Role of SIRT1 in Vascular Complications of Diabetes

Rokhsana Mortuza
The University of Western Ontario

Supervisor
Dr. Subrata Chakrabarti
The University of Western Ontario

Graduate Program in Pathology

A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

© Rokhsana Mortuza 2014
ROLE OF SIRT1 IN VASCULAR COMPLICATIONS OF DIABETES

(Thesis format: Integrated Article)

by

ROKHSANA MORTUZA

Graduate Program in PATHOLOGY

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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

© Rokhsana Mortuza 2014
Abstract

Chronic diabetic complications are significant causes of morbidity. In diabetes, as the cellular changes are similar to aging, we investigated the role of sirtuins (SIRTs) in chronic diabetic complications.

We examined glucose and aging-induced changes in the endothelial cells (ECs), the primary targets of chronic diabetic complications. ECs in high glucose showed evidences of early senescence, being more pronounced in microvascular ECs in association with decreased SIRT1 and FOXO1-dependent reduction in antioxidant gene expressions. A regulatory relationship of deacetylator SIRT1 and histone acetylator p300 was identified. Renal and retinal tissues of diabetic animals showed similar results.

Having established such changes, we investigated microRNA-mediated regulation of glucose-induced SIRT1 alteration. We examined the effect of miRNA-195 (miR-195), a SIRT1-targeting miRNA, on the development of diabetes-induced changes in the ECs and retina. High glucose caused increased miR-195 levels and decreased SIRT1 expression in the ECs. We showed that miR-195 binds to the 3’ untranslated region of SIRT1 and transfection with miR-195 antagonist or over-expression of SIRT1 prevented above changes, whereas transfection with miR-195 mimic produced glucose-like effects. miR-195 expression was upregulated in retinas of diabetic rats and intravitreal injection of miR-195 antagonist ameliorated
reduction of SIRT1 and associated changes in diabetes. These studies identified a novel mechanism whereby miR-195 regulates SIRT1-mediated tissue damage.

Based on results above, we examined whether in diabetes, SIRT1 alteration mediate specific effects in the target organs affected in diabetic complications. We focused on endothelin1 (ET-1) and transforming growth factor beta 1 (TGF-β1), two key mediators of increased extracellular matrix protein production. In ECs exposed to high glucose, there were increased ET-1, TGF-β1, p300 and collagen Iα(I) along with SIRT1 downregulation. Such changes were corrected by knockdown of p300 or SIRT1 overexpression. In the kidneys and retina of diabetic mice, similar biochemical alterations were seen along with increased vascular permeability in retina and microalbuminuria of the kidney. In the transgenic animals with SIRT1 overexpression, such diabetes-induced abnormalities were prevented.

Overall these studies identified SIRT1-mediated accelerated aging phenomena in diabetes causing cellular injuries leading to renal and retinal injury. SIRT1 may lend itself as a target to prevent organ damages in diabetes.

Keywords

Endothelial cells, SIRT1, FOXO1, p300, miR-195, ET-1, TGFβ-1, FN, Diabetic complications, Diabetic retinopathy, Diabetic nephropathy, Cellular aging
Co-Authorship Statement

Manuscript 1:


**Rokhsana Mortuza** Proposed ideas, designed and conducted all experiments, performed data analysis and drafted the manuscript

Shali Chen Provided technical support

Biao Feng Assisted with animal experiment

Subhrojit Sen Assisted with animal experiment

Subrata Chakrabarti Supervisor, conceptually designed the study, edited and finalized manuscript

Manuscript 2:


**Rokhsana Mortuza** Proposed ideas, designed and conducted all experiments, performed data analysis and drafted the manuscript

Biao Feng Assisted with animal experiment and one adenoviral experiment

Subrata Chakrabarti Supervisor, conceptually designed the study, edited and finalized manuscript
Manuscript 3:

Mortuza R, Biao Feng, Chakrabarti S (2014). SIRT1 Causes Renal and Retinal injury in Diabetes through Endothelin 1 (ET-1) and Transforming Growth Factor β1 (TGF-β1). (To be submitted).

Rokhsana Mortuza Proposed ideas, designed and conducted all experiments, performed data analysis and drafted the manuscript

Biao Feng Assisted with animal experiment

Subrata Chakrabarti Supervisor, conceptually designed the study, edited and finalized manuscript

Dedication

I dedicate this thesis to my family.
Acknowledgments

I express my deep acknowledgment and profound sense of gratitude to my supervisor and mentor Dr. Subrata Chakrabarti for his close and constant guidance, encouragements and helpful advices throughout the period of my studies. Through his unequivocal wisdom, knowledge and friendly attitude, he has taught me not only how to be a great researcher but also many qualities of life that have moulded and strengthen my character. I genuinely thank him for giving me the freedom and flexibility to do what interests me. He is an inspiring professor, amazing scientist, a role model physician and above all a wonderful human being. I am forever thankful for his patient supervision during this journey and am truly in debt for this degree to him.

I would like to extend my deepest gratitude to each and every member of my advisory committee: Dr. Chandan Chakraborty, Dr. Edith Arany and Dr. Daniel Hardy for their invaluable suggestions, generous time and tremendous support. Their genuine interest in my research, continuous encouragement and always willingness to help, made the difficult task possible.

I sincerely thank Charlie and Francis for training me with technical skills and to all my lab colleagues for providing a wonderful, supportive and friendly lab atmosphere. I am grateful to all pathology staff for their help and enthusiasm in my work during this program.
Last but not the least, I would like to thank my family for their prayers, unconditional love, sacrifices and support. Ammu and Abbu, receive my deepest gratitude. You have always been nothing but affectionate and supportive through every stages of my life. To my wonderful husband Shamim, thank you for your unwavering love, support and encouragement throughout this journey. My beautiful daughter Zara you are a true blessing from God. Every day is new and magnificent because of you. Merry and Fahim thank you for your well wishes, prayers and motivation. I am fortunate and blessed to have you all in my family and could not possibly have completed this without you.

Finally I THANK the Most Beneficient Most Merciful Almighty GOD for giving me the ability to accomplish this.
Table of Contents

Abstract ......................................................................................................................... ii
Co-Authorship Statement ............................................................................................... iv
Dedication ...................................................................................................................... vi
Acknowledgments ......................................................................................................... vii
Table of Contents ......................................................................................................... viii
List of Tables ................................................................................................................ xiv
List of Figures .............................................................................................................. xv
List of Abbreviations ................................................................................................... xviii

Chapter 1 ..................................................................................................................... 1

1 Introduction ............................................................................................................. 1

1.1 Diabetes mellitus (epidemiology and its economic impact) ................. 1

1.1.1 Type 1 DM ........................................................................................................ 2
1.1.2 Type 2 DM ........................................................................................................ 2
1.1.3 Gestational DM ............................................................................................. 3

1.2 Diabetic complications ..................................................................................... 3

1.2.1 Diabetic retinopathy ..................................................................................... 3
1.2.2 Diabetic nephropathy ................................................................................... 4

1.3 Endothelial cell dysfunction .......................................................................... 4

1.4 Alteration of extra cellular matrix proteins ............................................. 5

1.5 p300 production and histone acetylation in diabetes .......................... 7

1.6 SIRTUINS ......................................................................................................... 8

1.6.1 SIRT1 ............................................................................................................ 10
1.6.1.1 SIRT1 in pancreas ................................................................. 11
1.6.1.2 SIRT1 in adipose tissues ................................................... 11
1.6.1.3 SIRT1 in brain hypothalamus .............................................. 12
1.6.1.4 SIRT1 in hepatic glucose and lipid metabolism .................... 12
1.6.1.5 SIRT1 in inflammation ......................................................... 13
1.6.1.6 SIRT1 and circadian rhythm ................................................. 14
1.6.1.7 SIRT1 regulate insulin sensitivity .......................................... 15
1.6.1.8 SIRT1 in endothelial cells (ECs) and atherosclerosis .......... 16
1.7 FOXO1 ............................................................................................... 17
1.8 SIRT1 regulation by miRNA ....................................................... 18
1.9 Similarites of diabetic complications with aging ......................... 20
1.10 Rationale ...................................................................................... 22
1.11 Hypothesis .................................................................................. 22
1.12 Specific aims ............................................................................... 22
1.13 References .................................................................................. 23

Chapter 2 .......................................................................................... 38

2 High glucose induced alteration of SIRTs in endothelial cells causes rapid aging in a p300 and FoxO regulated pathway .............................. 38

2.1 Introduction .................................................................................. 39
2.2 Materials and methods ................................................................. 40
  2.2.1 Cell culture ................................................................................ 40
  2.2.2 Animal experiments ................................................................. 42
  2.2.3 Ethics statement ....................................................................... 42
  2.2.4 SA β-gal staining of cells and frozen tissue sections ............. 43
  2.2.5 Phase contrast microscopy and morphometrical analysis .... 43
  2.2.6 mRNA extraction and cDNA synthesis ................................. 43
2.2.7 mRNA analysis with quantitative Real Time RT-PCR ......................... 44
2.2.8 Nuclear fraction isolation ................................................................. 45
2.2.9 FOXO1 DNA binding activity measurement ..................................... 45
2.2.10 ROS, MnSOD and LDH analysis ................................................... 45
2.2.11 Telomerase activity ................................................................. 46
2.2.12 Western blot ........................................................................... 46
2.2.13 SIRT1 enzyme activity analysis .................................................. 46
2.2.14 SIRT1 gene silencing .................................................................. 46
2.2.15 p300 gene silencing ................................................................. 47
2.2.16 Statistical analysis ................................................................. 47

2.3 Results .......................................................................................... 48
2.3.1 High glucose accelerates aging process in the ECs ......................... 48
2.3.2 High glucose exaggerates downregulation of SIRTs in aging .......... 55
2.3.3 SIRT1 activators reduce glucose induced accelerated aging .......... 59
2.3.4 SIRT1’s actin in ECs is mediated through FOXO1 ......................... 60
2.3.5 FOXO1 inhibitor or SIRT1 silencing in NG mimics the HG effects .. 61
2.3.6 SIRT1 and p300 have a balancing role on each other ..................... 63
2.3.7 Diabetes causes accelerated aging in kidney .................................. 66
2.3.8 SIRT1 expression is reduced in retina with type 1 and type 2 diabetes ......................................................................................... 69

2.4 Discussion ...................................................................................... 69
2.5 References ...................................................................................... 73

Chapter 3 .......................................................................................... 78

3 miR-195 regulates SIRT1 mediated changes in Diabetic Retinopathy .... 78
3.1 Introduction ...................................................................................... 79
3.2 Materials and methods ..................................................................... 81
3.2.1 Cell culture ........................................................................................................ 81
3.2.2 mRNA extraction and quantitative analysis .................................................. 82
3.2.3 miRNA extraction and analysis ................................................................. 82
3.2.4 miRNA mimic and antagonir transfection .............................................. 82
3.2.5 Adenoviral forced expression of SIRT1 and SIRT1 gene silencing. 83
3.2.6 Luciferase reporter assay for targeting SIRT1-3’UTR .............................. 83
3.2.7 MnSOD, FN ELISA and SIRT1 enzyme activity assay .............................. 83
3.2.8 Animal experiments .................................................................................... 84
3.2.9 In situ hybridization .................................................................................... 85
3.2.10 Immunohistochemistry ............................................................................ 85
3.2.11 SA β-gal staining, phase contrast and fluorescent microscopy ......... 85
3.2.12 Western blot ............................................................................................. 86
3.2.13 Statistical analysis ..................................................................................... 86
3.3 Results .............................................................................................................. 86

