      iv) Continuing Care Visits
5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
      http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Coeeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care
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A

- Left common carotid artery
- Left subclavian artery
- Aortic arch
- Ascending aorta
- Aortic sinus
- Diaphragm
- Transverse aortic arch

B

- Tunicia externa (adventitia)
- Tunicia media
- Tunicia intima
- Vascular smooth muscle cell
- Elastic

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Graduate Research Assistant
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Undergraduate Research Assistant
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Peer Reviewed Publications


Presentations


Awards

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2019 - 2021 Western Graduate Research Scholarship (WGRS)
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2017 Propel Entrepreneurship Grant
Scholarship to fund a patent search
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2017 Deans Undergraduate Research Opportunities Program
Scholarship to fund undergraduate summer research
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Community Involvement

2021 - Present Premier Level Dragon Boat Paddler
Outer Harbour Warriors Club, Toronto, Ontario

2020 - Present Sr. Youth Programming Volunteer
BGCLondon, London, Ontario
Created and led a cooking program for youth aged 8 to 17