Interaction Of CircularRNA And MicroRNA-9-5p In The Mediation Of Cardiac Fibrosis In Diabetic Cardiomyopathy

Honglin Wang, Western University

Supervisor: Chakrabarti, Subrata, The University of Western Ontario
Co-Supervisor: Feng, Biao, The University of Western Ontario

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Pathology and Laboratory Medicine
© Honglin Wang 2023

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Cardiovascular Diseases Commons

Recommended Citation

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact wlsadmin@uwo.ca.
Abstract

Diabetic cardiomyopathy (DCM) is a chronic diabetic complication affecting the heart. It is a significant and independent contributor to heart failure and characterized by cardiac fibrosis and left ventricular hypertrophy. Endothelial cells are the first cells to be damaged by hyperglycemia, leading to epigenetic modifications and subsequent signaling and transcription derangements that lead to endothelial dysfunction and therefore cardiac fibrosis. The role of the two noncoding RNA microRNA-9-5p and circRNA_012164 are investigated using in vivo and in vitro models of hyperglycemia. CircRNA_012164 is upregulated in the hearts of diabetic mice and in cells exposed to high glucose. This results in an inhibition of miR-9 levels, which leads to increased expression of ACTA2, COL1A1, FN1 and FSP1, genes related to cardiac fibrosis. Knockdown of circRNA_012164 recovers miR-9 levels and protects against endothelial dysfunction and fibrosis. Establishment of the circRNA_012164/miR-9 regulatory axis sheds new light on the role of ncRNA in chronic diabetic complications.

Keywords

Diabetes, diabetic complications, diabetic cardiomyopathy, cardiac fibrosis, epigenetics, noncoding RNA, microRNA, circularRNA, endothelial dysfunction
Summary for Lay Audience

Diabetic cardiomyopathy (DCM) is a consequence of long-term diabetes that affects the heart. It can lead to heart failure and is characterized by heart muscle stiffening and remodeling of structures in the heart. The high blood sugar seen in diabetes harms endothelial cells, the type of cells lining blood vessels in the heart. This damage causes changes in how genes are switched ‘on’ or ‘off’, leading to irregular behaviour in the cells and leading to problems in the heart. One of the most prominent problems this results in is the accumulation of fibrous proteins which affect heart function and structure, these changes lead to DCM.

This project investigated the role of two RNA molecules, microRNA-9-5p and circRNA_012164, in DCM. Both live mice and cells were used. Previous research has found that microRNA-9 activity in the heart is reduced in diabetes and adding more microRNA-9 into cells, reduced genes that damage the heart. Our results show that circRNA_012164 becomes more active in diabetic hearts. This increased activity reduces the levels of microRNA-9. When circRNA_012164 levels rise and microRNA-9 levels drop, endothelial cells become more irregular, and the genes related to heart muscle problems get activated more. Once circRNA_012164 is reduced, genes related to heart muscle problems do not get more activated, even when blood sugar levels are high and microRNA-9 levels went back up. This shows that circRNA_012164
activation by high blood sugar is damaging to the heart, and reducing circRNA_012164 activity may protect the heart from developing DCM.

CircRNA_012164 is a new molecule that has never been studied before. This project has shed some light on how it, along with microRNA-9, can contribute to long-term problems in people with diabetes.
Acknowledgements

First and foremost, I must acknowledge and thank my supervisors, Drs Subrata Chakrabarti and Francis Feng. This project was only possible as a result of their guidance, teaching, and patience. I am grateful to have had you for my supervisors, thank you for taking me on.

Thank you to Dr. Christina Castellani for the comments and advice. To Dr. Zia A. Khan for giving me new questions to think about and for always supporting Pathology trainees. To Dr. Patti Kiser for making my time as a TA so wonderful. Thank you as well to PaLM administrative staff, who were always there to remember the things I did not.

Thank you as well to all the members of the Chakrabarti lab, past and present, for the experiences you have passed onto me. In particular I would like to thank Eric Wang, thank you for the training, advice, and moral support; but most importantly, for doing the down clues.

To Daniel, thank you for keeping me happy, fed, and warm for the many years. Thank you to my cottage girls, and thank you to all my friends for our hikes, brunches, and games.

Finally, and most importantly, I would like to thank my parents for fostering in me a lifelong love of learning and supporting me through my education. I literally would not be here without you.
Table of Contents

Abstract ii

Keywords ii

Summary for Lay Audience iii

Acknowledgements v

Table of Contents vi

List of Tables x

List of Figures xi

List of Appendices xiii

List of Acronyms xiv

Chapter 1: Introduction 1

1.1 Diabetes and epidemiology 2

1.2 Diabetic complications 3

1.2.1 Diabetic retinopathy 6

1.2.2 Diabetic nephropathy 7

1.2.3 Diabetic peripheral neuropathy 8

1.2.4 Diabetic cardiomyopathy 9
Chapter 2: Materials and Methods

2.1 Animals

2.1.2 Generation of miR-9 transgenic mice

2.1.3 Induction of diabetes in mice

2.2.1 Transfection

2.3 RNA Isolation and qRT-PCR Analysis

2.4 Protein Isolation and Analysis

2.5 Histology
Chapter 3: Results

3.1 Aim 1. Establish changes in microRNA-9 and circRNA_012164 in an in vivo model of diabetic cardiomyopathy.
   3.1.1 Clinical monitoring of transgenic and diabetic mice
   3.1.1 microRNA-9 and circRNA_012164 are altered in the murine heart in diabetes.
   3.1.2 MiR-9 regulates EndMT in the heart in diabetes.

3.2 Aim 2. To observe the effect of high glucose on levels of circRNA_012164 and downstream fibrosis genes in cardiac endothelial cells.
   3.2.1 Optimizing cell growing conditions
   3.2.2 Establish changes in gene expression in response to hyperglycemia

3.3 Aim 3. To establish that a regulatory relationship exists between microRNA-9, circRNA_012164 and downstream fibrotic genes.
   3.3.1 Mechanistic effect of circRNA_012164 in hyperglycemic endothelial dysfunction
   3.3.2 Regulatory interaction of circRNA_012164 and microRNA-9 in endothelial cells mediates glucose induced ECM protein production.
## Chapter 4: Discussion

4.1 Discussion

4.2 Future Directions

4.2 Limitations

References

Appendix 1: Animal Ethics Approval

Curriculum Vitae
List of Tables

Table 1.1 MicroRNA of note in diabetic cardiomyopathy 22

Table 1.2 CircularRNA of note in various diabetic complications 26

Table 2.1 Sequences of designed siRNAs targeted to the backsplice junction of circRNA_012164 38

Table 2.2 Primer sequences for qPCR Analysis 41

Table 3.1 Clinical monitoring of mice at 2 months following streptozotocin treatment 45
List of Figures

Figure 1.1 Schematic representation of hyperglycemic damage to endothelial cells resulting in fibrosis 14

Figure 1.2 Schematic representation of circularRNA biosynthesis and role as microRNA sponge. 24

Figure 2.1 Vector map of vector used to generate endothelial specific microRNA-9 overexpressing transgenic mice. 31

Figure 2.2 RT-qPCR analyses of cells from cardiac tissues of endothelial specific microRNA-9 overexpressing transgenic mice. 32

Figure 2.3 Flowchart of mouse treatment groups 35

Figure 2.4 Flowchart of mouse cardiac endothelial cell treatment groups 39

Figure 3.1 Echocardiogram images of experimental mice 46

Figure 3.2 Differential expression of microRNA-9 in diabetic and non-diabetic mice. 47

Figure 3.3 Percent change of circRNA_012164 levels and β-actin (ACTB) levels following RNase R incubation. 48

Figure 3.4 Differential expression of circRNA_012164 in diabetic and non-diabetic mice. 50
Figure 3.5 Masson’s Trichrome staining of murine left ventricular tissue demonstrating interstitial fibrosis 52

Figure 3.6 Inhibition of diabetes-induced changes in fibrotic gene expression by miR-9 overexpression in vivo. 54

Figure 3.7 Reduction of diabetes-induced fibrotic protein levels by miR-9 overexpression in vivo. 55

Figure 3.8 Optimal cell growth conditions for immortalized mouse cardiac endothelial cells 57

Figure 3.9 Hyperglycemic-induced changes in EndMT-related gene expression in mouse cardiac endothelial cells 59

Figure 3.10 Assessment of designed siRNA targeted to the backsplice junction of circRNA_012164. 61

Figure 3.11 Hyperglycemia-induced changes in gene expression in mouse cardiac endothelial cells with circRNA_012164 knockdown. 62

Figure 3.12 Knockdown of miR-9 negates the protective effects of circRNA_012164 knockdown in mouse cardiac endothelial cells. 65

Figure 4.1 Schematic representation of the regulatory axis of circRNA_012164 and microRNA-9-5p in hyperglycemia-mediated fibrosis and EndMT 72
List of Appendices

Appendix 1 Animal Ethics Approval 88
## List of Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCM</td>
<td>Diabetic cardiomyopathy</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>DKA</td>
<td>Diabetic ketoacidosis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end products</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>DR</td>
<td>Diabetic retinopathy</td>
</tr>
<tr>
<td>NPDR</td>
<td>Non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>PDR</td>
<td>Proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>BRB</td>
<td>Blood retinal barrier</td>
</tr>
<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>DPN</td>
<td>Diabetic peripheral neuropathy</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
</tbody>
</table>
ET-1  Endothelin 1
EndMT  Endothelial mesenchymal transition
ncRNA  Noncoding RNA
RISC  RNA-induced silencing complex
DCCT  Diabetes Control and Complications Trial
EDIC  Epidemiology of Diabetes Interventions and Complications
ChIP  Chromatin immunoprecipitation
M9TG  MicroRNA-9 transgenic
STZ  Streptozotocin
MCEC  Mouse cardiac endothelial cell
Chapter 1 Introduction
1.1 Diabetes and epidemiology

Diabetes is endemic in the world and a major cause of death and disability to the more than 400 million people living with diabetes\textsuperscript{1,2}. Diabetes is broadly categorized into two types, type 1 and type 2. Gestational diabetes also exists as a separate type which manifests exclusively in some pregnant women. Type 1 diabetes (T1DM) is typically thought of as an autoimmune disease resulting in the destruction of the insulin producing β islet cells in the pancreas\textsuperscript{3}. In contrast, type 2 diabetes (T2DM), which accounts for 95% of diabetic patients, develops through a complex interplay of genetics, age and an injurious lifestyle\textsuperscript{3}. Rates of diabetes continues to rise in many countries, and independently of geography, disproportionately affecting lower-income individuals\textsuperscript{3-7}. In the next 20 years, the number of people living with diabetes may rise to nearly 700 million if prevention methods are not adopted, with increasing prevalence of both of type 1 and type 2 diabetes\textsuperscript{2}. While T1DM results from a lack of endogenous insulin, T2DM is considered to result from low insulin receptor expression or faulty signal transduction, leading to insulin insensitivity\textsuperscript{3}. In either type, the impotence of insulin causes dysfunction in the insulin dependent glucose uptake transporter, GLUT4, which is responsible for the transport of glucose from the blood into adipose tissue and skeletal muscle\textsuperscript{8,9}. Without the function of GLUT4, blood sugar remains chronically high\textsuperscript{9}. Glycemic control is the standard
approach for the management of diabetes. Despite this, chronic diabetic complications driven by hyperglycemic damage will arise in the late stages of diabetes\textsuperscript{10}.