3.3.1 High glucose causes upregulation of miR-195 and SIRT1
downregulation in the ECs .............................................................................. 87
3.3.2 Glucose-induced SIRT1 reduction in the ECs is mediated by miR-195....................................................................................... 90
3.3.3 miR-195 binds to 3’UTR of SIRT1 mRNA ............................................. 92
3.3.4 miR-195 antagonir prevents HG-induced reduction of
SIRT1regulated antioxidant levels ................................................................. 93
3.3.5 miR-195 antagonir averts FN upregulation in ECs................................. 94
3.3.6 SIRT1 forced-expression prevents FN upregulation in ECs ........... 97
3.3.7 miR-195 is overexpressed in the retina in diabetes .................. 99
3.3.8 Diabetes induced SIRT1 downregulatin in the retina and and its
downstream effects are mediated by miR-195............................................... 101
3.3.9 miR-195 antagonir prevents SIRT1 mediated EC senescence in
hyperglycemia .................................................................................................. 104
3.4 Discussion .............................................................................................. 107

3.5 References ............................................................................................. 110

Chapter 4 ......................................................................................................... 116

4 SIRT1 causes renal and retinal injury in diabetes through endothelin 1 (ET-1) and transforing growth factor beta 1 (TGF-β1) ........................................... 116

4.1 Introduction ............................................................................................ 117

4.2 Materials and methods ........................................................................ 119

4.2.1 Cell culture .......................................................................................... 119

4.2.2 Animal experiments ........................................................................... 119

4.2.3 SIRT1 enzyme activity assay ............................................................... 120

4.2.4 mRNA extraction and cDNA synthesis .............................................. 121

4.2.5 mRNA analysis with Real Time RT-PCR ........................................... 121

4.2.6 Adenoviral overexpression of SIRT1 and SIRT1 gene silencing ..... 122

4.2.7 p300 gene silencing and p300 overexpression ................................... 123

4.2.8 ET-1, TGF-β1 & FN ELISA ............................................................... 123

4.2.9 Total ROS/RNS assay ......................................................................... 123

4.2.10 Endothelial permeability assay ........................................................ 124

4.2.11 Western blotting ............................................................................... 124

4.2.12 Statistical analysis ............................................................................. 124

4.3 Results ..................................................................................................... 125

4.3.1 SIRT1 regulate ET-1 and TGF-β1 expressions in high glucose .... 125

4.3.2 SIRT1 regulate ET-1 and TGF-β1 expressions via p300 ................. 128

4.3.3 SIRT1 overexpression prevent glucose-induced increased endothelial permeability and collagen Iα(I) expression ............................................. 131

4.3.4 SIRT1 regulate ET-1 and TGF-β1 expressions in the kidney and retinal of diabetic mice ................................................................. 134

4.3.5 SIRT1 overexpression prevents diabetes-induced micro-albuminuria and FN upregulation in tissues ................................................................. 137
4.4 Discussion ........................................................................................................ 139
4.1 References ...................................................................................................... 142

Chapter 5 ............................................................................................................. 145

5 Thesis summary and future directions ............................................................ 145
  5.1 Overall findings .......................................................................................... 146
  5.2 Limitations and future directions ................................................................. 150
  5.3 References ................................................................................................... 152

Appendix-Experimental methods and materials .............................................. 156

Copyright permission ........................................................................................ 174

Curriculum vitae ................................................................................................. 178
List of Tables

Table 2.1 The primers sequences for Real Time RT-PCR ........................................44
Table 3.1 Fold changes of SIRT1 targeting miRNAs .............................................87
Table 4.1 Oligonucleotide sequences for Real Time RT-PCR .............................122
List of Figures

Figure 2.1 Aging signs in microvascular endothelial cells (HMEC) with HG start as early as passage 1 ...................................................................................................49

Figure 2.2 Accelerated aging and associated changes in retinal endothelial cells (BREC) with increasing passages with HG treatment .............................................51

Figure 2.3 Accelerated aging in large vessel endothelial cells (HUVEC) with HG is delayed ....................................................................................................................52

Figure 2.4 Increased oxidative stress and reduced TERT mRNA expression observed in endothelial cells with HG .................................................................54

Figure 2.5 SIRT (1-7) mRNA reduction in HG in microvascular endothelial cells (HMECs) parallels the accelerated aging .................................................................56

Figure 2.6 SIRT 1-7 mRNA analysis with Quantitative Real Time RT-PCR at various passages in BREC showed significant reduction in HG treated cells ......................57

Figure 2.7 SIRT (1-7) mRNA reduction with increasing passage number is augmented with HG treatment in HUVECs ..............................................................58

Figure 2.8 Chemically induced activation of SIRT1 reduces oxidative stress in HG treated endothelial cells ....................................................................................59

Figure 2.9 SIRT1 knockdown or FOXO1 inhibition in NG induces signs of early aging mimicking the HG treatment .................................................................62

Figure 2.10 SIRT1 and p300 regulate each other in microvascular endothelial cells .........................................................................................................................65

Figure 2.11 Diabetes causes accelerated aging in mice kidney .......................67
Figure 2.12 Oxidative stress and associated changes are present in the kidneys and retinas of diabetic animals .................................................................68

Figure 3.1 miR-195 regulates SIRT1 in ECs in hyperglycemia .................................88

Figure 3.2 miR-195 regulates glucose-induced SIRT1 mediated aging changes in HMECs ........................................................................................................91

Figure 3.3 Plasmid map showing site of SIRT1-3’UTR (wt/mut) insertion in the vector (pMIR-Report-Luciferase plasmid) in regards to the luciferase assay ..........93

Figure 3.4 SIRT1 forced-expression prevents glucose-induced FN upregulation in ECs ........................................................................................................95

Figure 3.5 SIRT1 knockdown in NG increases FN protein levels in HRECs ..........96

Figure 3.6 SIRT1 forced-expression showed preventative effect against glucose-induced damage in HMECs .................................................................98

Figure 3.7 miR-195 is overexpressed in the retina in diabetes ...............................100

Figure 3.8 miR-195 antagonir treatment can ameliorate diabetes induced reduction of antioxidant levels in retinal tissues ..............................................102

Figure 3.9 miR-195 antagonir prevents diabetes-induced increased FN protein and vascular leakage in retina .................................................................103

Figure 3.10 miR-195 inhibition halts glucose-induced SIRT1 mediated cellular senescence in ECs .........................................................................................105

Figure 4.1 SIRT1 regulates ET-1 and TGF-β1 expression in ECs ...........................126

Figure 4.2 SIRT1 regulates ET-1 and TGF-β1 via p300 .........................................129

Figure 4.3 SIRT1 overexpression prevents glucose-induced increased endothelial permeability and collagen Iα(I) expression .......................................132
Figure 4.4 SIRT1 overexpressing diabetic mice shows reduced p300 expressions in the kidney and retina ..........................................................................................................................135

Figure 4.5 SIRT1 regulates ET-1 and TGF-β1 expressions in kidney and retina of diabetic mice ...........................................................................................................................................136

Figure 4.6 SIRT1 transgenic mice shows improved renal function and reduced oxidative stress and FN upregulation with diabetes .........................................................138
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>adenosine triphosphate binding cassette transporter 1</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end products</td>
</tr>
<tr>
<td>AgRP</td>
<td>agouti related peptide</td>
</tr>
<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’ adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>one way analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein-1</td>
</tr>
<tr>
<td>aP2</td>
<td>adipose tissue marker protein</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>BM</td>
<td>basement membrane</td>
</tr>
<tr>
<td>BMAL1</td>
<td>aryl hydrocarbon receptor nuclear translocator-like</td>
</tr>
<tr>
<td>c-AMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CLOCK</td>
<td>circadian locomotor output cycles kaput</td>
</tr>
<tr>
<td>CR</td>
<td>calorie restriction</td>
</tr>
<tr>
<td>CREB</td>
<td>c-AMP response element binding protein</td>
</tr>
<tr>
<td>DM</td>
<td>diabetes mellitus</td>
</tr>
</tbody>
</table>
DN  diabetic nephropathy
DR  diabetic retinopathy
DYRK1A  dual specificity tyrosine-phosphorylation-regulated kinase 1A
EC  endothelial cell
ECM  extracellular matrix
ED  endothelial dysfunction
EDBFN  extra domain B fibronectin
ELISA  enzyme-linked immune adsorbent assay
eNOS  endothelial nitric oxide synthase
ER  endoplasmic reticulum
ERCC1  DNA excision repair protein 1
ERCC4  DNA repair endonuclease XPF
ERK5  extracellular signal-regulated kinase 5
ET-1  endothelin-1
ETC  electron transport chain
FA  fatty acid
FN  fibronectin
FOXO1  forkhead box protein O1
FXR  farnesoid X receptor
GDM  gestational diabetes mellitus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAT</td>
<td>histone acetyl transferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>histone deacetylase</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HepG2</td>
<td>hepatocellular carcinoma cell line</td>
</tr>
<tr>
<td>HG</td>
<td>high glucose (25mmol/L D-glucose)</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia inducible factor 1 alpha</td>
</tr>
<tr>
<td>HMVEC</td>
<td>human dermal microvascular endothelial cell</td>
</tr>
<tr>
<td>HREC</td>
<td>human retinal microvascular endothelial cell</td>
</tr>
<tr>
<td>hTERT</td>
<td>human telomerase reverse transcriptase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IDDM</td>
<td>insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>IFN γ</td>
<td>interferon gamma</td>
</tr>
<tr>
<td>IκB</td>
<td>inhibitor of κB</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IRS1</td>
<td>insulin receptor substrate 1</td>
</tr>
<tr>
<td>IRS2</td>
<td>insulin receptor substrate 2</td>
</tr>
<tr>
<td>Lox-1</td>
<td>lectin-type oxidized low density lipoprotein receptor 1</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mM</td>
<td>mmol/L</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>MyoD</td>
<td>member of myogenic regulatory factor proteins</td>
</tr>
<tr>
<td>Nampt</td>
<td>nicotinamide phosphoribosyl transferase</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa B</td>
</tr>
<tr>
<td>NG</td>
<td>normal glucose (5mmol/L)</td>
</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NPDR</td>
<td>non-proliferative diabetic retinopathy</td>
</tr>
<tr>
<td>OSM</td>
<td>osmotic control (25 mmol/L L-glucose)</td>
</tr>
<tr>
<td>OxLDL</td>
<td>oxidized low density lipoprotein</td>
</tr>
<tr>
<td>PARP</td>
<td>poly ADP ribose polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDK4</td>
<td>pyruvate dehydrogenase kinase 4</td>
</tr>
</tbody>
</table>
PDR    proliferative diabetic retinopathy
Pdx-1  pancreatic and duodenal homeobox 1
PER2   period circadian protein homolog 2
PGC-1α  peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PKC    protein kinase C
POMC   pro-opiomelanocortin
PPARα  peroxisome proliferator-activated receptor alpha
PPARγ  peroxisome proliferator-activated receptor gamma
PTP1B  protein-tyrosine phosphatase 1B
rDNA   ribosomal DNA
ROS    reactive oxygen species
RT-PCR reverse transcriptase polymerase chain reaction
siRNA  small interfering RNA
SIRTs  sirtuins
SIRT1  silent information regulator protein 1
SIRT2  silent information regulator protein 2
SIRT3  silent information regulator protein 3
SIRT4  silent information regulator protein 4
SIRT5  silent information regulator protein 5
SIRT6  silent information regulator protein 6
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIRT7</td>
<td>silent information regulator protein 7</td>
</tr>
<tr>
<td>SREBPs</td>
<td>sterol regulatory element-binding proteins</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>TERT</td>
<td>telomerase reverse transcriptase</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor beta 1</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UCP2</td>
<td>uncoupling protein 2</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>WAT</td>
<td>white adipose tissue</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

1.1 Diabetes mellitus (epidemiology and its economic impact)

Diabetes mellitus (DM) is a multifactorial and genetically heterogeneous disease which is characterized by impaired glucose homeostasis, reduced insulin activity and insulin resistance leading to elevated blood glucose levels [1, 2]. The elevated blood glucose causes symptoms of frequent urination, increased thirst and hunger in patients [1, 2].