1.2 Diabetic complications

Diabetic complications are either acute or chronic. Acute diabetic complications result from sudden severe imbalances in homeostasis. These acute complications are dangerous and often life-threatening if untreated. Diabetic ketoacidosis (DKA) and acute hypoglycemia are generally associated with T1DM, while hyperglycemic hyperosmolar state (HHS) are seen more in T2DM\textsuperscript{11}. DKA develops due to the generation and buildup of acidic ketone bodies, resulting from the metabolism of lipids as a response to lack of insulin signaling and intracellular glucose\textsuperscript{12}. Left untreated, DKA leads to blood acidification and can result in cerebral edema, coma, or death\textsuperscript{12}. Acute hypoglycemia happens when blood glucose suddenly drops below normal levels. When this happens, the brain is unable to receive enough glucose to sustain function, which may lead to seizures and coma\textsuperscript{13}. T2DM patients generally do not suffer from DKA due to being able to sustain enough insulin signaling to avoid ketogenesis\textsuperscript{14}. However, hyperglycemia also causes discrepancies in osmolarity, which results in compensatory fluid release from cells and resultant severe dehydration\textsuperscript{14}. Though a relatively rare acute complication, it results in the highest mortality rates\textsuperscript{15}. 
Chronic diabetic complications resulting from diabetes may be categorized as micro- or macrovascular, affecting capillaries and small vessels or larger vessels respectively\textsuperscript{16}. Microvascular complications include diabetic retinopathy, diabetic nephropathy, diabetic neuropathy and diabetic cardiomyopathy\textsuperscript{17-19}. Endothelial cells, being the cell type most commonly in contact with circulating blood, are among the first to be damaged in diabetic hyperglycemia\textsuperscript{17,20,21}. Endothelial cells express GLUT1, a glucose transporter not dependent on insulin, but on the amount of circulating of glucose in the blood, with increased blood glucose levels necessitating increased glucose trafficking into the cell\textsuperscript{21,22}. As such, the increased glucose results in overloaded glucose metabolism pathways and subsequent damage\textsuperscript{21}. Cells with elevated glucose levels compensate for the increased load in a variety of ways that ultimately contribute to cell damage and dysfunction, primarily through oxidative damage\textsuperscript{23,24}. Glucose is metabolized through the glycolytic pathway, and excess glucose overtaxes the pathway, leading to the compensatory activation of alternative harmful pathways by glycolytic intermediates\textsuperscript{17,18,23}. The encumbered glycolytic pathway also results in the generation of damaging reactive oxygen species (ROS) to cope with excessive oxidative respiration\textsuperscript{23,24}. ROS contribute to oxidative stress, mediating the production and secretions of pro-inflammatory cytokines\textsuperscript{24-26}. These pathways are the polyol, hexosamine, protein kinase C (PKC) and advanced glycation end-product (AGE) pathways\textsuperscript{27-31}. The polyol
pathway reduces glucose to sorbitol, which is then oxidized to fructose, this process consumes nicotinamide adenine dinucleotide phosphate (NADPH)\textsuperscript{26,27}. NADPH is required to synthesize glutathione, a potent cellular antioxidant\textsuperscript{26}. The sequestering of NADPH by the polyol pathway renders the cell increasingly susceptible to oxidative stress and damage\textsuperscript{27}. The hexosamine pathway similarly contributes to NADPH depletion. The hexosamine pathway takes an intermediary of the glycolytic pathway, fructose-6-phosphate and converts it to glucosamine-6-phosphate\textsuperscript{28}. This molecule then inhibits the activity of glucose-6-phosphate dehydrogenase, a key molecule in maintaining the levels of NADPH\textsuperscript{28}. The hexosamine pathway also results in the production of uridine diphosphate N-acetyl glucosamine, which promotes excessive protein glycosylation and altering protein function\textsuperscript{28}. The AGE pathway promotes protein modification that is ultimately pathogenic\textsuperscript{29,32}. Excess glucose leads to protein glycation, a non-enzymatic process that alters proteins pathologically\textsuperscript{29,32}. Glycated proteins include various structural and functional proteins, and modifications may result in protein dysfunction and ultimately the generation of further ROS\textsuperscript{29}. The PKC pathway begins with the activation of PKC by diacylglycerol, driven by high levels of yet another glycolytic intermediate, dihydroxyacetone phosphate\textsuperscript{30,31}. PKC activation promotes fibrotic and proinflammatory gene expression changes, as well as endothelial permeability by reducing the expression levels of endothelial nitric oxide synthase (eNOS)\textsuperscript{26,30}. The activation of
PKC promotes endothelial dysfunction in myriad ways\textsuperscript{26,30,31}. A brief description of specific chronic complications involving the microvasculature follows.

1.2.1 Diabetic retinopathy

Diabetic retinopathy (DR) is the most common eye disease and cause of blindness in working-aged adults, currently affecting 100 million people worldwide\textsuperscript{33}. It results in diabetic macular edema, neovascular glaucoma and retinal detachment. In addition, DR increases the risk for developing cataracts\textsuperscript{34}. DR has two stages, non-proliferative (NPDR) which develops into proliferative (PDR), and macular edema may develop in any stage of DR\textsuperscript{35–37}. NPDR is characterized by increased vascular permeability, capillary non-perfusion and other microvascular abnormalities\textsuperscript{35–37}. The dysfunction of endothelial cells is crucial to the development NPDR and progression of PDR\textsuperscript{38}. The damage sustained by endothelial cells result in the dysfunction and breakdown of both the blood-retinal barrier and retinal vasculature\textsuperscript{35,39}. Endothelial dysfunction and death leads to a leaky endothelium, resulting in increased vascular permeability\textsuperscript{35,39}. Basement membrane thickening, observed in DR, is yet another indication of endothelial dysfunction\textsuperscript{39}. Both the signaling derangement of damaged endothelial cells along with the increased vascular permeability and subsequent immune infiltration contribute to the production and deposition of extracellular matrix proteins into the basement.
membrane. The eventual damage and disruption to retinal vessels results in overexpression of angiogenic factors, leading to neovascularization and commencing the final stage of DR. The blood-retinal barrier (BRB) maintains balance in the retina and is composed of endothelial cells. Breakdown of the BRB due to endothelial damage and death results in the accumulation of fluid in the retina, resulting in macular edema and its associated vision loss. Neovascularization in PDR results in vascular leakage from newly formed vessels to further accumulate in the retina.

1.2.2 Diabetic nephropathy

Diabetic nephropathy (DN) is the leading cause of kidney failure in the world, in addition, it has strong associations with the development of cardiovascular complications. The primary manifestations of DN are proteinuria and failure of renal filtration. The glomerulus is the structure in the kidney most critical to kidney function, and glomerular scarring, glomerulosclerosis, leads to end-stage kidney disease. Various cells in the glomerulus overexpress GLUT1 in diabetes and suffer from hyperglycemic damage as a result. This promotes an inflammatory environment that drives immune infiltration and fibrosis. Among these cells are endothelial cells, which maintain the selective barrier that drives reabsorption and filtration. The glomerular basement membrane is also of key importance, and it is the production and deposition of excess
extracellular matrix proteins into the basement membrane that results in both glomerulosclerosis and proteinuria\textsuperscript{44}. With the thickened glomerular basement membrane, there is a paradoxical increase in membrane permeability, this is due a lack of organization with non-uniformity in the membrane\textsuperscript{44}. Due to the increased permeability, large proteins can pass from circulation into the filtrate, which results in tubular damage and increases the severity of interstitial inflammation. This, of course, leads to further fibrosis and reduced kidney function\textsuperscript{44,45}. The reduced kidney function results in a reduction of glomerular filtration rate, which is associated with increased mortality and morbidity with cardiovascular disease\textsuperscript{46}. Patients with diabetes already face higher risk of cardiovascular complications, and the development of diabetic nephropathy only compounds upon the issue.

1.2.3 Diabetic peripheral neuropathy

Diabetic peripheral neuropathy (DPN) manifests as a loss of sensation in the distal regions of the body\textsuperscript{47}. This renders diabetic patients particularly susceptible to infections of the lower limb, with diabetic patients being 15-30 times more likely to receive a lower-limb amputation due to infection of distal wounds\textsuperscript{48}. Originally considered to be a disease with a neurological origin, it is now being understood as a microvascular complication of diabetes\textsuperscript{47,49}. Hyperglycemic damage changes the endothelial cells lining the peripheral nerve vasculature\textsuperscript{47,49}. Microvessels
of neurovasculature show thickened basement membranes and disrupted endothelial cells\textsuperscript{47}. Due to hyperglycemic damage, the endothelial cells making up the vasculature around peripheral nerves will malfunction and show signs of disrupted endothelium\textsuperscript{47}. In addition, vascular tone is disrupted in diabetes as a result of deranged eNOS and NO balance, leading to inappropriate vasoconstriction and vasodilation, further disrupting blood flow\textsuperscript{50}. Peripheral nerves have limited vascular supply, and any disruption to the vasculature renders the peripheral nerves especially susceptible to ischemia and subsequent death\textsuperscript{47,49,50}.

### 1.2.4 Diabetic cardiomyopathy

Type 2 diabetes, in particular, is often associated with and comorbid with obesity, coronary artery disease, hypertension, and increased risk of stroke\textsuperscript{51}. However, diabetes itself is an independent risk factor for cardiovascular disease, with each 1\% increase in glycated hemoglobin increases the risk of heart failure in both T1DM and T2DM\textsuperscript{52}. The cardiovascular system suffers both micro- and macrovascular complications from diabetes\textsuperscript{16,19,53}. Atherosclerosis being the primary macrovascular complication of diabetes, thought to arise from chronic inflammation and injury to arterial walls\textsuperscript{19,54–56}. Atherosclerosis results in the restriction of blood flow, leading to hypertension and coronary artery disease, exacerbating risk for heart failure\textsuperscript{55}. Diabetic cardiomyopathy (DCM) is the microvascular cardiovascular complication of diabetes, and it
presents as ventricular dysfunction independent coronary atherosclerosis and hypertension. It presents clinically with structural and functional deviations, including left ventricular hypertrophy, cardiac fibrosis and remodeling. Patients with T1DM who do not have hypertension or coronary artery disease suffer from cardiac dysfunction up to five times more often than non-diabetic cohorts. One study has found that 16.7% of diabetic patients meet the criteria for diabetic cardiomyopathy. Although other mechanistic changes may also play specific roles, microvascular pathology remains the primary contributor. DCM is characterized by interstitial and perivascular fibrosis, structural changes, tissue remodeling, cardiac dysfunction, ventricular hypertrophy, and cardiomyocyte death. Endothelial damage and dysfunction lie at the heart of DCM pathogenesis, with endothelial cells contributing to cardiac fibrosis and remodeling. As such, DCM is responsible for long-term changes that compound upon the already increased burden on cardiovascular health suffered by diabetic patients.

1.3 Cardiac fibrosis

Cardiac fibrosis is the first notable structural change in the progression of DCM. It is characterized by interstitial and perivascular fibrosis that result in stiffness of the heart, loss of contractility, arrhythmic events, and will lead to cardiac remodeling and heart failure. Cardiac fibrosis most commonly results after myocardial infarction, however aging,
hypertension, and diabetic cardiomyopathy also result in development of cardiac fibrosis. The interstitial fibrosis is associated with accumulation of the extracellular matrix proteins, type I and III collagen in the left and right ventricle. Myocardial fibrosis in diabetes is in addition, observed along with the thickening of the capillary basement membrane, indicating endothelial dysfunction. Fibroblasts are the primary matrix-producing cells in the heart, and as such are the primary contributing cell type to cardiac fibrosis. In myocardial infarction, the primary investigative area of focus for cardiac fibrosis, fibroblasts are activated through TGFβ signaling, and subsequently differentiate into myofibroblasts. Fibroblasts are abundant in the heart, making up to 20% of the cells, but myofibroblasts are extremely rare. Myofibroblasts function differently from fibroblasts by the expression of contractile proteins such as α-SMA, by increased proliferation, and by the mass production of extracellular matrix proteins, collagen and fibronectin. Immune cell infiltration is also observed in cardiac fibrosis, especially macrophages and lymphocytes, which acquire a fibrogenic phenotype which contributes to fibrosis and remodeling. Furthermore, inflammatory immune cell signaling further activate fibroblasts, resulting in more myofibroblasts and production of extracellular matrix proteins. Though cardiac fibrosis has primarily been examined in the context of myocardial infarction, hyperglycemia also independently leads to cardiac fibrosis. Hyperglycemia directly leads to the accumulation of AGEs that crosslink
extracellular matrix proteins. AGEs may accumulate in the extracellular matrix and contribute to Receptor for AGE (RAGE) signaling\textsuperscript{80}. In fibroblasts, RAGE signaling stimulates inflammatory gene synthesis and stimulates proliferation and matrix protein production\textsuperscript{80,81}. As explained previously, endothelial damage from hyperglycemia is a large and significant driving force behind development of fibrosis. Hyperglycemic damage to endothelial cells further contributes to inflammatory signaling, matrix protein production, endothelial to mesenchymal transition, fibroblast activation and proliferation, and immune infiltration and will be explored next.

1.4 Endothelial dysfunction in diabetic cardiomyopathy

As previously mentioned, endothelial cells suffer oxidative damage and undergo various signaling and transcriptional changes under hyperglycemic conditions. Endothelial dysfunction is defined by the dysregulation of nitric oxide and the prevalence of oxidative stress, both of which occur in hyperglycemic damage\textsuperscript{82}. In DCM, PKC pathway activation reduces the transcription of eNOS and inhibits eNOS signaling, while increasing the expression of VEGF, TGFβ, and NF-κB\textsuperscript{17,21,83–86}. In addition, PKC signaling results in endothelial cells producing collagen and fibronectin independent of fibroblasts\textsuperscript{17,87}. eNOS produces the vasodilator nitric oxide (NO), crucial to the maintenance of vascular
Endothelin-1 (ET-1), a potent vasoconstrictor and promotes fibroblasts to produce collagen 1 and 3, the two types of collagen previously mentioned to be enriched in cardiac fibrosis. TGFβ and NF-κB further promote the differentiation of fibroblasts to myofibroblasts, as well as immune cell recruitment through the expression of leukocyte adhesion molecules. Immune cell infiltration into the interstitium is also abetted by the increased expression of VEGF, which causes increased vascular permeability. The feedback loop of these multiple intertwined pathways, all promoting fibrogenesis, inflammation and immune recruitment, result in a spiderwebbing of fibrotic protein production centered around hyperglycemia-driven endothelial dysfunction.

Endothelial cells additionally contribute directly to fibrosis contribution to the fibroblast pool. In response to hyperglycemic damage, endothelial cells may differentiate into mesenchymal cells through a process called endothelial-to-mesenchymal transition (EndMT). EndMT is regulated by signaling pathways involved in inflammatory signaling, such as TGFβ, Notch, and Wnt, which are dysregulated in various disease states. Hyperglycemic damage is also capable of triggering pro-EndMT signaling. The mesenchymal-like cells take on the traits of bone marrow derived mesenchymal cells, increase expression of extracellular matrix proteins, decreased endothelial junction proteins, increased motility, and pseudo-multipotency. As such, they are able to differentiate into fibroblasts as well as migrate to the interstitium, with
Figure 1.1 Hyperglycemic damage to endothelial cells results in the generation of reactive oxygen species, which results in endothelial dysfunction. Dysfunctional endothelial cells contribute to the development of fibrosis in a myriad of ways.
the gaps left by the migrated mesenchymal cells also contributing to vascular permeability\textsuperscript{98}. These fibroblasts of endothelial origin may then be activated as resident cardiac fibroblasts and contribute to the production of matrix proteins\textsuperscript{98}. While EndMT is a physiologic process which exists during cardiac development, and the transition of endothelial cells and subsequent migration of mesenchymal cells is crucial in formation of heart valves, it is also a contributor to the pathogenesis of various other cardiovascular diseases, primarily atherosclerosis in diabetes, as well as in cancer and fibrosis of other organs\textsuperscript{16,96,99–102}. Several molecular changes, as well as the development of pathogenic EndMT, are regulated by epigenetic processes.

1.5 Epigenetics

Many alterations to gene expression happen throughout a cell’s life. Epigenetic changes are stable and heritable changes to gene expression without a change in the underlying genome\textsuperscript{103}. Epigenetic modifications include three different categories, histone modification, DNA methylation and noncoding RNA (ncRNA)\textsuperscript{104}. Histones are proteins which organize strands of DNA, packaging DNA into its chromatin structure\textsuperscript{104}. In addition to their capacity as organizational molecules, they also function as regulators of gene expression. Histones can be post-translationally modified to alter the structure of chromatin, affecting access of translational machinery to areas of the genome\textsuperscript{105}. Several families of
enzymes make changes to histones. Histone deacetylases, as their name would suggest, remove acetyl groups from histones, rendering DNA less accessible\textsuperscript{105}. Histone acetyltransferases are their counterpart, acetylating histones to promote transcription\textsuperscript{105}. In addition, histones may be modified with methylation and ubiquitination, along with others such modifications\textsuperscript{106}. DNA itself can be epigenetically modified through DNA methylation. In DNA methylation, a methyl group is covalently added to a cytosine residue\textsuperscript{104}. These methyl groups change the structure of DNA without changing the genetic sequence\textsuperscript{104}. DNA methylation may act both to promote and inhibit the recognition of DNA by different proteins\textsuperscript{107}. In general, DNA methylation leads to gene repression, but may also promote gene expression\textsuperscript{108}. Noncoding RNAs (ncRNAs) are a group of RNA molecules which are transcribed but not translated and are generally classified as infrastructural and regulatory ncRNAs\textsuperscript{109}. Infrastructural ncRNA includes transfer, ribosomal and small nuclear\textsuperscript{109}. Regulatory ncRNA includes microRNA (miRNA), long non-coding RNA (lncRNA), small interfering RNAs (siRNA) and circular RNA (circRNA)\textsuperscript{110-113}. ncRNA’s capacity as epigenetic regulator is fulfilled primarily by regulatory RNA classes, and each class of ncRNA has different structures and function differently as a result. For example, miRNA are on average 22 nucleotides long and function through binding to the 3’ UTR of target mRNA, leading to degradation in conjunction with a RNA-induced silencing complex (RISC)\textsuperscript{114}. In contrast, circRNA are covalently closed RNA molecules which
function as a sponge to sequester miRNA and bind with proteins\textsuperscript{115}. While certain epigenetic changes are conserved and occur through the natural life cycle of an organism, the epigenome of different individuals varies as greatly as the genomic structure itself\textsuperscript{116,117}. Environmental stimuli are most culpable in epigenetic changes\textsuperscript{103,117}. A person’s lifestyle and environment are strongly linked to their epigenome and may influence the epigenome of their offspring\textsuperscript{116,117}. The epigenome serves as marker for differentiation between healthy and diseased states, and many epigenetic modifications are capable of driving disease\textsuperscript{117}. Abnormal epigenomic profiles have been found in various cancers, autoimmune diseases, neurodegenerative disease, and diabetes\textsuperscript{16,118-123}.