As of 2013 it is estimated that 382 million people worldwide have diabetes [3-8]. This is affecting in equal rates in both men and women [3-8]. Diabetes related complications are the 8th leading cause of death worldwide and half the people who die from diabetic complications are under the age of 60 [3-8]. By 2035, the number of people with diabetes is predicted to rise to 592 million [3-8]. Diabetes occurs throughout the world, but is more predominant in the developed countries (specially type 2). However the greatest increase in rates is expected to occur in the developing countries in Asia and Africa [3-8]. Such increase in rates in developing countries is probably due to urbanization and lifestyle changes, specially a western-style diet. This suggests an environmental effect, but there is little understanding of such mechanism at present [3-8]. According to
International Diabetes Federation, the economic cost of diabetes globally in 2013 was estimated to be $548 billion [8]. In Canada, according to data from the Public Health Agency (PHA) of Canada’s Economic burden of illness in Canada (EBIC) the estimated cost for diabetes cares is above $7 billion per year [9, 10].

There are three main types of DM. Type 1 DM, type 2 DM and gestational DM.

1.1.1 Type 1 DM

Type I DM results from the body's failure to produce insulin and was previously referred to as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes [1, 2]. Type 1 DM is caused by autoimmune destruction of the insulin producing beta cells in the pancreatic islets. The subsequent loss of insulin causes hyperglycemia [1, 2]. These patients require exogenous insulin injections for survival [1, 2].

1.1.2 Type 2 DM

Type 2 DM (previously known as non-insulin dependent diabetes mellitus, NIDDM) results from resistance to insulin [1, 2]. This is a condition in which cells fail to use insulin properly and sometimes also with an absolute insulin deficiency [1, 2]. This typically develops in adults. The etiology of type 2 diabetes is unknown. However, physiological, genetic and environmental factors such as obesity, family history, pollution are known to predispose someone to this disease [1-10]. Most of the population with diabetes belongs to this group [1-10].
1.1.3 Gestational DM

Gestational diabetes occurs in some pregnant women, without a previous history of diabetes during third trimester. This is caused by impaired function of the insulin receptors due to interference with pregnancy related hormones leading to high blood glucose level [1, 2]. GDM usually resolves after the birth of the baby however these patients have a high risk of developing type 2 DM later in life [1, 2].

1.2 Diabetic complications

Despite the etiologic type of diabetes, chronic hyperglycemia in advanced form of the disease causes serious health problems affecting multiple organs causing conditions such as retinopathy, nephropathy, cardiomyopathy, and neuropathy [11]. Such damages to the eye, kidney, heart and nerves are caused by micro- and macro vascular dysfunctions [12, 13]. Hyperglycemia causes structural and functional alteration to the vascular system thus affecting the target organs [11-13]. In the present study we have focused on diabetic retinopathy and nephropathy.

1.2.1 Diabetic retinopathy (DR)

DR is a slowly progressive disease of the eye which if left untreated can cause blindness [14]. Hyperglycemia induced oxidative stress in diabetes is the primary stimuli that triggers a cascade of events leading to DR [14-17]. DR causes vascular structural and functional changes in the retina. DR is classified
into two phases, non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR) [14-17]. In NPDR micro vessels in the retina become weak and leaky forming microaneurysms and causing retinal hemorrhages, which leads to decreased vision [14-16]. A characteristic feature of PDR is neovascularization in which new blood vessels grow to reperfuse ischemic areas of the retina [14-16]. These new fragile blood vessels hemorrhage easily and in advanced stage cause scarring, tractional retinal detachment and eventually blindness [14-16].

1.2.2 Diabetic nephropathy (DN)

DN is a progressive kidney disease caused by microangiopathy of the kidney glomeruli [11, 12]. DN is characterized by nephrotic syndrome (increased permeability of the capillary wall causing microalbuminuria, hypoalbuminemia, hyperlipidemia and edema) and diffuse glomerulosclerosis [11, 12]. It is a major cause of dialysis in Western countries [1-10]. In spite of the improvements in therapeutic modalities, diabetes and its complications account for significant morbidity and mortality throughout the world [1-10]. The root cause of all vascular damage in chronic diabetic complication is endothelial cell (EC) dysfunction [11-13].

1.3 Endothelial cell dysfunction

Endothelial dysfunction (ED) is the initiating and perpetuating factor in the development of vascular complications [11-13]. Loss of proper endothelial function is a characteristic feature of several vascular diseases. It is often
regarded as a key early event in the development of atherosclerosis during early stages of diabetes [11-13, 18, 19]. In diabetes, the most prevailing mechanism of endothelial dysfunction is an increase in oxidative stress which can alter cellular homeostasis via several mechanisms [12, 13]. In response to high ambient glucose levels, several metabolic pathways get activated. These include the polyol pathway, non-enzymatic formation of advanced glycation end-product (AGE), activation of protein kinase C (PKC) and hexosamine pathway [12, 13]. It has been shown that in diabetes hyperglycemia increases the proton gradient in the mitochondrial electron transport chain (ETC) causing oxidative stress and overproduction of superoxides [11-13]. Ultimately these pathways lead to an increased production of vasoactive factors (VEGF, ET-1), growth factors (TGF-β1) and cytokines [11-14, 18, 19]. These factors lead to an alteration of hemodynamic parameters such as blood flow and permeability [11-14, 18, 19]. In addition, autocrine and paracrine properties of these factors lead to increased production of extracellular matrix (ECM) proteins [20-22]. Imbalance in the production and the degradation of ECM proteins like fibronectin (FN) and collagen lead to structural alterations such as basement membrane (BM) thickening [11-13, 20-22]. Outlined below are some specific changes seen in diabetic complications that are of interest in this research.

1.4 Alteration of extracellular matrix proteins

The mechanism of increased ECM protein synthesis is of great interest for the development of therapeutic modalities, as ECM protein deposition remains
the single most common finding in chronic diabetes [11]. Increased syntheses of ECM proteins such as FN and collagen have been demonstrated in diabetic angiopathy [19-22]. FN is a high molecular weight glycoprotein and a major component of the ECM proteins. FN plays an important role in various cellular events such as adhesion, growth, migration, differentiation and is crucial during embryogenesis and wound healing [23]. Altered FN expression, organization and degradation have been found to be associated with a number of pathologies including fibrosis and cancer [23]. Our lab has shown that diabetes leads to increased FN in all target organs of diabetic complications [11, 19-22]. We have also shown that EDB+FN (an embryonic splice variant of FN, absent in adult tissues) is upregulated in the ECs and retinas in diabetes [24]. EDB+FN upregulation is dependent on ET-1 and TGF-β1 and provides outside-in signalling, causing EC proliferation and differentiation [24]. EDB+FN gene silencing prevented glucose-induced VEGF expression and reduced the proliferative capacity of ECs [24].

Expression of FN in ECs exposed to high glucose is regulated by the activation of transcription factor NF-κB [25]. NF-κB plays a pivotal role in the regulation of several genes. In a non-stimulated cell, NF-κB dimers are sequestered in the cytoplasm bound to a group of inhibitor proteins named IκB. There are two classes of NF-κB (P50/52 or P65/relB c-rel) [26]. The main form of activated NF-κB is heterodimer P50/P65 [26]. Upon stimulation, IκB is phosphorylated by IKK and eventually degraded leaving NF-κB free to be translocated to nucleus [26]. NF-κB in nucleus binds to the promoters of specific
genes affecting its expression [26]. Function of transcription factor(s) such as NF-κB depends on transcriptional co-activators. For example, association of NF-κB with transcriptional co-activators p300, is essential, and without such association, transcription factors remains silent, even following nuclear translocation [25, 26].

1.5 p300 production and histone acetylation in diabetes

EP300 or p300 is a transcriptional coactivator protein (part of the p300-CBP coactivator family) which interacts with numerous transcription factors to increase the expression of various target genes [27]. It has five protein interaction domain and an intrinsic histone acetyltransferase (HAT) domain [27]. P300 regulates gene expression by relaxing the chromatin structure at the gene promoter through their HAT activity [27]. P300 also increase gene expression by acting as an adaptor molecule or recruiting the basal transcriptional machinery to the promoter [27]. Mutation in p300 gene causes severe mental retardation whereas loss of one copy of the genes prevents normal development. P300 mutation has also been found to be associated with several types of cancers [27]. P300 has been found to be increased in response to hyperglycemia in ECs and tissues in diabetes [28]. It regulates AP-1 and NF-kB [28]. Studies in our lab showed increased p300 production in hyperglycemia leads to augmented histone acetylation, activation of multiple transcription factors and increased expression of vasoactive factors and ECM proteins in the ECs [28-30]. P300 plays a vital role on FN, ET-1 and VEGF overexpression in the cardiac, retinal, and renal tissue in diabetes [28-30]. DNA repair enzymes ERCC1 (DNA Excision Repair
Protein 1) and ERCC4 (DNA Repair Endonuclease XPF) increase FN expression in a p300-dependent manner [31]. MAPK, PKC, and Akt pathways have been shown to mediate overexpression of p300 in response to oxidative stress [28-31]. Inhibitors of p300 have potential therapeutic value. Silencing or inhibition of p300 ameliorated the structural and functional damages of diabetic nephropathy and cardiomyopathy [28-31]. The HAT activity of p300 is balanced by histone deacetylases (HDACs). By deacetylating histones HDACs move chromatin to a more repressed state controlling gene expressions [28]. Three classes of HDACs have been identified. Class I HDACs reside in the nucleus, while class II HDACs shuttle between the nucleus and cytoplasm. Sirtuins are class III HDACs [32-38].