1.5.1 Epigenetics and contribution to metabolic memory in diabetes and diabetic complications

The Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) were landmark studies in the field of diabetic complications, showing the persistent damage from high blood glucose. DCCT gave over 1,400 patients with T1DM either conventional therapy or intensive glucose management therapy. In 1994 the EDIC study treated both groups to intensive therapy and has continued to monitor patients to the present day\textsuperscript{124}. During EDIC, the hemoglobin $A_{1c}$ (HbA\textsubscript{1c}) levels were normalized between the two groups, yet even after 18 years, the patients who received conventional
treatment were at a significantly higher risk of developing nephropathy, retinopathy, and neuropathy\textsuperscript{125,126}. The findings of the DCCT and EDIC drew attention to the concept of metabolic memory, that the effects of hyperglycemia on cells throughout the body persisted even after sustained normoglycemia. The persistent nature of these changes has indicated epigenetic factors, and researchers have since found several differences in the epigenome between those in the conventional and intensive therapy groups. Monocytes analyzed from the DCCT show that patients who received conventional therapy and experienced progression of either retinopathy or nephropathy showed hyperacetylation of histone 3 lysine 9 (H3K9) and therefore activation of promoters associated with the inflammatory NF-\(\kappa\)B pathway\textsuperscript{127}.

Aside from the DCCT and EDIC, researchers have experimentally shown that even transient high glucose causes epigenetic changes which last long-term into normal glycemia, both \textit{in vitro} and in mouse models\textsuperscript{128}. Genes and pathways associated with endothelial dysfunction have been observed in conjunction with H3K9 and H3K14 hyperacetylation and aberrant CpG methylation profiles\textsuperscript{129}. In the kidneys, podocytes exposed to hyperglycemia induced persistent hyperacetylation and hypermethylation of H3K4 in genes contributing to insulin resistance and podocyte dysfunction even after glucose re-normalization\textsuperscript{130}. ChIP assays of diabetic mice showed increased histone methylation of H3K4 in conjunction with increased expression of collagen type 1 and TGF\(\beta\)\textsubscript{1}\textsuperscript{131}. In
addition, levels of miR-311, a miRNA associated with fibronectin production were found upregulated \textit{in vitro} and in mouse diabetic nephropathy models\textsuperscript{132}. DNMT1 is overexpressed in diabetic retinopathy and has shown to hypermethylate a gene which regulates mitochondrial function and apoptosis. DNMT1 was induced by hyperglycemia, and inhibition of DNMT1 protected mitochondria from damage in human retinal endothelial cells\textsuperscript{133,134}. Several miRNA have also been found to be differentially expressed in diabetic retinopathy, and many have been experimentally found to be mechanistically significant in the pathogenesis of diabetic retinopathy\textsuperscript{135}. In the heart, histone modifications play a role in cardiac remodeling in diabetic cardiomyopathy, with silencing of HDAC3s to ameliorate cardiac function and reverse cardiac remodeling\textsuperscript{136,137}.

1.5.2 Epigenetics and cardiac fibrosis

The pathogenesis of cardiac fibrosis is well characterized as it is resultant of various injuries. As previously described, cardiac fibrosis manifests early into diabetic cardiomyopathy and is a result of hyperglycemic damage\textsuperscript{71}. Myocardial infarcts and hypertensive heart disease also result in cardiac fibrosis\textsuperscript{68,138}. The development of cardiac fibrosis regardless of injury is heavily regulated through epigenetic means, especially regarding the activation of resident cardiac fibroblasts as well as the transition of
endothelial cells to mesenchymal cells (EndMT) which further contribute to fibrosis\textsuperscript{139}.

The acetyltransferase p300 promotes fibrosis in cardiac myoblasts treated with hyperglycemia. p300 also regulates the synthesis of type 1 collagen by cardiac fibroblasts, and knockdown of p300 prevents synthesis of type 1 collagen in the presence of TGF\textbeta\textsuperscript{140,141}. Cardiac endothelial cells treated with hypoxia indicate hypermethylation of the promotor for RASCAL1, an EndMT inhibitor. The hypermethylation of RASCAL1 has been found to be replicated in heart tissue from end-stage heart failure patients\textsuperscript{142,143}. Angiotensin II, a vasoconstrictor, has been known to induce the expression of ET-1, previously mentioned as a inducer of fibrosis\textsuperscript{144}. Evidence now shows that Angiotensin II induces the methylation of H3K4/3, promoting the transcription of ET-1\textsuperscript{144}. In cardiac fibroblasts, miR-489 suppresses expression of HDAC2, which in turn promotes the expression of $\alpha$-SMA and type 1 collagen\textsuperscript{145}. Hypoxia was also found to upregulate miR-143, miR-145 and miR-21, all of which were found to promote downstream fibrotic genes in cardiac fibroblasts\textsuperscript{146,147}. In contrast, expression of the EndMT regulator miR-126-3p was reduced in cardiac ischemia, and overexpression of miR-126-3p reduced cardiac fibrosis\textsuperscript{148}. 

20
1.5.3 microRNA in diabetic cardiomyopathy

Many epigenetic changes have been observed in fibrotic tissue following ischemia, hypertension, and hyperglycemia, however the best example of the commonalities between different diseases and epigenetic categories is well-characterized through miR-200. MiR-200 is an inhibitor of EndMT, and it was found to be downregulated in both ischemia and hyperglycemia, and in both fibroblasts and endothelial cells\textsuperscript{149-151}. MiR-222 and miR-9-5p have all been found to be EndMT regulators that are downregulated in endothelial cells in hyperglycemia\textsuperscript{152,153}. On the other hand, miR-27a-3p and miR-21 are both upregulated in hyperglycemia and are promoters of EndMT, and inhibition of both are able to reverse EndMT\textsuperscript{154,155}. MiR-27a-3p overexpression resulted in increased ROS, reduced NO and increased TGF\textbeta\textsuperscript{ signaling}. Aside from EndMT, miR-133a was found to cause cardiomyocyte hypertrophy in T1DM and treatment of cardiomyocytes with miR-133a mimics was able to prevent hypertrophy\textsuperscript{156}. Hundreds of other miRNA have been found through genome-wide analyses to be persistently differentially expressed in animals treated to high glucose, and only a small subset have been experimentally explored\textsuperscript{157}. The subsequent sections detail the role of miRNA and circRNA relevant to this research.
Table 1.1 MicroRNA of note in diabetic cardiomyopathy

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Change in DCM</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-200b¹⁵⁸</td>
<td>↓</td>
<td>EndMT inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>VEGF inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGFβ1 inhibition</td>
</tr>
<tr>
<td>miR-222¹⁵²</td>
<td>↓</td>
<td>EndMT inhibition</td>
</tr>
<tr>
<td>miR-29a¹⁵⁹</td>
<td>↓</td>
<td>Collagen I and III regulation</td>
</tr>
<tr>
<td>miR-9-5p¹⁵³</td>
<td>↓</td>
<td>EndMT inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TGFBR2 inhibition</td>
</tr>
<tr>
<td>miR-27a-3p</td>
<td>↑</td>
<td>EndMT promotion</td>
</tr>
<tr>
<td>miR-21¹⁴⁷</td>
<td>↑</td>
<td>EndMT promotion</td>
</tr>
<tr>
<td>miR-133a¹⁵⁶</td>
<td>↓</td>
<td>TGFβ1 inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cardiomyocyte hypertrophy</td>
</tr>
<tr>
<td>miR-146a¹⁴⁶</td>
<td>↓</td>
<td>NF- κB inhibition</td>
</tr>
</tbody>
</table>
1.5.4 microRNA in other diabetic complications

Many of the miRNA listed above in cardiac complications have also been indicated in diabetic complication of other organs. For example, miR-9-5p is downregulated in diabetic retinopathy as well, and in both organs, interacts with the binding sites of TGFβ receptor type II and NF-κB\textsuperscript{153,160}. MiR-200 has been shown to be differentially expressed in diabetic retinopathy, nephropathy, and promotes inflammation in aortic endothelial cells in hyperglycemia, contributing to atherosclerosis\textsuperscript{150,161,162}. MiR-21 is also upregulated in the kidneys and retinas by diabetes, contributing to nephropathy and retinopathy\textsuperscript{163,164}.

1.5.5 circRNA in diabetic complications

CircularRNA are a novel class of ncRNA which have only recently been seen biologically significant. They are formed through backsplicing, and serve as transcriptional regulators and as miRNA sponges (Figure 1.2)\textsuperscript{165}. As many miRNAs have been established to be critical regulators of diabetic complications, circRNA should also be explored in the same capacity. CircRNA’s circular structure sets itself apart from other ncRNA, as its two tails are covalently linked, it is highly resistant to endonuclease activity and has a half-life many times greater than other ncRNA. Their unique structure makes them targets to be explored as biomarkers and as therapeutic agents. Their expression profiles have been categorized in
**Figure 1.2** CircularRNA are created through backsplicing, resulting in the creation of a backsplice junction. The backsplice junction may be targeted to differentiate circRNA from linear counterparts. CircRNA function through microRNA sponging through complementary binding.
various cancers, neurological diseases, and autoimmune diseases, but comparatively few experiments have probed their mechanistic significance, a sampling of circRNA with experimentally examined significance is found in Table 1.2. Many circRNA have predicted significance based upon the miRNAs they have binding affinity for. An assay done assessing the differential expression of circRNA in diabetic mice showed over 600 circRNAs which are both upregulated in diabetic murine hearts\textsuperscript{166}. Of the circRNA identified, several are predicted to have binding affinity for several miRNA previously mentioned as significant in diabetic complications.
Table 1.2 CircularRNA of note in various diabetic complications

<table>
<thead>
<tr>
<th>CircularRNA</th>
<th>Change in diabetic complication</th>
<th>MicroRNA target</th>
</tr>
</thead>
<tbody>
<tr>
<td>CircCOL1A2\textsuperscript{167}</td>
<td>Upregulated in DR</td>
<td>MiR-29b</td>
</tr>
<tr>
<td>CircZNF532\textsuperscript{159}</td>
<td>Upregulated in DR</td>
<td>MiR-29a-3p</td>
</tr>
<tr>
<td>Circ-PSEN1\textsuperscript{168}</td>
<td>Upregulated in DR</td>
<td>MiR-200b-3p</td>
</tr>
<tr>
<td>CircRNA_010383\textsuperscript{169}</td>
<td>Downregulated in DN</td>
<td>MiR-135a</td>
</tr>
<tr>
<td>Circ_0080425\textsuperscript{170}</td>
<td>Downregulated in DN</td>
<td>MiR-24-3p</td>
</tr>
<tr>
<td>CircHIPK3\textsuperscript{171}</td>
<td>Upregulated in DCM</td>
<td>MiR-26b-5p</td>
</tr>
</tbody>
</table>
1.6 Rationale

While the role of miR-9 in diabetic complications has been shown, its upstream regulators remain unknown, especially the interactive role of specific circular RNAs, which were shown earlier to be altered in the heart in diabetes.

Hence, to gain a better insight of the molecular mechanisms of miR-9 in diabetic heart disease, especially with respect to its regulation by specific circRNA an in-depth study is needed. Hence, this study was designed to investigate the role of circRNA_012164 in mediating fibrosis in diabetic cardiomyopathy through its interactive role with miR9 to mediate pathogenetic effects.

1.7 Hypothesis

We hypothesize that circRNA_012164 expression will be altered in response to hyperglycemia and in diabetes and will play a role in conjunction with miR-9 in the pathogenesis of cardiac fibrosis in DCM.

1.8 Specific Aims

2. To observe the effect of high glucose on levels of circRNA_012164 and downstream fibrosis genes in cardiac endothelial cells.

3. To establish that a regulatory relationship exists between microRNA-9, circRNA_012164 and downstream fibrotic genes.
Chapter 2 Materials and Methods
2.1 Animals

All animal experiments were performed in accordance with the Canadian Council on Animal Care. All experiments were approved by the University of Western Ontario Council on Animal Care Committee.

2.1.2 Generation of miR-9 transgenic mice

The mice used were miR-9 overexpressing transgenic (m9TG) mice with EC-specific promoter previously generated in our laboratory. A cDNA fragment containing miR-9 was inserted into a pg52pSPTg.T2FpAXK (pg52) plasmid which contained a Tie2 promoter, a promoter primarily expressed in endothelial cells, as well as an enhancer and a SV40 PolyA signal. The fragment containing Tie2-miR9 was excised using restriction enzyme SalI and injected into C57BL/6 mouse blastocysts (Figure 2.1). The blastocysts were transferred into pseudopregnant female mice. Transgenic founders were identified using PCR of genomic DNA from mouse tail-tips. M9TG mice used were confirmed to overexpress miR-9 with PCR. Transgenic mice were assessed for cardiac function through echocardiogram and miR-9 overexpression did not alter cardiac function. No other phenotypic or behavioural abnormalities were observed. Endothelial cells were further isolated from hearts of M9TG mice to assess endothelial specificity of miR-9 overexpression (Figure 2.2).
**Figure 2.1** Vector map of vector used to generate endothelial specific microRNA-9 overexpressing transgenic mice. Adapted from Wang E, Feng B, Chakrabarti S. MicroRNA 9 Is a Regulator of Endothelial to Mesenchymal Transition in Diabetic Retinopathy. Invest Ophthalmol Vis Sci. 2023 Jun 1;64(7):13. doi: 10.1167/ iovs.64.7.13. PMID: 37279396; PMCID: PMC10249683.
Figure 2.2 RT-qPCR analyses of cells from cardiac tissues of endothelial specific microRNA-9 overexpressing transgenic mice. MicroRNA-9 overexpression is significant only in endothelial cells. Adapted from Feng B, Liu J, Wang E, Su Z, Chakrabarti S. Endothelial derived miRNA-9 mediated cardiac fibrosis in diabetes and its regulation by ZFAS1. PLoS One. 2022 Oct 14;17(10):e0276076. doi: 10.1371/journal.pone.0276076. PMID: 36240130; PMCID: PMC9565427.
2.1.3 Induction of diabetes in mice

MiR9TG and C57BL/6 mice at 8 weeks were randomly divided into diabetic and control groups. The animals were given intraperitoneal injections with streptozotocin (STZ) in sodium citrate buffer (pH 4.5, 50 mg/kg). Littermates were given equal volumes of buffer only injections. Mice were injected once per day for 5 consecutive days to induce destruction of pancreatic beta cells to generate the T1DM model. Diabetes was confirmed by measuring blood glucose levels (>16.7 mmol/L) following final STZ injection. For 2 months after confirmation of diabetes, mice were weighed and checked for blood glucose weekly. Diabetic mice were not treated with insulin to maintain blood glucose levels and to control for potential confounding factors resulting from exogenous insulin. After the 2 months, echocardiography was performed to assess cardiac function. Immediately following echocardiography, mice were sacrificed using isoflurane and cardiac tissues were collected for future assessment.

2.1.4 Echocardiography

Echocardiography was performed on mice to assess left ventricular function. Hair was removed from the chest with Nair™ hair removal cream (Church & Dwight Co., Inc., NJ, USA). Mice were anesthetized with 0.2 mL/20 g ketamine hydrochloride (Bioniche). Aquasonic 100 ultrasound transmission gel (Parker Laboratories, Inc., NY, USA) was placed on the
Figure 2.3 Endothelial specific microRNA-9 overexpressing transgenic mice were generated from C57BL/6 mice. The miR-9 overexpressing gene is linked to TIE2 promoter, a promoter almost exclusively expressed in endothelial cells. B6 and M9TG mice at 8 weeks were randomly divided into diabetic and control groups. The mice in the diabetic group were injected with streptozotocin (STZ) in sodium citrate buffer (pH 4.5, 50 mg/kg) and control groups were injected with an equal volume of buffer only. Injections were given daily for 5 consecutive days. Diabetes was confirmed by measuring blood glucose levels (>16.7 mmol/L) following final STZ injection.
chest and abdominal area on mice. VisualSonics scanner was used and images were acquired in M-mode in Vevo 2100 Imaging System Cardiac Package software (FUJIFILM VisualSonics, ON, Canada). Images 1.5 to 5 seconds in duration were taken, and representative images were selected based on clarity. Ventricular parameters were taken, ejection fraction, fractional shortening and E/A ratios were calculated to assess left ventricular function.

2.2 Cell Culture

Mouse Cardiac Endothelial Cells (#CLU510, Cedarlane Laboratories, ON, Canada) were used. As this was a cell line novel to the lab, a series of cell culture protocol optimization was necessary. Following cell passaging into 6 well plate, three different cell counts (50,000, 100,000, 150,000) were treated with either 2% or 5% bovine serum endothelial growth medium (DMEM, Thermo Fisher Scientific Inc., IL, USA) with 1% amoxicillin (Wisent Inc. QC, Canada). Cells were then counted using Trypan Blue Solution, 0.4% (Thermo Fisher Scientific Inc., IL, USA) and a hemacytometer. Varying amounts of cells were plated in 6 well plates. Treatment groups were: i) 50,000 cells, 2% serum medium; ii) 100,000 cells, 2% serum medium; iii) 150,000 cells, 2% serum medium, iv) 50,000 cells, 5% serum medium; v) 100,000 cells, 5% serum medium; vi) 150,000 cells, 5% serum medium.
For subsequent experiments, cells were passaged at 100,000 cells per well and incubated with 2% medium until reaching 60% confluency. The cells were serum-starved for 18 hours, then exposed to normoglycemia (5 mmol/L) or hyperglycemia (30 mmol/L) of D-glucose for 48 hours. The endothelial cells were either treated with TRIzol reagent (Invitrogen Canada Inc, ON, Canada) for mRNA analysis or trypsinized for protein analysis.

2.2.1 Transfection

MCECs were transfected with siRNA targeted circRNA_012164 (50 nM) using Lipofectamine 2000 (Invitrogen Canada Inc, ON, Canada). Scrambled siRNA was used as a control. SiRNA were designed based on complementarity to the backsplice junction of circRNA_012164. Two separate siRNA were designed and assessed for transfection efficiency and the most effective siRNA was used for experiments. In double transfection experiments, MCECs were transfected simultaneously with a miRNA-9 antagomir. Cells were incubated with OPTI-MEM and Lipofectamine 2000 for 6 hours. Cells were then recovered in 1% serum medium for 6 hours, following which, they proceeded to be serum starved, treated to glucose, and collected as previously described.
Table 2.1 Sequences of designed siRNAs targeted to the backsplice junction of circRNA_012164

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Sense 5'-3'</th>
<th>Antisense 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>circRNA_012164_1</td>
<td>UGCCAAGCCCCAAGGUCACCAAGCUU</td>
<td>GCUUUGGUGACCUUGGCUUUGGCAUU</td>
</tr>
<tr>
<td>circRNA_012164_2</td>
<td>GCCCAAGGUCACCAAGCCCAAGAUU</td>
<td>CUUUGGCGUUUGGUGAAGCUUGGCUU</td>
</tr>
</tbody>
</table>
Figure 2.4 Treatment groups for mouse cardiac microvascular cells. Cells are first either transfected with non-specific scrambled siRNA, with siRNA targeted to the backsplice junction of circRNA_012164, or with both si-circRNA_012164 and a microRNA-9 antagomir. Cells are then serum starved for 18 hours, after which they are treated for 48 hours with either 5 mmol/L (normal glucose, NG) or 30 mmol/L (high glucose, HG) glucose.
2.3 RNA Isolation and qRT-PCR Analysis

RNA was isolated from mouse heart tissue and MCECs as previously described. 50-100 mg of tissue was homogenized in TRIzol reagent). Chloroform was added and mixed thoroughly. Following centrifugation, the aqueous phase was collected, and RNA was precipitated with isopropanol. RNA was then quantified with QuickDrop (Molecular Devices, LLC. CA, USA) and cDNA was subsequently synthesized from 2 ug RNA using reverse transcription kit (Thermo Fisher Scientific Inc., IL, USA) and a thermal cycler (MJ Research PTC-100 Programmable Thermal Cycler). The mRNA levels of ACTA2, COL1A1, FN1, and FSP1 were quantified using LightCycler™ (Roch Diagnostics Canada, QC, Canada). The reaction mixture consisted of SYBR(R) Green Taq Ready Mix (Sigma-Aldrich, ON, Canada), forward and reverse 10 uM primers, cDNA template and H2O. The data was normalized to the housekeeping gene β-actin to account for variances in amount of template and reverse transcriptase efficiencies.