1.6 SIRTUINS

Silent information regulator proteins or sirtuins (SIRTs) are class III histone deacetylases (HDACs) and known to regulate epigenetic gene silencing and suppress recombination of rDNA [32-38]. Initially they were identified in yeast and were found to increase lifespan hence also known as the longevity gene. In mammals they have a range of molecular functions and have emerged as important proteins in aging, calorie restriction, inflammation, stress resistance, cellular differentiation, DNA repair and metabolic regulation [32-38]. SIRTs have a conserved 275 amino acid catalytic core domain and a unique N-terminal and/or C-terminal sequences. SIRTs deacetylase not only histones, but also many cellular proteins and transcription factors [38]. In addition to protein deacetylation, studies have shown that some SIRTs also function as intracellular
regulatory proteins with mono ADP ribosyltransferase activity [32-38]. Unlike other known protein deacetylases, which only hydrolyze acetyl-lysine residues, sirtuins couple lysine deacetylation to NAD hydrolysis yielding O-acetyl-ADP-ribose, the deacetylated substrate and NAD (itself an inhibitor of SIRT activity) [38]. SIRTs require nicotinamide adenine dinucleotide (NAD$^+$) for their deacetylase or ADP-ribosyl transferase activity, linking their function strictly to cellular energy levels [32-38]. In chronic diabetic complications, lack of available NAD$^+$ due to activation of polyol pathway and PARP (poly ADP ribose polymerase), further reduces activity of SIRTs [32-38]. Furthermore glucose induced reduced nicotinamidephosphoribosyl transferase (Nampt) also decreases available NAD$^+$ and reduce SIRT activity [32-38]. In mammals, the SIRTs represent a small gene family with seven members designated SIRT1 through SIRT7 localizing in nucleus (1, 2, 6, 7), cytoplasm (1, 2) and the mitochondria (3, 4, 5). SIRT1, SIRT6, and SIRT7 are localized in the nucleus, where they act to deacetylate histones thereby influencing gene expression epigenetically [32-38]. SIRT1 further deacetylates specific transcription factors and enzymes to influence their activities. SIRT2 was originally described as a cytosolic molecule; however recent data show that SIRT2 is also found in the nucleus where it functions to modulate cell cycle control. SIRT3, SIRT4, and SIRT5 are localized in mitochondria regulating the activities of metabolic enzymes and moderate oxidative stress in this organelle [32-38]. In general, SIRT3–5 switch cells to favor mitochondrial oxidative metabolism, along with the
induction of accompanying stress tolerance [32-38]. In this study we have examined the role of SIRT1 in diabetic complications.

1.6.1 SIRT1

SIRT1 the leading member of the SIRT family resides both in the nucleus and in the cytoplasm [39]. It has been found to regulate many central pathways related to cellular senescence, metabolism, inflammation, insulin secretion, tumorigenesis, cell cycle regulation, stress resistance and various diseases [39-43]. SIRT1 plays a critical role in metabolic health by deacetylating many target proteins in numerous tissues, including liver, heart, muscle, adipose tissue and endothelium [39-43]. SIRT1 also exerts important systemic effects via the hypothalamus [44]. In response to different environmental stimuli, SIRT1 links the cellular metabolic status to the chromatin structure and the regulation of gene expression, thus modulating a variety of cellular processes such as energy metabolism and stress response [44]. Current studies have shown that SIRT1 controls both glucose and lipid metabolism in the liver, promotes mobilization of fat and stimulates differentiation of brown and white adipose tissue, controls pancreatic insulin secretion, influences obesity-induced inflammation in macrophages and modulates the activity of circadian clock in metabolic tissues [39-44].
1.6.1.1 SIRT1 in pancreas

SIRT1 has been shown to be a positive regulator for pancreatic insulin secretion, which consecutively triggers glucose uptake and utilization [45]. Increased dosage of SIRT1 in mice pancreatic beta cells improve glucose tolerance and enhances insulin secretion [45-47]. Deletion of SIRT1 impairs glucose-stimulated insulin secretion [45, 46]. SIRT1 has been shown to promote insulin secretion through transcriptional repression of uncoupling protein 2 (UCP2) [46]. Activation of SIRT1 in animals protects against obesity and insulin resistance [48]. Overexpression of SIRT1 has been found to have a protective effect against glucose intolerance [49-52].

1.6.1.2 SIRT1 in adipose tissues

SIRT1 has been shown to repress PPARγ in white adipose tissue (WAT) suppressing the expression of adipose tissue marker protein such as aP2 [53]. Genetic ablation of SIRT1 in adipose tissues leads to increased adiposity and insulin resistance [54]. Treatment of mice with SIRT1 activator resveratrol has been shown to protect against obesity and metabolic derangements [47-52]. In addition to WAT, studies have also implicated a role of SIRT1 in the differentiation and function of brown adipose tissue (BAT) [55]. SIRT1 promote BAT differentiation through repression of the MyoD-mediated myogenic gene expression and PGC-1α mediated mitochondrial gene expression [55]. SIRT1 in propiomelanocortin (POMC) neurons selectively control perigonadal WAT to BAT like remodeling to increase energy expenditure in female mice [56]. A recent
study indicates that SIRT1 can also regulate the brown remodeling of WAT in response to cold exposure by deacetylation of PPARγ [57].

1.6.1.3 SIRT1 in the hypothalamus

Recent studies have shown that both calorie restriction (CR) and fasting enhance SIRT1 expression and activity in the hypothalamus [58]. Mice lacking SIRT1 in the brain fail to mediate changes in pituitary signaling and physical activity in response to CR [58, 59]. Brain-specific SIRT1 transgenic mice display enhanced neural activity in the hypothalamus [58-60]. Inhibition of hypothalamic SIRT1 activity has been found to increase acetylation of FOXO1 (forkhead box protein O1), leading to increased POMC and decreased AgRP (agouti-related peptide) expressions, thereby decreasing food intake and weight gain [61]. AgRP specific deletion of SIRT1 alleviates the inhibitory tone on the POMC neurons causing decreased food intake and body weight [61]. Deletion of SIRT1 in POMC neurons in mice causes a blunted response to leptin signaling and reduced energy expenditure leading to obesity [61]. Intra-cerebro-ventricular treatment of resveratrol, a known SIRT1 activator normalizes hyperglycemia and improves hyperinsulinemia in obese mice with diabetes [62].

1.6.1.4 SIRT1 in hepatic glucose and lipid metabolism

SIRT1 is an important regulator of hepatic glucose metabolism [63]. Hepatic SIRT1 is a key modulator of gluconeogenesis in response to fasting [64]. Fasting increases SIRT1-mediated deacetylation and activation of PGC-1α,
resulting in increased fatty acid oxidation and improved glucose homeostasis [63]. SIRT1 also plays an important role in hepatic fatty acid metabolism [65]. Adenoviral knockdown of SIRT1 reduces expression of fatty acid β-oxidation genes in the liver [65]. Specific deletion of the exon 4 of the mouse hepatic SIRT1 gene, results in an inactive SIRT1 protein which impairs fatty acid β-oxidation through the PPARα/PGC-1α pathway [66, 67]. This increases the susceptibility of mice to dyslipidemia, hepatic steatosis, inflammation, and endoplasmic reticulum (ER) stress [68]. Overexpression of SIRT1 in mice liver attenuates hepatic steatosis and ER stress restoring glucose homeostasis [68]. SIRT1 also regulates hepatic cholesterol and bile acid through direct modulation of the liver X receptors (LXRs) and farnesoid X receptor (FXR) [69-71]. Systemic deletion of SIRT1 in mice results in decreased serum high density lipoprotein (HDL) levels [72]. SIRT1 also regulate hepatic lipid metabolism through deacetylation of SREBPs (sterol regulatory element binding proteins), critical regulators of lipid and cholesterol genesis [73].

1.6.1.5 SIRT1 in inflammation

Researchers have identified SIRT1 as an important repressor of inflammation in multiple tissues and cells including the macrophages [74-76]. In mice, overexpression of SIRT1 causes suppression of the inflammatory response and insufficiency of SIRT1 induces systemic inflammation [77]. Deletion of SIRT1 in hepatocytes results in increased local inflammation [78]. Several recent studies indicate that SIRT1 suppress the activity of NF-κB, the central regulator
of cellular inflammatory response [79, 80]. SIRT1 deacetylates the RelA/p65 subunit of NF-κB, leading to decreases in the NF-κB transcriptional activity thus reducing expression of pro-inflammatory cytokines [80]. Overexpression of SIRT1 in mice leads to reduced NF-κB activity, while knockdown of SIRT1 in the mouse macrophages increases LPS-stimulated TNFα (tumor necrosis factor α) secretion [79, 80]. Moreover, smoking decreases SIRT1 levels in human macrophages leading to increased activation of NF-κB mediated pro-inflammatory response by these cells [80]. In a macrophage-specific knockout mouse SIRT1 attenuates NF-κB mediated gene transcription thus predisposing mice to the development of insulin resistance and metabolic disorders [80]. The activity and expression of SIRT1 are also under control of systemic inflammation [79]. It has been shown that interferon gamma (IFNγ), represses the transcription of SIRT1, thereby disrupting metabolism and energy expenditure [81].

1.6.1.6 SIRT1 and circadian rhythm

Circadian rhythm is the biological 24 hour oscillation present in all living beings and work in part through chromatin modification and epigenetic control of gene expression [82]. Studies have shown that SIRT1 interacts with CLOCK-BMAL1 (circadian locomotor output cycles kaput-aryl hydrocarbon receptor nuclear translocator-like, dimer transcription factor) to directly regulate the amplitude and duration of circadian clock controlled gene expression through deacetylation of PER2 (perdio carccadian protein homolog 2) and/or BMAL1 [83]. More over circadian regulation of SIRT1 activity is regulated by the cellular NAD
levels [84]. Phosphorylation of SIRT1 by DYRK1A (dual specificity tyrosine-phosphorylation-regulated kinase 1A), an essential clock component activates its NAD dependent deacetylases activity in response to various environmental stresses [85-87]. These studies show a feedback loop involving CLOCK-BMAL1, Nampt, NAD and SIRT1, providing an important connection between physiological rhythm and cellular metabolism [85-87].

1.6.1.7 SIRT1 regulates insulin sensitivity

SIRT1 has been shown to directly regulate pancreatic insulin secretion thus improving systemic insulin sensitivity [45]. In skeletal muscle SIRT1 has been shown to stimulate mitochondrial fatty acid oxidation through PGC-1α thereby promoting insulin sensitivity [88, 89]. Moreover, SIRT1 improves systemic insulin sensitivity through interaction with AMP-activated protein kinase (AMPK) increasing fatty acid oxidation and mitochondrial biogenesis [90]. SIRT1 directly regulates the secretion of adiponectin from adipocytes through deacetylation of FOXO1, improving insulin sensitivity in the liver and muscle tissues [91-94]. SIRT1 also represses the expression of PTP1B (protein-tyrosine phosphatase 1B) which is a negative regulator of insulin signaling, thereby improving insulin sensitivity under insulin-resistant conditions [95]. SIRT1 interact with insulin receptor substrates, IRS-1 and IRS-2 modulating insulin signaling [96]. Genetic deletion of SIRT1 in adipose tissues, myeloid cells and liver or systemic loss of a single copy of the SIRT1 gene has been found to cause the development of insulin resistance and signs of metabolic syndrome [97].
Activation of SIRT1 by its activators such as the resveratrol and SRT1720 in animals protected against high-fat-induced obesity and insulin resistance [49-51].