2.4 Protein Isolation and Analysis

The tissues were lysed and ultrasonicated in RIPA buffer (MilliporeSigma, Canada) following medium aspiration. Total protein was collected and the concentration was measured by using BCA kit (Thermo Fisher Scientific Inc., IL, USA). ELISA for mouse FN1 (Boster Bio, CA, USA) and COL1A1( Novus Biologicals, ON, Canada) was performed according to the
Table 2.2 Primer sequences for qPCR Analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’S-3’</th>
<th>Reverse 5’S-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>CCTCTATGCCAACACAGTGC</td>
<td>CATCGTACTCCTGCTTGCTG</td>
</tr>
<tr>
<td>ACTA2</td>
<td>CTAICTGCGAGCGTGAGATTGT</td>
<td>GTTTCGTGGATGCCGCTGA CT</td>
</tr>
<tr>
<td>COL1A1</td>
<td>CACCCTCAAGAGCCTGAGTC</td>
<td>GTTGGGGCTGATGTACCAGT</td>
</tr>
<tr>
<td>FN1</td>
<td>CGGTAGGACCTTCTATTCTCT</td>
<td>GATACATGACCCCTTCCATT</td>
</tr>
<tr>
<td>FSP1</td>
<td>AAGTTGCTCATCACCCTTCTG G</td>
<td>GTCCACCTTCCACAAATACT C</td>
</tr>
<tr>
<td>CircRNA 012164-1</td>
<td>CTAAGCCGGTAAGCCAAAG GC</td>
<td>TCTTGACCTTCTTGGGCTTG G</td>
</tr>
<tr>
<td>CircRNA 012164-1</td>
<td>CTAAGCCGGTAAGCCAAAG GCTG</td>
<td>TCTTGACCTTCTTGGGCTTG GT</td>
</tr>
</tbody>
</table>
manufacturer’s instructions.

2.5 Histology

Mouse heart tissues were collected, left ventricles were fixed in 10% formalin, embedded in paraffin and cut to 5 μm thick sections on positively charged slides. The sections were deparaffinized in xylene and stained with hematoxylin and eosin as well as Masson’s trichrome stain.

2.6 Statistical Analysis

The data are expressed as mean +/- SEM and were analyzed by Bonferroni/Dunn test for multiple comparisons. Differences were considered to be statistically significant at values of p < 0.05. Analyses were done and graphs were generated using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, Boston, MA, USA).
Chapter 3 Results
3.1 Aim 1. Establish changes in microRNA-9 and circRNA_012164 in an in vivo model of diabetic cardiomyopathy.

3.1.1 Clinical monitoring of transgenic and diabetic mice

Diabetic animals showed lower body weight, hyperglycemia, increased thirst, and increased urinary volume (Table 3.1). Non-transgenic diabetic mice later showed left ventricular changes characteristic of diabetic cardiomyopathy as assessed with echocardiogram (Figure 3.1).

3.1.1 microRNA-9 and circRNA_012164 are altered in the murine heart in diabetes.

To corroborate and authenticate assay findings and previous laboratory investigations, miR-9 and circRNA_012164 levels were assessed. In keeping with our previous findings, it was confirmed that microRNA-9 was significantly inhibited by diabetes in the murine heart (Figure 3.2). Next, circRNA_012164 levels were evaluated. As no previous work has been done on the molecule, primers needed to be tested. To this end, two different primers were designed and tested with the selection process prioritizing minimal self-annealing tendencies. The primer additionally needed to be validated for circularRNA specificity. To this effect, samples were tested with RNase R to degrade linear RNA. RNase R treatment degraded ACTB significantly when compared to degradation of circRNA_012164 (Figure 3.3), suggesting that the target of the designed
Table 3.1 Clinical monitoring of mice at 2 months following streptozotocin treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean body weight (g)</th>
<th>Mean blood glucose (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 non-diabetic</td>
<td>31.3 ± 3.1</td>
<td>9.1 ± 1.2</td>
</tr>
<tr>
<td>B6 diabetic</td>
<td>24.2 ± 3.9</td>
<td>25.5 ± 1.5</td>
</tr>
<tr>
<td>M9TG non-diabetic</td>
<td>33.2 ± 2.7</td>
<td>7.9 ± 1.7</td>
</tr>
<tr>
<td>M9TG diabetic</td>
<td>25.2 ± 1.8</td>
<td>27.0 ± 2.6</td>
</tr>
</tbody>
</table>
Figure 3.1 Echocardiogram images of A) non-diabetic C57BL/6, B) diabetic C57BL/6 mice, C) nondiabetic endothelial specific microRNA-9 overexpressing mice, D) diabetic microRNA-9 overexpressing mice. Mice were assessed 2 months after induction of diabetes with streptozotocin. Cardiac function was further assessed through quantifying percent change in E) ejection fraction, F) fractional shortening, and G) E/A ratio between the 4 groups.
Figure 3.2 Differential expression of microRNA-9 in diabetic and non-diabetic mice. Diabetes was induced via intraperitoneal injections of streptozotocin, 50 mg/kg/day in citrate buffer in C57BL/6 mice. Age-matched control littermates were given vehicle injections. At 2 months, mice were sacrificed, and hearts were collected. Expression of miR-9 was compared between diabetic and control mice to establish baseline changes in microRNA-9. Expression was normalized to U6 and error bars are representative of the standard error of the mean. Statistical significance was calculated by Student’s t-test. (* $p < 0.05$). $n = 8$. 
Figure 3.3 Percent change of circRNA_012164 levels and β-actin (ACTB) levels following RNase R incubation. Samples were either incubated with 10 ug RNase R or equal amount reaction buffer for 2 hours at 37°C. Difference in amount of RNA was determined as reduction between control and RNase R treatment. Error bars are representative of the standard error of the mean. Statistical significance was calculated by Student’s t-test. (* $p < 0.05$). n = 3.
primer was not degraded with exonuclease, indicating primer was circularRNA-specific. CircRNA_012164 was found to be significantly upregulated in diabetic hearts, validating assay findings (Figure 3.4).

3.1.2 MiR-9 regulates EndMT in the heart in diabetes.

We then proceeded to directly examine the role of miR9 in the heart in diabetes. To this extent we used a novel tool, endothelial-specific microRNA-9 overexpressing (m9TG) mice. These mice had previously been generated in our laboratory. Given miR-9's potent inhibitory role in Endothelial-to-Mesenchymal Transition (EndMT), a critical process in cardiac valve development, an evaluation of cardiac function was deemed necessary to account for any potential confounding factors contributing to cardiac dysfunction.

M9TG mice showed no specific phenotypic changes. The clinical changes in the m9TG mice were similar to wild type mice both in diabetic and non-diabetic groups (Table 3.1). Furthermore, echocardiography was performed to assess the ejection fraction, fractional shortening, and E/A ratio in four groups: i) B6 nondiabetic mice, ii) B6 diabetic mice (littermates to previous), iii) m9TG nondiabetic mice, and iv) m9TG diabetic mice (littermates to previous) (Figure 3.1). Ejection fraction measures the volume of blood pumped by the heart per beat relative to the total volume. In B6 mice, the normal ejection fraction falls within the
Figure 3.4 Differential expression of circRNA_012164 in diabetic and non-diabetic mice. Diabetes was induced as previously in C57BL/6 mice. Age-matched controls were given vehicle injections. At 2 months, mice were sacrificed, and hearts were collected. Expression of circRNA_012164 was compared between diabetic and control mice to establish baseline changes in circRNA_012164. Expression was normalized to β-actin (ACTB) and error bars are representative of the standard error of the mean. Statistical significance was calculated by Student’s t-test. (* p < 0.05). n = 7.
range of 50-65%, with no significant differences observed between the groups. Fractional shortening quantifies the percentage change in the left ventricle during the cardiac cycle, with normal mouse levels typically ranging from 40-45%. Again, no significant differences were found among the groups. The E/A ratio evaluates the ratio of peak velocity blood flow during left ventricular relaxation in early diastole (the E wave) to the peak velocity flow in late diastole caused by atrial contraction (the A wave). A normal E/A ratio is close to 2, whereas an E/A ratio below 1 indicates left ventricular dysfunction indicative of early DCM. B6 diabetic mice exhibited E/A ratios that were significantly lower than the other groups, while these variations were subclinical, they are indicative of early-stage left ventricular dysfunction. These findings suggest that m9TG mice do not differ significantly from B6 mice in terms of cardiac function, except for their potential protective effects in the context of diabetes.

Additionally, Masson's trichrome stains were performed using heart tissue from the four aforementioned groups of mice (Figure 3.5). Areas of blue staining represent interstitial collagen deposition and fibrosis. While the observed effect was not dramatic, there were more apparent areas of blue staining in the hearts of B6 diabetic mice compared to the other groups. The relatively subdued effect may be attributed to the fact that the mice were sacrificed at 2 months, prior to the onset of immune infiltration and the development of more pronounced fibrosis.
Figure 3.5 Masson’s Trichrome staining of murine left ventricular tissue demonstrating interstitial fibrosis. Diabetes was induced in C57BL/6 mice and endothelial-specific miR-9 overexpressing mice. At 2 months, mice were sacrificed, and hearts were collected and stained with Masson’s Trichrome. The four resultant treatment groups are A) B6 non-diabetic control, B) B6 diabetic, C) endothelial-specific miR9-overexpressing non-diabetic control, D) endothelial-specific miR9-overexpressing diabetic. Arrows indicate regions of blue staining indicating interstitial collagen deposition.
We then expanded our studies to examine changes in the heart in diabetes at specific molecular level. Gene expression associated with the molecules altered in fibrosis were examined in murine hearts from all mice, encompassing both diabetic and non-diabetic conditions (Figure 3.6). This analysis included the assessment of COL1A1 and FN1, representing extracellular matrix proteins, and FSP1, serving as a fibroblast activation marker. ACTA2 was omitted from cardiac tissue analysis due to its expression in cardiac muscle cells, rendering it difficult to discern any diabetes-related changes in ACTA2 expression.

All the fibrotic genes studied (COL1A1, FN1, FSP1) exhibited significant upregulation in diabetic hearts, a phenomenon that was mitigated by the overexpression of miR-9 (Figure 3.6). These findings strongly suggest that miR-9 plays a mechanistic role in safeguarding against the development of cardiac fibrosis in diabetic cardiomyopathy.

Furthermore, to validate that mRNA expression levels corresponded with observable changes in phenotype, we conducted protein analyses for two key ECM proteins, collagen type 1a1 and fibronectin (Figure 3.7). The levels of both collagen 1a1 and fibronectin were significantly elevated in B6 diabetic mouse hearts, a trend that was effectively reversed in m9TG mice.
Figure 3.6 Inhibition of diabetes-induced changes in fibrotic gene expression by miR-9 overexpression in vivo. Gene expression of the fibrotic genes A) COL1A1, B) FN1, and C) FSP1 were evaluated in harvested left ventricular tissue of diabetic and non-diabetic, B6 and EC-specific miR-9 overexpressing mice. MiR-9 overexpression was able to reverse diabetes induced overexpression of fibrotic genes. Expression was normalized to β-actin (ACTB) and error bars are representative of the standard error of the mean. Statistical significance was calculated by one way ANOVA and post-hoc Tukey’s test (* p < 0.05). n = 7.
Figure 3.7 Reduction of diabetes-induced fibrotic protein levels by miR-9 overexpression *in vivo*. Protein levels were assessed using ELISA in harvested left ventricular tissue of diabetic and non-diabetic, B6 and EC specific miR-9 overexpressing mice. MiR-9 overexpression was able to rescue overexpression of fibrotic proteins A) collagen type 1 and B) fibronectin, and return protein levels to baseline non-diabetic. Statistical significance was calculated by one way ANOVA and post-hoc Tukey’s test (* p < 0.05). n = 3.
3.2 Aim 2. To observe the effect of high glucose on levels of circRNA_012164 and downstream fibrosis genes in cardiac endothelial cells.

3.2.1 Optimizing cell growing conditions

The CLU510 cells utilized in this study were immortalized mouse cardiac endothelial cells (MCECs), a cell line previously untested and unused in our laboratory. Consequently, it became imperative to optimize the growth conditions to ensure the cells retained their endothelial characteristics.

Given their immortalized nature, these cells exhibit limited contact inhibition, and if allowed to grow uncontrolled, they tend to become overconfluent. To avoid introducing confounding variables during subsequent treatments, it was necessary to address passaging issues, particularly during treatments lasting 4-5 days. Thus, careful consideration was given to determining the appropriate serum concentration and initial cell count.

Through experimentation, it was established that cultivating 100,000 cells per well in a 6-well flask using a 2% serum medium were optimal conditions. This regimen ensured that the cells grew at a rate allowing them to reach 90% confluency by the end of the experiment, as shown in Figure 3.8.
Figure 3.8 Optimal cell growth conditions for immortalized mouse cardiac endothelial cells determined to be 100,000 cells plated with 2% serum medium. Cells were counted using trypan blue and passed into counts of A,D) 50,000 B,E) 10,000 and C,F) 15,000 per well, and grown with A,B,C) 2% serum medium and D,E,F) 5% serum medium. Cells were grown for 5 days following passaging into wells and confluence observed.
3.2.2 Establish changes in gene expression in response to hyperglycemia

MCECs were grown as previously described. MCECs were subjected to treatment with either 5 mM (NG, mimicking normoglycemia) or 30 mM (HG, mimicking hyperglycemia) D-glucose. L-glucose was used as an osmotic control. No differences were observed due to osmolarity, as such, subsequent experiments did not include osmotic controls. The levels of miR-9, circRNA_012164, ACTA2, COL1A1, FN1, and FSP1 were assessed (Figure 3.9). The alterations witnessed in vivo were replicated in vitro and manifested within endothelial cells.

Notably, there was an observable elevation in the levels of circRNA_012164 in response to high glucose. Furthermore, the expression of ECM protein-coding genes COL1A1 and FN1 exhibited a significant increase in response to high glucose. Similarly, the levels of ACTA2 and FSP1, recognized markers of mesenchymal cells and fibroblasts respectively, also experienced an upswing. Particularly noteworthy was the heightened expression of ACTA2, a clear indication that endothelial cells had initiated the transition toward a mesenchymal phenotype.
Figure 3.9 Hyperglycemic-induced changes in EndMT-related gene expression in mouse cardiac endothelial cells. Cells were incubated with 5 mM (normoglycemic, NG) or 30 mM (hyperglycemic, HG) for 48 hours following serum starvation. Expression of A) miR-9, B) circRNA_012164 and the downstream genes C) ACTA2 D) COL1A1, E) FN1, and F) FSP1 were assessed to quantify degree of fibrosis and endothelial signaling derangement. Expression of miR-9 was normalized to U6 while all else was normalized β-actin (ACTB). Error bars are representative of the
standard error of the mean. Statistical significance was calculated by Student’s t-test. (* p < 0.05). n = 6.

3.3 Aim 3. To establish that a regulatory relationship exists between microRNA-9, circRNA_012164 and downstream fibrotic genes.

3.3.1 Mechanistic effect of circRNA_012164 in hyperglycemic endothelial dysfunction

To gain a mechanistic understanding and establish a causational, rather than incidental, relationship between circRNA_012164, miR-9, and downstream fibrotic genes, we conducted knockdown experiments. SiRNAs were designed specifically targeting the backsplice junction of circRNA_012164 and were assessed for their efficacy in reducing circRNA_012164 levels (Figure 3.10). Subsequently, siRNA-1 was selected for use in our future experiments. These experiments were performed in aforementioned mouse cardiac microvascular endothelial cells. The knockdown of circRNA_012164 proved to be effective in rescuing the observed increase in markers of fibrosis and EndMT, while also restoring miR-9 levels to those seen in normoglycemic conditions (Figure 3.11). These findings suggest that circRNA_012164 indeed functions as a sponge for miR-9, and that its knockdown prevents the inhibition and degradation of miR-9. Interestingly, aside from its protective role in hyperglycemia, the knockdown of circRNA_012164 had no significant impact on basal levels of all genes, except for FN1 (Figure 3.11).
**Figure 3.10** Assessment of designed siRNA targeted to the backsplice junction of circRNA_012164. Two siRNA were designed, both were tested for transfection efficiency in mouse cardiac endothelial cells. Expression was normalized to β-actin (ACTB) and error bars are representative of the standard error of the mean. Statistical significance was calculated by one way ANOVA and post-hoc Tukey’s test (* p < 0.05). n = 6.
Figure 3.11 Hyperglycemia-induced changes in gene expression in mouse cardiac endothelial cells with circRNA_012164 knockdown. Cells were transfected using Lipofectamine 2000 with siRNA targeted to the backsplice junction of circRNA_012164 to induce knockdown. Cells were then incubated with 5 mM (normoglycemic, NG) or 30 mM (hyperglycemic, HG) for 48 hours following serum starvation. Expression of A) miR-9, B) circRNA_012164 and the downstream genes C) ACTA2 D) COL1A1, E) FN1, and F) FSP1 were assessed to quantify degree of fibrosis.
and endothelial signaling derangement. HG treatment increased both expression of circRNA_012164 and endothelial markers of fibrosis and EndMT, while inhibiting expression of miR-9. CircRNA_012164 knockdown shows significant reduction in fibrotic gene expression and rescued miR-9 expression levels. Expression of miR-9 was normalized to U6 while all else was normalized β-actin (ACTB). Error bars are representative of the standard error of the mean. Statistical significance was calculated by one way ANOVA and post-hoc Tukey’s test (* p < 0.05). n = 6.
3.3.2 Regulatory interaction of circRNA_012164 and microRNA-9 in endothelial cells mediates glucose induced ECM protein production.

To establish a direct regulatory pathway, MCECs were transfected with both siRNA targeted to circRNA_012164 and an antagomir targeting miR-9. Previously, the knockdown of circRNA_012164 had been shown to prevent the development of endothelial fibrogenesis and mesenchymal transition, possibly by restoring miR-9 levels to those observed under normoglycemic conditions. Co-transfection with both si-circ012164 and miR-9 antagonir resulted in a complete reversal of all protective effects of circRNA_012164 knockdown, returning fibrotic gene levels to control hyperglycemic levels (Figure 3.12).
Figure 3.12 Knockdown of miR-9 negates the protective effects of circRNA_012164 knockdown in mouse cardiac endothelial cells. Cells were transfected using Lipofectamine 2000 with either only siRNA targeted to the backsplice junction of circRNA_012164 to induce knockdown or with both miR-9 antagonir and si-circ012164. Cells were then incubated with 5 mM (normoglycemic, NG) or 30 mM (hyperglycemic, HG) for 48 hours following serum starvation. Expression of downstream genes A) ACTA2 B) COL1A1, C) FN1, and D) FSP1 were
assessed to quantify degree of fibrosis and endothelial signaling derangement. HG treatment increased expression of all fibrotic markers. Knockdown of circRNA_012164 showed a reduction in fibrotic markers. Cotransfection with miR-9 antagomir negated the protective effect of circRNA_012164 knockdown. Expression of miR-9 was normalized to U6 while all else was normalized β-actin (ACTB). Error bars are representative of the standard error of the mean. Statistical significance was calculated by one way ANOVA and post-hoc Tukey’s test (* p < 0.05). n = 6.
Chapter 4 Discussion
4.1 Discussion

The aim of this study was to investigate novel epigenetic mechanisms to develop a better understanding of the pathogenic mechanisms involved in diabetic cardiomyopathy. DCM is an independent risk factor of heart failure in diabetes and manifests clinically as a loss of contractility with cardiac fibrosis and remodeling\textsuperscript{66}. Several mechanisms have been identified as contributing to the development of DCM. Previous research done in the lab has found the involvement of multiple epigenetic mechanisms, including histone acetylation, long-noncoding RNA, and other miRNAs\textsuperscript{153,158,172-174}.

The novelty of this study lies in identifying the regulatory role of circularRNA on microRNA. CircRNAs are a class of ncRNA molecule with newly identified significance both as biomarkers of disease and therapeutic targets\textsuperscript{165}. They function through sponging and therefore inhibit the actions of miRNA and proteins\textsuperscript{165}. A previous assay done had identified several differentially expressed circRNA in the heart in diabetes and their predicted miRNA targets\textsuperscript{166}. In this study, we focused on circRNA\textsubscript{012164}, which was upregulated in the heart in diabetes. CircRNA\textsubscript{012164} was predicted to regulate microRNA-9, which has previously been identified as a regulator of diabetic complications in multiple organs, including DCM. Our investigation established regulatory
pathways in various models, both in *in vitro* and *in vivo*, using both genetically modified and chronically diabetic mice.

Overall, our results indicate the existence of the circRNA_012164/microRNA-9 regulatory axis in the pathogenesis of cardiac fibrosis in DCM. Cardiac fibrosis manifests as increased production of ECM proteins such as collagen type I and fibronectin\(^{175}\). Both endothelial cells and cardiac fibroblasts contribute to cardiac fibrosis in DCM\(^{91}\). Fibroblasts produce the majority of ECM proteins in cardiac fibrosis\(^{138}\). ECs are the most abundant cells in the heart and the first to be damaged from exposure to chronic hyperglycemia in diabetes, and the resultant damage response causes both production of ECM proteins and enabling immune infiltration\(^{91,176}\). ECs are capable of both activating fibroblasts through signaling derangement and differentiating into fibroblasts through EndMT, a process by which transcription derangement leads to ECs taking on a mesenchymal phenotype\(^{91}\).