1.6.1.8 SIRT1 in endothelial cells and atherosclerosis

SIRT1 is prominently expressed in ECs of growing blood vessel sprouts [98]. Loss of endothelial SIRT1 activity impaired the ability of ECs to form new vessels in response to ischemic stress [98]. Inactivations of SIRT1 impaired endothelial migration and sprout elongation and caused defects in vessel navigation, whereas overexpression of SIRT1 augmented such process [98]. In the vasculature of rodent models SIRT1 mediates vasodilatation via eNOS-derived nitric oxide (NO) and scavenging reactive oxygen species (ROS) [99]. In the ECs SIRT1 plays an anti-inflammatory function by downregulating the expression of various pro-inflammatory genes (P-Selectin, ICAM-1 and VCAM-1) through the NF-κB signaling pathway [100]. SIRT1 in macrophages suppresses the expression of Lox-1 which is a scavenger receptor for oxidized low-density lipoproteins (oxLDL), preventing macrophage foam cell formation in atherosclerosis [101]. Also, SIRT1 has been shown to regulate the activity of Liver X-receptor (LXR), promoting ABCA1-driven reverse cholesterol transport in plaque macrophages [101]. SIRT1 exerts antithrombotic properties by suppressing the expression of endothelial tissue factor (coagulation factor III) [102, 103]. SIRT1 deacetylase p53 in ECs preventing its activation and causing protection against vascular senescence in hyperglycemia [104, 105]. Overexpression of SIRT1 diminishes atherogenesis in mice and improves
vascular function [105]. Deletion of one SIRT1 allele increased atherosclerosis in hypercholesterolemic mice [101].

1.7 FOXO1

Some of the SIRTs activities are carried out through deacetylation of the FOXOs, i.e., forkhead family ‘O’ group of transcription factors [106]. Among the FOXO family, FOXO1 is best characterized and plays important roles in oxidative stress resistance, cell survival and cell death [107]. FOXO1 has a highly conserved DNA binding domain subjected to posttranslational modifications such as acetylation, phosphorylation and ubiquitination [108]. Such modifications can either increase or decrease the transcriptional activity of FOXO1 [108]. FOXO1 acetylation by HAT such as p300 causes attenuation of its DNA binding ability and facilitates its phosphorylation by Akt, which leads to its export from the nucleus; whereas deacetylation increases FOXO1’s nuclear retention and transcriptional activity [106-108]. During oxidative stress FOXO1 is known to increase the expression of antioxidant genes (manganese superoxide dismutase, catalase) and thus promotes the scavenging of reactive oxygen species preventing DNA damages [109-112]. FOXO1 has important roles in systemic homeostasis. In mice, loss of FOXO1 is embryonically lethal [113]. FOXO1 also plays a role in regulating cardiac glucose and fatty acid (FA) metabolism [114]. FOXO1 upregulates gluconeogenesis by increasing hepatic glucose-6-phosphatase and phosphoenolpyruvate carboxykinase [115]. In skeletal muscle and in HepG2 cells, FOXO1 increase pyruvate dehydrogenase kinase (PDK4)
expression thereby regulating glucose oxidation [116]. In chronic hyperglycemia, advanced glycated end products and their receptor activation cause modification of FOXO1 which enhance its transcriptional function independent of the phosphorylation state [117]. In diabetes, there is a decreased check on FOXO1 activity by the insulin signaling pathway [118]. Under conditions of hypoxia/ischemia, transcription factors like hypoxia inducible factor (HIF-1α) are shown to interact with FOXO1 and enhance their transcriptional function [119]. In the liver, following FOX1 activation, important enzymes of gluconeogenesis like glucose-6-phosphatase, fructose-1,6-biphosphatase and phosphoenolpyruvate carboxykinase are increased [120]. FoxO1 is also known to enhance lipogenesis and lipid induced steatosis in liver [120]. In beta cells FOXO1 inhibit pancreatic and duodenal homeobox factor 1 (Pdx1) causing damage of beta cell function [121]. Due to their ability to promote cell cycle arrest by activation of p27kip1 (a G1 cyclin inhibitor) and c-myc (transcription factor), FOXO1 also have a tumor suppressive role [122]. Through the activation of pro-apoptotic proteins like Bim and caspases, they are known to increase tumor cell death [122]. Another important role of FOXO1 in inflammation and sepsis has been explored through their ability to co-ordinate with TLR4 (Toll-like receptor 4) and NF-κB [123].

1.8 SIRT1 regulation by miRNAs

miRNAs are small, non-protein coding RNAs, approximately 19-24 nucleotides in length and highly conserved among species [124]. They regulate gene expression at the post transcriptional level through interaction with their target mRNA 3’ untranslated region (UTR) [125]. miRNAs have significant effects
on the regulation of gene expression. The transcription of the miRNA occurs through RNA polymerase II [124-126]. The processing to precursor miRNAs (hairpin shaped, 70-100 nucleotides) in the nucleus is mediated by RNAse III, Dorsha and DGCR8. Following synthesis, they are exported to the cytoplasm by exportin 5 [126]. In the cytoplasm, they are further processed by Dicer into the functionally active mature miRNA. miRNA binds to the specific mRNA targets and causing degradation of specific mRNA or translational repression [124-126]. Several investigators have demonstrated importance of miRNA in diverse cellular processes [124-131]. miRNAs also play important roles in controlling histone modification. A significant number of miRNA coding regions are located in the intron of the protein coding gene and are usually co-regulated with their host genes [124-126].

Epigenetic mechanisms play a fundamental role in the regulation of miRNAs [124-131]. In mammals, it is estimated that over one third of genes are regulated by miRNAs and on average one miRNA may regulate 100-200 different target genes, and a single gene may have several targets sites for various miRNA [124-126]. miRNAs are important players in a variety of cellular processes [128-137]. We have recently identified alterations of several miRNAs in diabetes [133-137].

Sixteen miRNAs have been found to regulate SIRT1 [138]. These miRNAs regulate SIRT1 in brain, liver, pancreas, cardiac metabolism and tumorigenesis in various disease and normal conditions [138]. With respect to this project, we have identified by array studies significant upregulation of miRNA195 (a SIRT1
targeting miRNA) in the ECs exposed to glucose and in the retinal tissue in diabetes.

1.9 Similarities of diabetic complications with aging

A large number of structural and functional changes seen in diabetic complications are also found in normal cellular aging [139-158]. Cellular aging process or senescence is a phenomenon where cells demonstrate a reduced ability to multiply. It is characterized by reduced ability to respond to stress, increased susceptibility to diseases or infection, increased secretion of pro-inflammatory cytokines, chemokines, growth factors, ECM proteins and increased homeostatic imbalance [139-158]. The biological basis of aging is still unknown however increased oxidative stress and DNA damage are known to induce senescence in cells [139-158].

It is proposed that aging and longevity are determined by a complex mixture of environmental and genetic factors [139-158]. Researchers have demonstrated that selected alterations in specific genes can extend lifespan in model organisms and laboratory settings [49-51, 159, 160]. Increased DNA repair and telomerase activity, reduced oxidative damage and cell apoptosis can extend life span [139-158]. DNA damage is thought to be a common cause of aging [142, 150-158]. DNA damage caused by extrinsic factors such as, radiation, chemical or viral infection can prevent cell division or cause apoptosis in cells [142, 150-158]. It can often affect the stem cell pools hampering regeneration. However intrinsic causes such as ROS/RNS (reactive oxygen
species/reactive nitrogen species) are considered as one of the major drivers of DNA damage and aging [139-158].

Three metabolic pathways have been identified which influence the rate of aging: caloric restriction, the activity levels of the electron transport chain in the mitochondria and the insulin/IGF-1-like signaling pathway [161]. It may be possible that these three pathways affect aging separately as simultaneously targeting those leads to additive increases in lifespan [161]. The SIRT family of genes has been shown to have a significant effect on the lifespan of several species [32-34, 40]. In addition, diet has been shown to substantially affect lifespan in many animals including prevention or delay of many age-related diseases. In several model species caloric restriction has been found to lead to longer lifespans mediated by the nutrient-sensing function of the mTOR (mammalian target of rapamycin) pathway [140, 159-161]. This is thought to prevent spikes of glucose concentration in the blood, leading to improved insulin signaling [161]. Evidence in both animals and humans suggests that resveratrol, a known SIRT activator may be a caloric restriction mimetic [49-51, 161]. As more genetic connections to aging are being discovered, it is increasingly being considered similar to other genetically influenced conditions and potentially treatable. Similar to aging in diabetes, glucose induced oxidative damage is a key mediator of cellular damage. Such oxidative damage causes activation of several metabolic pathways ultimately converging on the nucleus. We and others have shown oxidative stress induced DNA damage in diabetes [11-13]. Ultimately such process causes aberrant expressions of cytokines, growth
factors, ECM proteins and structurally manifesting as changes such as cell loss, vascular sclerosis etc [11-13, 17-21, 24, 25, 28-31]. Hence tissue damage in both aging and diabetes may in part be mediated by similar mechanisms.

1.10 Rationale

As discussed above, changes in ECs and organs in diabetes such as increased oxidative stress, increased AGE, DNA damage, ECM protein production, cognitive decline, atherosclerosis, small vessel sclerosis are also observed in normal aging. Hence it is possible that high glucose in diabetes actually accelerates the aging process in cells and tissues. The mechanism of such process was investigated in this research.

1.11 Hypothesis

Diabetes, through oxidative damage, causes alteration of specific nuclear enzymes leading to an accelerated aging process. Such changes lead to alteration of vasoactive molecules and ECM proteins and causes development of chronic complications.

1.12 Specific aims

1. **Aim 1** - to investigate accelerated aging in endothelial cells and in tissues in response to hyperglycemia and examine the role of SIRTs in such process

2. **Aim 2** - to investigate SIRT1 regulation by microRNA 195 in diabetic animals
3. **Aim 3**-to investigate the effect of SIRT1 overexpression in the organ damage in diabetic animals

Aim 1 was investigated in chapter 2. Aim 2 was investigated in chapter 3. Aim 3 was investigated in chapter 4.

### 1.13 References


44. Li X (2013) SIRT1 and energy metabolism. Acta Biochim Biophys Sin (Shanghai) 45:51-60.


142. Matsui-Hirai H, Hayashi T, Yamamoto S et al. (2011) Dose-dependent modulatory effects of insulin on glucose-induced endothelial senescence


Chapter 2

2 High glucose induced alteration of SIRTs in endothelial cells causes rapid aging in a p300 and FOXO regulated pathway

Rokhsana Mortuza, Sali Chen, Biao Feng, Subhrojit Sen and Subrata Chakrabarti. Department of Pathology, Schulich School of Medicine & Dentistry, Western University, London ON Canada.

In this study our aim was to investigate accelerated aging in endothelial cells and in tissues in response to hyperglycemia and examine the role of SIRTs in such process. We examined 3 different ECs of various origins as well as kidney and retinal tissues of type 1 diabetic mice for this investigation. We also looked at the possible mechanism behind such glucose-induced rapid aging process.

A version of this chapter have been published: Mortuza R, Chen S, Feng B, Sen S, Chakrabarti S. High glucose induced alteration of SIRTs in endothelial cells causes rapid aging in a p300 and FOXO regulated pathway. PLoS One. 2013;8(1):e54514. doi: 10.1371/journal.pone.0054514. As per the journal policy, authors retain ownership of the copyright for their content. No permission is required from the publishers.
2.1 Introduction

Diabetes and its complications account for significant morbidity and mortality throughout the world [1-3]. The major factor in the development of chronic diabetic complications is vascular EC dysfunction [4]. The prevailing mechanism leading to EC dysfunction is an increase in reactive oxygen species (ROS) formation [5]. In response to high ambient glucose levels and subsequent oxidative stress, ECs elaborate large amount of vasoactive factors, growth factors and cytokines [6, 7]. Such factors lead to increased production of extracellular matrix (ECM) proteins causing structural alterations [6-8]. Interestingly, several such changes seen at the cellular and tissue level in diabetes are similar to the changes seen in normal aging process [9-13].

Oxidative stress causes DNA damage and alters transcriptional machinery both in aging and in diabetes [4, 6, 14, 15]. We have previously shown that glucose induced oxidative stress causes histone acetylation by p300, which regulates several transcripts in diabetes [6, 16]. p300, a transcriptional coactivator with an intrinsic histone acetyltransferase (HAT) activity, regulates numerous transcription factors [6, 16, 17]. Acetylation by p300 and other HATs are balanced by histone deacetylases (HDACs).

Silent information regulator 2 proteins or sirtuins (SIRTs) belong to Class III HDACs and regulates epigenetic gene silencing and suppress recombination of rDNA [18-20]. In mammals, SIRTs have a range of molecular functions and have emerged as important proteins in aging and metabolic regulations [18, 21].
SIRTs represent a small gene family with seven members designated as SIRT1-7, known to be modulated by oxidative stress [22].

Some of the SIRTs activity is carried out through deacetylation of the FOXOs, forkhead family ‘O’ group of transcription factors [23-25]. Among the FOXO family, FOXO1 is best characterized and plays important roles in cell survival, oxidative stress resistance and cell death [26-29]. FOXO1 has a highly conserved DNA binding domain subjected to posttranslational modifications such as phosphorylation, acetylation and ubiquitination. These modifications can either increase or decrease the transcriptional activity of FOXO1 [17]. FOXO1 acetylation by HAT such as p300, leads to attenuation of its DNA binding ability and facilitates its phosphorylation by Akt, leading to its export from the nucleus; whereas deacetylation increases FOXO1’s transcriptional activity [17, 24].

The purpose of this study was to investigate whether high glucose causes accelerated aging process in ECs through alteration of SIRTs. We further investigated whether the effects of SIRTs are mediated through FOXO1 and if such process is regulated by histone acetylase p300. We carried out these studies in various ECs as well as in the diabetic animals.

2.2 Materials and methods

2.2.1 Cell culture

Dermal-derived human microvascular EC (HMEC) was obtained from Lonza, Inc. (Walkersville, MD) and grown in EC basal medium 2 (EBM-2,
complete). Human umbilical vein ECs (HUVECs) were obtained from Lonza and cultured in EC growth medium (EBM complete, Walkersville, MD). Bovine retinal microvascular ECs (BRECs) were obtained from VEC Technologies (Rensselaer, NY) and grown in a defined EC growth medium (MCDB-131 complete). We have previously described the culture conditions of these three cells [30, 31]. No insulin was present in any media.

For the long term continuous exposure to glucose, ECs were cultured in 12 well plates (Corning, Acton, MA) and treated with 5mM glucose (NG) or 25mM glucose (HG, D-glucose) or osmotic control (OSM, 25 mM L-glucose). Upon confluence cells were propagated & maintained in the same treatment condition until they stopped proliferating completely. During each passage subculture cells from each treatment group were stained for SA β-gal and collected for RNA analysis. Cell lysates were collected with RIPA (Millipore, Billerica, MA) buffer with protease inhibitor (Roche, Laval, Canada) for ROS and MnSOD analysis. Total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL). Cells were monitored daily and images taken for morphological and growth analysis. All experiments were conducted with 6-10 biological replicates.

To test the effect of SIRT1 activation on accelerated aging in diabetes, cells were treated with 10 µM resveratrol (Sigma, Oakville, ON, Canada) dissolved in ethanol or 25 µM BML278 (Enzo, Farmingdale, NY) in DMSO for 72 hr in HG following subculture in HMEC P1 (P1 = passage 1). To investigate the
effect of FOXO1 inhibition, cells were treated with 0.1 µM FOXO1 inhibitor AS1842856 (Millipore, Billerica, MA) in DMSO similarly.

2.2.2 Animal experiments

Male C57BL/6 mice (20-25g), were obtained (Charles River, Wilmington, MA) and diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg, in citrate buffer, pH 5.6). Age- and sex-matched mice were used as controls and given equal volumes of citrate buffer [32]. db/db (Lepr<sup>db</sup>, DBA/J) mice (8 weeks, Jackson Laboratory, CA) were used as type 2 model of Diabetes [32]. The animals were monitored daily as described by us previously [32]. The animals were killed at 2 & 4 months following the development of diabetes (n = 10/group). Retinal and renal cortical tissues were dissected out and snap frozen in liquid nitrogen. All tissues were stored at −80°C until further analysis. Urinary albumin (Exocell, Philadelphia, PA) and serum creatinine (Arbor assays, Ann Arbor, MI) were measured as per the instructions.

2.2.3 Ethics statement

Animal experiments were performed in accordance with regulations specified by the Canadian Council of Animal Care. The investigation was in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publ. no. 85-23, revised 1996). All protocols were approved by the University of Western Ontario Animal Care and Veterinary Service.
2.2.4 SA β-gal staining of cells and frozen tissue sections

Cells from each passage or tissue slides were fixed and stained with SA β-gal staining according to the manufacturer’s instructions (abcam, Cambridge, MA). Tissue slides were counterstained with H&E for orientation purpose.

2.2.5 Phase contrast microscopy and morphometrical analysis

Images of SA β-gal stained cells were photographed with phase contrast inverted microscope (Meiji Techno, TC5400, Santa Clara, CA) with 20X objective and SPOT Basic software. Morphometrical analysis of the images was done by ImageJ software (NIH, Bethesda, MD). Images (10 per sample) of the tissue slides were recorded by an Olympus BX51 microscope (Olympus, Center Valley, PA) with Northern Eclipse software (Empix Inc, Cheektowaga, NY).

2.2.6 mRNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis from cells and tissues samples has been described by us previously [30]. RNA concentration was assessed on a spectrophotometer (Pharmacia Gene Quant, GE, Mississauga, ON, Canada). First-strand cDNA was made by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA) as per the manufacturer instruction.
2.2.7 mRNA analysis with quantitative Real Time RT-PCR

Real-time RT-PCR was performed by LightCycler™ (Roche Diagnostics, Laval, Canada) to quantify the mRNA expression of SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6, SIRT7, TERT (telomerase reverse transcriptase) and p300 using the Qiagen One Step RT-PCR kit (SYBR Green I detection platform). All primers were either ordered or custom made from Sigma (Table 2.1). The data were normalized to housekeeping gene β-actin/18s mRNA to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures. The details of the method have been described by us previously [30].

Table 2.1 The primers sequences for Real Time RT-PCR.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TERT</td>
<td>CGTGGTTTCTGTGTGGTGTC</td>
</tr>
<tr>
<td></td>
<td>CCTTGTCGCCTGAGGAGTAG</td>
</tr>
<tr>
<td>FOXO1</td>
<td>AAGAGCGTGCCCCTACTTCAA</td>
</tr>
<tr>
<td></td>
<td>CTGTTGTTGTCATGGATGC</td>
</tr>
<tr>
<td>SIRT3</td>
<td>CATGAGCTGCACTGACTGTT</td>
</tr>
<tr>
<td></td>
<td>GAGCTTGCCGTTCACTAGG</td>
</tr>
<tr>
<td>SIRT4</td>
<td>CAGCAAGTCCTCTCTCTGGAC</td>
</tr>
<tr>
<td></td>
<td>CCAGCCTACGAAGTTTCTCG</td>
</tr>
<tr>
<td>SIRT6</td>
<td>AGGATGTCGGTGAATTACGC</td>
</tr>
<tr>
<td></td>
<td>AAAGGTGTTGTCGAACCTTG</td>
</tr>
<tr>
<td>SIRT2</td>
<td>GACTTTCCGCTCTCCATCCAC</td>
</tr>
<tr>
<td></td>
<td>GGAGTCGCCCTTGTCTCTTC</td>
</tr>
<tr>
<td>SIRT5</td>
<td>CCAATTTGTCCCAAGTGTGTTG</td>
</tr>
<tr>
<td></td>
<td>CGGAACGGCTGGAGGTACAGAC</td>
</tr>
<tr>
<td>SIRT7</td>
<td>AGGGAGAAGCGTTAGTGCTG</td>
</tr>
<tr>
<td></td>
<td>GGAAACGCAGAGGGATACAGAC</td>
</tr>
<tr>
<td>SIRT1</td>
<td>GCAGATAGTAGAGCCGGCTTG</td>
</tr>
<tr>
<td></td>
<td>TCTGGCATGTCCCACTATCA</td>
</tr>
<tr>
<td>P300</td>
<td>GGGACTAAACCAATGGTGTTG</td>
</tr>
</tbody>
</table>
2.2.8 Nuclear fraction isolation

Nuclear fractions were isolated from ECs and tissues as per the kit instruction (Active Motif, Carlsbad, CA). Protein concentrations in the samples were measured by BCA protein assay (Pierce, Rockford, IL).

2.2.9 FOXO1 DNA binding activity measurement

FOXO1 DNA binding ELISA was conducted on the collected nuclear fraction as per the manufacturer’s instruction (Active Motif, Carlsbad, CA). The plates were read at 450 nm using a plate reader (Multiskan, Thermo Fisher, Canada).

2.2.10 ROS, MnSOD and LDH analysis

Total ROS level in the EC lysates were measured as per the manufacturer instructions using a commercially available kit (Cell Biolabs Inc., San Diego, CA). The plates were read with a fluorescent plate reader (Biotek, Winooski, VT) at excitation 480 nm and emission 530 nm. MnSOD ELISA was done on cell and tissue lysates as per the manufacturer instruction (abcam, Cambridge, MA). The plates were read at 450 nm using a plate reader (Multiskan, Thermo Fisher, Canada). Total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL). Quantification of intracellular lactate dehydrogenase (LDH) in the cell lysates was used as an additional measure of cell growth. The assay
was conducted as per the manufacturer instruction (Caymen Chemical Company, Ann Arbor, MI).

2.2.11 Telomerase activity

Telomerase activities of the samples were measured using a commercially available kit following instructions provided by the manufacturer (Allied Biotech Inc., Vallejo, CA).

2.2.12 Western blot

200µg of protein was used for the western blot analysis according to the standard protocol established at our lab [6] using p300, Ac-FOXO1 & β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

2.2.13 SIRT1 enzyme activity analysis

Enzyme assay for SIRT1 activity was performed as per the manufacturer instructions (Sigma, Oakville, CA). The plates were read with a fluorescent spectrophotometer (Biotek, Winooski, VT) at excitation 340 nm and emission 430 nm.

2.2.14 SIRT1 gene silencing

To silence SIRT1, transfection of small interfering RNA (siRNA) was performed using N-TER nanoparticle siRNA transfection system (Sigma, Oakville, Canada) according to the manufacture's protocol. SIRT1 siRNA1 was
purchased from Dharmaco Inc. (Lafayette, CO) and SIRT1 siRNA2 was purchased from Santa Cruz Biotecnology (Santa Cruz, CA). Cells were incubated with or without glucose after transfection for 24 hr and sample collected for mRNA analysis. To test the effect of SIRT1 siRNA on resveratrol, the siRNA was added 48 hr post treatment with resveratrol in the cultured cells and samples collected 24 hr post transfection. Transfection efficiency was assessed by real-time RT-PCR.