Levels of miR-9 were found to be downregulated in the heart in diabetes, consistent with previous studies\(^{153,160}\). In addition, an increase in transcripts of genes encoding the ECM proteins collagen type 1 (COL1A1) and fibronectin (FN1), as well as an increase in the fibroblast marker, FSP1. We further show the potency of miR-9’s regulatory role using an EC-specific miR-9 overexpressing transgenic mouse model. Through endothelial-specific expression of miR-9, levels of COL1A1 and FN1 were
both significantly reduced in diabetes and restored to baseline non-diabetic. In addition, FSP1 was also significantly reduced and restored to baseline. This is consistent with previous studies that miR-9 has an effect on mediating and preventing the development of cardiac fibrosis in response to diabetes\textsuperscript{153}.

While miR-9 has been quantified previously as being a regulator of genes contributing to various diabetic complications through interactions with proteins and other ncRNA, little is known about the mechanisms behind regulation of miR-9\textsuperscript{153,160}. The IncRNAs ZFAS1 has been shown to regulate miR-9, with it potentially affecting miR-9 levels through trimethylation of H3K27\textsuperscript{153}.

CircRNAs are one molecule known to function through regulating miRNA. In diabetic retinopathy, circRNA_0005015 levels were found to be upregulated and facilitated endothelial angiogenesis by promoting EC proliferation, migration and tube formation through inhibition of miR-519d-3p\textsuperscript{177}. In DCM, circRNA_010567 was found upregulated in diabetic mouse myocardium and in cardiac fibroblasts, and promoted fibrosis through interactions with miR-141\textsuperscript{178}. Our study identifies circRNA_012164 as an upstream regulator of miR-9. CircRNA_012164 was found to be upregulated in diabetes, confirming results of the previously conducted assay.
The role of circRNA_012164 and miR-9 was further established in ECs. CircRNA_012164 was found to be upregulated in isolated ECs in response to hyperglycemic treatment. A knockdown of circRNA_012164 resulted in a recovery of levels of miR-9 in cells treated with hyperglycemia. MiR-9 has previously been established as a repressor of EndMT, and the prevention of miR-9 depletion resulted in a reduction in transcripts associated with fibrosis and EndMT. In particular, alpha-smooth muscle actin (ACTA2), a marker for mesenchymal cells and activated myofibroblasts was found to be reduced in circRNA_012164 knockdown. The reduction of the mesenchymal marker indicates that inhibition of circRNA_012164 prevents hyperglycemia induced EndMT. The protective effect of circRNA_012164 knockdown against fibrosis and EndMT was negated through miR-9 knockdown, indicating a circRNA_012164/miR-9 regulatory axis, and that the fibrosis-promoting capacity of circRNA_012153 is in part mediated through miR-9 inhibition (Figure 4.1).

Knockdown of circRNA_012164 did not significantly affect levels of either miR-9 or fibrotic genes in normoglycemic conditions. This indicates that circRNA_012164 may suppress the protective effects of miR-9 through sponging in hyperglycemia, but remain inactive through other means when in a normoglycemic condition. Regulation of circRNA_012164 and indeed other circRNA have yet to be fully researched.
**Figure 4.1** Schematic representation of the regulatory axis of circRNA_012164 and microRNA-9-5p in hyperglycemia-mediated fibrosis and EndMT.
ECs affect the surrounding cells of the heart in dramatic ways. EC signaling activates cardiac fibroblasts into myofibroblasts, amping up production of ECM proteins. EC death and dysfunction leads to vascular permeability as well as immune cell recruitment, resulting in immune infiltration into the interstitium, leading to further inflammation and fibrosis. ECs also produce ECM proteins when activated by damage and inflammation, or after undergoing EndMT and taking on a mesenchymal and eventually myofibroblast phenotype. The role of circRNA_012164 and miR-9 have been established mechanistically in cardiac endothelial cells, however their regulatory effect may be even greater when considering the exponential role of ECs in diabetic complications.

4.2 Future Directions

Levels of circRNA_012164 changed in cardiac tissue in response to diabetes a comparable amount as in endothelial cells treated with high glucose. This suggests that other cells in the heart may also express circRNA_012164. Previous studies have shown that miR-9 is downregulated in cardiomyocytes and in diabetic hearts, and that miR-9 inhibits cardiomyocyte death\(^{179}\). Should circRNA_012164 be expressed in cardiomyocytes as well, that indicates a potential future avenue for exploration.
MiR-9 has also been found to be differentially expressed in diseases aside from diabetic complications. It has a role in various cancers and neurologic disorders\textsuperscript{180-182}. CircPLEKHM3 inhibits miR-9 function, which is protective in ovarian cancer\textsuperscript{180}. Similarly, miR-9 is a promoter of hepatocellular carcinoma, and through suppression of miR-9, circMTO1 suppresses hepatocellular carcinoma\textsuperscript{181}. MiR-9 has been implicated as mediating major depressive disorder and suppression of miR-9 by circDYM in microglial cells led to improved depression-like behaviour\textsuperscript{182}. The expression profiles of these circRNA have yet to be investigated in diabetes, and may play a role in regulating miR-9 as well.

In addition, the assay by Patil et. al in 2021 show several other circRNA which bind to various miRNA of interest. Five other circRNA, circRNA\textsubscript{35663}, circRNA\textsubscript{34936}, circRNA\textsubscript{40807}, circRNA\textsubscript{36135} and circRNA\textsubscript{42623} have been found to both be upregulated in diabetic murine hearts and bind to miR-9. Other upregulated circRNA, like circRNA\textsubscript{39888}, bind to miR-200b, another miRNA of interest in diabetes. Investigation into these differentially expressed circRNA may further illustrate a more comprehensive network of interlinked regulatory molecules that contribute to a disease process as complicated as DCM.

Altogether, long term studies must also be conducted in the future to ensure the generalizability of research. Diabetic cardiomyopathy along with other chronic complications arise due to long-term diabetes and
hyperglycemia. *In vitro* and *in vivo* experiments are unable to fully capture the complexity of a system exposed to years of chronic hyperglycemia.

### 4.2 Limitations

Due to this study being a Master's research project, many limitations exist due to both time and scope constraints. A major limitation of this study was the lack of capacity for overexpression of circRNA_012164, in addition, levels of circRNA_012164 were not manipulated in vivo in any way. The manipulation of circRNAs in vivo and methods for transfecting circRNA mimics into cells are currently under preliminary investigation, however none are possible within the scope of this project.

The mouse model used also presents limitations. Only male mice were used for in vivo studies, limiting the generalizability for findings. Male and female cardiovascular health outcomes are known to be different, and the effect of subject sex was not accounted for in this study. As diabetes was induced through streptozotocin injections to destroy pancreatic β cells, the mouse model is specific to type 1 diabetes mellitus. Despite this limitation, it is worth noting that DCM is a complication seen in both T1DM and T2DM. Due to the nature of the transgenic model, m9TG mice may potentially have variable number of copies of the gene. The potential dose effect of multiple miR-9
overexpressing genes was not accounted for in results, nor were copy numbers identified in individuals throughout the results process.

Diabetic cardiomyopathy is a chronic complication of diabetes, as such, signs and symptoms develop gradually over long periods of time. In our model of diabetes, mice were sacrificed 2 months after confirmation of diabetes to avoid external systemic insulin injection, which may act as a confounding variable. In addition, cells were incubated for 48 hours in hyperglycemia, a timeline which is not replicative of chronic hyperglycemia and may lead to reduced applicability to full systems.
References


39. Sorrentino, F. S., Matteini, S., Bonifazzi, C., Sebastiani, A. & Parmeggiani, F. Diabetic retinopathy and endothelin system:


74. The alpha-smooth muscle actin-positive cells in healing human myocardial scars. - PMC.  
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1887334/.


77. Urbina, P. & Singla, D. K. BMP-7 attenuates adverse cardiac remodeling mediated through M2 macrophages in prediabetic


144. Yu, L. et al. Histone Methyltransferase SET1 Mediates Angiotensin II–Induced Endothelin-1 Transcription and Cardiac Hypertrophy in


Appendix 1: Animal Ethics Approval

AUP Number: 2019-095
PI Name: Chakrabarti, Subrata
AUP Title: Vasoactive and Cardioactive Factors in Diabetic Cardiomyopathy
Approval Date: 12/01/2019

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2019-095:1: entitled "Vasoactive and Cardioactive Factors in Diabetic Cardiomyopathy " has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please 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:
   a) Western's Senate MAPPs 7.12, 7.10, and 7.15
      http://www.uwo.ca/univsec/policies_procedures/research.html
   b) University Council on Animal Care Policies and related Animal Care Committee procedures
      http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm

2) As per UCAC's Animal Use Protocols Policy,
   a) this AUP accurately represents intended animal use;
   b) external approvals associated with this AUP, including permits and scientific/departmental 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.
      e) http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
   a) be made familiar with and have direct access to this AUP;
   b) complete all required CCAC mandatory training; and
   c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,
   a) Practice will align with approved AUP elements;
   b) Unrestricted access to all animal areas will be given to ACVS 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: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

Dr. Timothy Regnault,
Animal Care Committee Chair

The University of Western Ontario
Animal Care Committee / University Council on Animal Care
London, Ontario Canada N6A 5C1
http://www.uwo.ca/research/services/animalethics/index.html
Curriculum Vitae

Education

2021 – present
Western University
MSc candidate
Pathology and Laboratory Medicine

2017 – 2021
Western University
BMSc
Pathology and Physiology

Publications/Manuscripts


In preparation

Teaching

2022 – 2023  Graduate Teaching Assistant
              One Health 4100
              Dr. Patti Kiser
              Western University

2021  Graduate Teaching Assistant
      Biology 1001
      Dr. Niki Sharan
      Western University

Abstracts and Presentations


---

2022


**Awards**

<table>
<thead>
<tr>
<th>2021 – 2023</th>
<th>Western Graduate Research Scholarship</th>
</tr>
</thead>
<tbody>
<tr>
<td>2017 – 2021</td>
<td>Dean’s Honour List</td>
</tr>
</tbody>
</table>