2.2.15 P300 gene silencing

To silence the p300 expression in ECs, p300 siRNA1 (Silencer, Ambion, Austin, TX) and p300 siRNA2 (Santa Cruz Biotechnology, Santa Cruz, CA) was used with siPORT Lipid transfection reagent (Ambion, Carlsbad, CA). The details of the transfection protocol has been described by us previously [6,16]. siRNA transfection efficiency was indirectly assessed by measuring p300 mRNA expression by real-time RT-PCR.

2.2.16 Statistical analysis

Data are expressed as mean ± SEM (n ≥ 6), normalized to controls. The statistical significance of the results was analyzed by one way or two way ANOVA followed by Tukey’s HSD post hoc correction and the two tailed Student’s t-test as appropriate (PASW Statistics 18, IBM, Canada). P value < 0.05 was considered statistically significant.
2.3 Results

2.3.1 High glucose accelerates aging process in the ECs.

Suspecting that hyperglycemia accelerates aging, we first investigated whether glucose causes rapid aging process in the ECs as they are the primary target of diabetic vascular complications. We examined three different ECs of various origins, at various passages following treatment with high glucose and used morphological alteration & senescence associated β-gal positivity as two biomarkers of aging.

We found aging changes in HMEC starts as early as passage 1, as seen from positive β-gal staining in HG treated cells compared to NG treated cells where it appeared in passage 4. By passage 5, all cells in HG showed such changes, whereas in NG it was present in only 1.5% cells (Figure 2.1A and B). Additionally, cell growth was slower in HG treated groups compared to the NG treated cells. By passage 5, the HG treated cells stopped proliferating completely, whereas the cells in NG continued to do so (Figure 2.1D-F). The morphology of the cells also changed as they got older. The older cells became larger and irregular in shape compared to the smaller spindle shaped young cells. This process was exaggerated in HG. The average cell size in HG was significantly larger than the controls (Figure 2.1C).
Figure 2.1 Aging signs in microvascular endothelial cells (HMEC) with HG start as early as passage 1. (A) SA-βgal staining showed increased β-gal positivity or senescent cells with HG treatment compared to the NG treated cells, with the increasing passage number. Arrow indicates β-gal positive (blue) cells. [Scale bar represent 100µm for all micrographs]. (B) Quantification of β-gal positivity (n=10 image per sample). Signs of cellular aging started to appear from passage 1 in HG. (C) Morphometric cell area analysis showed significant increase in cell size with HG treatment. Cell growth was slow with HG treatment compared to controls as seen from (D) days needed to be confluent in HG (90%, at which stage cells were subcultured to next passage), (E) cell count in HG (attached cells per microscopic field at 20X objective, n = 10 image) and (F) reduced
intracellular LDH activity in HG. (NG = normal glucose, 5 mM; HG = high glucose, 25 mM D-glucose; OSM = osmotic control, 25 Mm L-glucose; P = passage number). [*p<0.05 compared to NGP1; †p<0.05, ‡p<0.01 compared to respective NG passages for HG cultured cells].

We then examined retinal endothelial cells, a major target in diabetic retinopathy. We found similar changes. In HG, BREC showed increased β-gal positivity and slower growth (Figure 2.2A and D-F). Similar to HMEC, such aging started as early as passage 1 in HG treated cells whereas with NG it appeared at passage 4. At passage 8 complete growth arrest and 100% β-gal positivity was seen in HG compared to 35% positivity in NG treated cells (Figure 2.2B). Furthermore, morphologically such aging changes were associated with a large and irregular appearance (Figure 2.2C).
Figure 2.2 Accelerated aging and associated changes in retinal endothelial cells (BREC) with increasing passages with HG treatment. (A) SA-βgal staining showing increased β-gal positivity (A) with HG treatment. Arrow indicates β-gal positive cells. [Scale bar represent 100µm for all micrographs]. (B) Quantification of β-gal positivity. Aging signs appeared at passage 1 with HG. (C) Morphometric cell area analysis showed significant increase in cell size with HG treatment. Cell growth was slow with HG treatment compared to controls as seen from (D) days needed to be confluent (90%, at which stage cells were subcultured to next passage), (E) cell count (attached cells per microscopic field at 20X objective, n = 10 image) and (F) reduced intracellular LDH activity. (NG = normal glucose, 5 mM; HG = high glucose, 25 mM D-glucose; OSM = osmotic control, 25 mM L-
Interestingly, although HUVECs showed similar changes, such process was delayed. HUVECs were a better survivor in HG environment. They were able to grow up to 11 passages in HG (Figure 2.3A). They showed signs of aging with HG at passage 4 and with NG at passage 7. β-gal positivity reached 100% at passage 11 in HG compared to 60% in NG at the same passage (Figure 2.3B). In addition, similar to other ECs, aging was associated with large and irregular shape of these cells along with slow growth, (Figure 2.3C and D-F). Such changes were not seen with 25mM L-glucose (osmotic control) in any of the cell types (Figure 2.1, Figure 2.2 and Figure 2.3).
Figure 2.3 Accelerated aging in large vessel endothelial cells (HUVEC) with HG is delayed. (A) SA-βgal staining showed increased β-gal positivity (blue cells as indicated by arrow) with HG treatment compared to NG and osmotic control groups. Aging signs appeared later with HG in HUVEC. [Scale bar represent 100µm for all micrographs]. (B) Quantification of β-gal positivity shows increased percentage of aged cells in HG treated group. (C) Morphometric cell area analysis showed significant increase in cell size with HG treatment. Cell growth was slow with HG treatment compared to controls as seen from (D) days needed to be confluent in HG (90%, at which stage cells were subcultured to next passage), (E) cell count in HG (attached cells per microscopic field at 20X objective, n = 10 image) and reduced (F) intracellular LDH activity in HG. (NG = normal glucose, 5 mM; HG = high glucose, 25 mM D-glucose; OSM = osmotic control, 25 mM L-glucose; P = passage number). [*p<0.05, **p<0.01 compared to NGP1; †p<0.05, ‡p<0.01 compared to respective NG passages for HG cultured cells].

As reduction of TERT has been found in many models of cellular aging, we investigated possible alteration in TERT mRNA expression. We investigated them at an early stage, (P1) and at the end passage (P5 in HMEC, P8 in BREC and P11 in HUVEC). We noticed TERT mRNA was significantly reduced in all ECs with HG treatment along with accelerated aging (Figure 2.4A).
Figure 2.4 Increased oxidative stress and reduced TERT mRNA expression observed in endothelial cells with HG. (A) HMEC, BREC & HUVEC showed reduction of TERT (telomerase reverse transcriptase) mRNA in HG treated cells. mRNA levels are expressed as a ratio to 18s and normalized to NG P1. (B) Total ROS levels in HMEC, BREC & HUVEC showed significant increase with HG and aging. MnSOD levels in all cells at end passages (P5 in HMEC, P8 in BREC and P11 in HUVEC) showed significant reduction with HG treatment (C). (NG = 5 mM; HG = 25 mM glucose, P1 = passage 1). Data normalized to NG (P1) treatments of the respective ECs. [*p < 0.05 compared to NGP1; +p<0.05 compared to NGP5 for HMEC, NGP8 for BREC and NGP11 for HUVEC].
In order to see whether such accelerated aging is associated with oxidative stress, we measured total ROS level in the cell lysate from early and late passages between the treatment groups. As anticipated, we found with HG the ECs produced more ROS than with NG, depicting higher level of oxidative stress in hyperglycaemia, which augmented with the increased passage number or aging (Figure 2.4B).

Disruption of the scavenging of ROS in the mitochondria in aging is well known [33]. Manganese superoxide dismutase (MnSOD) is the primary mitochondrial ROS scavenging enzyme. It converts superoxide to hydrogen peroxide which is eventually converted to water by catalases and other peroxidases [29, 33, 34]. Our investigation of MnSOD in the final passages of the ECs showed a significant reduction of the enzyme in the aged cells (Figure 2.4C).

2.3.2 High glucose exaggerates down regulation of SIRTs in aging.

We then examined whether above changes are associated with alteration of SIRTs. We found mRNA expressions of all SIRT (1-7) genes were reduced with increasing passages, in all ECs (Figure 2.5A, Figure 2.6A & Figure 2.7A). Such reduction was faster and exaggerated in HG treated groups. There were 25-50% reductions in SIRT mRNA expressions with HG from as early as passage 1 in HMECs, which increased to 50% at passage 5 (Figure 2.5A). Similar reductions were observed in the BRECs (Figure 2.6A). HUVECs however, exhibited smaller reduction (15-30%) in earlier passages (Figure 2.7A).
Nonetheless by passage 11, mRNA levels in HG in HUVEC were reduced to almost half of NG treated cells (Figure 2.7A). Reduction in SIRT1 enzyme activities found in the ECs in final passages with HG treatment, were in keeping with the reduced mRNA levels (Figure 2.5B, Figure 2.6B & Figure 2.7B). As SIRT1 is one of the most important isoforms of the SIRT family which is associated with aging [5, 18, 24, 34], we focused on SIRT1 for the subsequent experiments. We carried out the following experiments in HMECs, as they are the major target of diabetic complications.

Figure 2.5 SIRT (1-7) mRNA reduction in HG in microvascular endothelial cells (HMECs) parallels the accelerated aging. (A) Quantitative Real Time RT-PCR of SIRTs in HMEC showed significant reduction in the SIRT mRNA levels in HG
treated cells. mRNA levels are expressed as a ratio to β-actin normalized to baseline controls, NG P0 (P0 = before start of treatment). [*p<0.05 compared to NGP0; †p<0.05 compared to respective NG passages for HG cultured cells]. (B) SIRT1 enzyme activity was reduced in HG in these endothelial cells (P5, data normalized to NG). (NG = 5 mM; HG = 25 mM glucose; P = passage number). [*p<0.05 compared to NGP5].

Figure 2.6 SIRT 1-7 mRNA analysis with Quantitative Real Time RT-PCR at various passages in BREC showed significant reduction in HG treated cells. (A) SIRT (1-7) mRNA expressions in BREC with increasing passages. mRNA levels are expressed as a ratio to β-actin and normalized to baseline controls, NG P0 (before treatment began). [*p<0.05 compared to NGP0; †p<0.05 compared to
respective NG passage for HG cultured cells]. (B) SIRT1 enzyme activity was reduced in HG in BREC (P8, data normalized to NG). (NG = 5 mM; HG = 25 mM glucose; P = passage number). [*p<0.05 compared to NGP8].

Figure 2.7 SIRT (1-7) mRNA reduction with increasing passage number is augmented with HG treatment in HUVECs. (A) SIRT (1-7) mRNA expressions are decreased in large vessel endothelial cells (HUVEC) with increasing passages and with HG it is escalated (analyzed by quantitative Real Time RT-PCR). mRNA levels are expressed as a ratio to β-actin and normalized to baseline controls, NG P0 (before treatment began). [*p<0.05 compared to NGP0; ±p<0.05 compared to respective NG passages for HG cultured cells]. (B) SIRT1 enzyme activity was reduced in HG in HUVEC (P11, data normalized to NG).
[*p<0.05 compared to NGP11]. (NG = 5 mM; HG = 25 mM glucose, P = passage number).

2.3.3 SIRT1 activators reduce glucose induced accelerated aging.

As SIRT1 enzyme is significantly reduced in HG induced oxidative stress and aging, we investigated whether SIRT1 activators can rescue such process. Both resveratrol and BML278 significantly increased SIRT1 enzyme activity (Figure 2.8C) and reduced the sign of aging in HG as seen from reduced β-gal positivity (Figure 2.8A and B). As expected, SIRT1 activators reduced total ROS levels in the HG treated cells compared to the controls (Figure 2.8D).
Figure 2.8 Chemically induced activation of SIRT1 reduces oxidative stress in HG treated endothelial cells. (A) SA β-gal staining of HMEC (P1) with resveratrol and BML278 treatment. β-gal positivity was reduced with SIRT1 activators in HG treated cells compared to controls. [Scale bar represent 100µm for all micrographs]. (B) Quantification of β-gal positivity. (C) SIRT1 activators significantly increased the enzyme’s activity and reduced (D) total ROS levels in HG. (E) FOXO1 DNA binding activity was reduced in the nuclear fractions of HMEC with SIRT1 activators along with increased (F) MnSOD levels. (G) Western blot analysis of acetylated FOXO1 shows HG induced increase in Ac-FOXO1 level was corrected with SIRT1 activators. HG caused (H) nonsignificant change in TERT mRNA expression, however (I) telomerase activity was significantly reduced in the ECs and such reductions were prevented with SIRT1 activators. mRNA levels are expressed as a ratio to β-actin. All data were normalized to NG. Osmotic controls with L-glucose had no effect. [*p<0.05, **p<0.01 compared to NG; #p<0.05 compared to HG].

2.3.4 SIRT1’s action in ECs is mediated through FOXO1.

To elucidate the mechanism of HG induced SIRT1 mediated oxidative stress and aging pathway, we looked into FOXO1, a major regulator of MnSOD and also a target of SIRT1 [17, 23, 24]. We observed FOXO1’s DNA binding activity in nuclear fractions of HMEC was reduced in HG and effectively rescued by the SIRT1 activators (Figure 2.8E). In parallel, investigation of MnSOD levels in these samples correlated with the reduced FOXO1 activity levels (Figure
Finally Western blot analysis demonstrated, HG induced increased acetylated FOXO1 (Ac-FOXO1) levels were efficiently reduced with SIRT1 activators (Figure 2.8G). Together these results supported SIRT1 regulated MnSOD pathway in the ECs during HG induced rapid aging.

We further examined TERT mRNA expressions and telomerase activity in the ECs with SIRT1 activators. In passage 1 although TERT mRNA was non-significantly reduced, telomerase activity were reduced significantly in HG and such reductions were prevented by SIRT1 activators (Figure 2.8H and I).

2.3.5 FOXO1 inhibitor or SIRT1 silencing in NG mimics the HG effects.

To investigate the role of FOXO1 in such pathway further, we treated HMECs with a commercially available FOXO1 inhibitor (AS1842856) in NG. As expected FOXO1 inhibitor reduced FOXO1 DNA binding (data not presented) and subsequently reduced the MnSOD levels (Figure 2.9B). This led to an increase in ROS levels and induced early senescence as seen from increased β-gal positivity (Figure 2.9A and C). In addition, FOXO1 inhibitor dampened the preventative effects of resveratrol on glucose-induced aging and oxidative stress, supporting the relationship of SIRT1 and FOXO1 (Figure 2.9A-C). Silencing of SIRT1 expression with siRNA (>50% reduction in mRNA expression, data not shown) in NG also showed similar effects and mimicked the HG effects (Figure 2.9D and E). These findings confirmed our earlier findings and the specificity of SIRT1 and FOXO1 in the rapid aging pathway in these ECs.
Figure 2.9 SIRT1 knockdown or FOXO1 inhibition in NG induces signs of early aging mimicking the HG treatment. (A) Early senescence was induced with FOXO1 inhibitor (AS1842856) in NG, and the rescue of senescence with resveratrol in HG disappeared with FOXO1 inhibitor, as evidenced by SA-β gal stain (HMECP1). (B) In NG, AS1842856 reduced MnSOD and increased (C) ROS levels; it also hindered the preventative effect of resveratrol in HG regarding these parameters. [*p<0.05, **p<0.01 compared to NG; †p<0.05 compared to HG; ‡p<0.05 compared to HG+Resveratrol]. (D) SIRT1 knockdown with siRNA induced senescence in NG as seen using SA-β gal stain (HMECP1). (E) Such knockdown also increased ROS levels. siRNA1 and siRNA2 represent separate experiments using two different siRNAs. (F) Glucose induced increased SA β-gal
positivity was reduced with resveratrol. However SIRT1 siRNA transfection prevented such effect. (G) Analysis of SIRT1 mRNA level showed such effect of resveratrol is mediated through SIRT1. mRNA levels are expressed as a ratio to 18s normalized to controls. (H) Glucose induced increased ROS production was prevented by resveratrol. However such preventative effects were partially lost with SIRT1 siRNA transfection. [*p<0.05, **p<0.01 compared to NG; †p<0.05 compared to HG; ‡p<0.05 compared to HG+Res.+scramble]. (NG = 5 mM; HG = 25 mM glucose, P1 = passage 1). [Scale bar represent 100µm for all micrographs].

We then performed some additional experiments using SIRT1 siRNA in ECs following SIRT1 activation with resveratrol. We treated HMECs with SIRT1 siRNA for 24 hrs following treatment with resveratrol for 48 hrs. We found the siRNA reversed the effect of resveratrol on mRNA expression, oxidative stress and aging (Figure 2.9F-H). This re-demonstrated the SIRT1 mediated aging in the ECs in hyperglycemia.

2.3.6 SIRT1 and p300 have a balancing role on each other.

In order to explore the relationship of SIRT1 with other molecules in HG induced accelerated aging, we investigated possible association between SIRT1 and p300. Since SIRT1 is a HDAC and actions of deacetylases are balanced by HATs, we examined p300, a well characterized HAT. To investigate such phenomenon, we knocked down SIRT1 in HMECs using siRNA and examined p300 mRNA expression and vice versa (Figure 2.10A and B). We were able to
achieve more than 50% reduction of the specific mRNAs following the transfections (data not shown). SIRT1 silencing increased p300 mRNA expression in both NG and HG treated cells (Figure 2.10A). On the other hand, p300 silencing had no significant effect on basal SIRT1 mRNA level but reversed glucose induced reduction of SIRT1 expression indicating a possible regulatory role on each other (Figure 2.10B). To explore this relationship further we performed additional experiments investigating the downstream molecules (Figure 2.10C-F). We found p300 silencing in HG lead to a significant increase in FOXO1 DNA binding activity and subsequent MnSOD level compared to controls (Figure 2.10C and D). In addition, glucose induced increase of ROS and β-gal positivity were also reduced with p300 siRNA (Figure 2.10E-G). These findings together indicate that both SIRT1 and p300 regulate oxidative stress pathway in ECs through FOXO1.
Figure 2.10 SIRT1 and p300 regulate each other in microvascular endothelial cells. (A) Quantitative Real Time RT-PCR analysis showed HG upregulated p300 mRNA and such effects are augmented by SIRT1 knockdown with siRNA. (B) HG induced downregulation of SIRT1 mRNA expression was corrected with p300 knockdown with siRNA. mRNA levels are expressed as ratio of 18s normalized to NG. (C) p300 siRNA reduced total ROS levels in HG by increasing (D) FOXO1’s DNA binding activity subsequently increasing (E) the MnSOD levels. (F, G) Microscopic photographs and quantitative analysis showed glucose induced increased SA β-gal positivity is effectively prevented by p300 siRNA. Data normalized to NG. (NG = 5 mM; HG = 25 mM D-glucose, OSM = 25 mM L-glucose, P1 = passage1). [*p<0.05 and **p<0.01 compared to NG; †P<0.05 and
+++p<0.01 compared to HG. Scale bar represent 100µm for all micrographs. siRNA1 and siRNA2 represent separate experiments using two different siRNAs].

2.3.7 Diabetes causes accelerated aging in kidney.

Finally, in order to investigate if the findings in vitro is reflected in vivo, we used a well-established animal model, STZ induced C57BL/6 mice. Diabetic animals showed high blood glucose (23.43 ± 3.27 mmol/L vs. controls 7.3 ± 0.93 mmol/L, p<0.001) and reduced body weight (22.50 ± 1.11 gm vs. controls 30.25 ± 2.04 gm, p<0.003). Urinary albumin concentration was increased (0.58 ± 0.07 µg/mL vs. 0.21 ± 0.07 µg/mL in control, p<0.009) along with increased serum creatinine in the diabetic animals (0.52 ± 0.08 mg/dL vs. controls 0.18 ± 0.04 mg/dL, p<0.02). Kidney tissues from diabetic mice showed increased β-gal positivity compared to the controls, supporting our in vitro findings (Figure 2.11A and B). This was further demonstrated as MnSOD levels, FOXO1 transcriptional activity, SIRT1 mRNA expression and enzyme activity were significantly reduced in kidneys of the diabetic animals along with an increased p300 protein levels in these tissues (Figure 2.11C-G).
Figure 2.11 Diabetes causes accelerated aging in mice kidney. (A) SA β-gal staining of kidney tissue of STZ induced diabetic mice at 2 and 4 months showing increased positivity with uncontrolled diabetes. Arrow indicates β-gal positive cells. (B) Quantification of β-gal staining of the kidney tissue. Data presented as β-gal positivity/view (n = 10 image). (C) Shows reduction of MnSOD levels in kidney tissues of the diabetic animals following 2 and 4 months. (D) mRNA analysis confirmed downregulation of SIRT1 expressions in the kidney tissues of these animals. mRNA levels are expressed as a ratio to 18s normalized to controls. (E) FOXO1 DNA binding and (G) SIRT1 enzyme activities are reduced in the kidney tissues in diabetes. (F) Western blot analysis showed increased p300 protein levels in the kidneys in diabetes. Data
normalized to controls. [*p<0.05 compared to control animals. Scale bar represents 100 µm for all micrographs].

We further examined renal tissues from type 2 diabetic mice that were hyperglycemic and obese (data not shown). We found reduced MnSOD level following 2 and 4 months of diabetes in the kidney tissues of these animals (Figure 2.12A). mRNA analysis further showed reduced SIRT1 expression in these tissues (Figure 2.12B). In addition FOXO1 DNA binding activity was reduced in the kidneys of these mice (Figure 2.12C).

Figure 2.12 Oxidative stress and associated changes are present in the kidneys and retinas of diabetic animals. (A) MnSOD levels were reduced in kidneys of *db/db* mice following 2 and 4 months of diabetes indicating increased oxidative
stress. (B, C) SIRT1 mRNA expression and FOXO1 activity in kidneys of db/db mice were reduced compared to controls. (D) SA β-gal staining of type 1 diabetic mice retina showed increased positivity in retinal blood vessels following 2 months of diabetes. [Scale bar represent 50 µm for all micrographs]. Arrow indicates retinal blood vessels. (E) Such changes were associated with reduced MnSOD level in these tissues. (F, G) Shows reduction of SIRT1 mRNA expressions in retinal tissues of both type 1 and 2 diabetic mice. mRNA levels are expressed as a ratio to 18s normalized to controls. [*p<0.05 compared to control animals].

2.3.8 SIRT1 expression is reduced in retina with type 1 and type 2 diabetes.

Furthermore, we examined SIRT1 mRNA expression in retinas of the type 1 and 2 diabetic animals. Retinal SIRT1 mRNA expressions were reduced in both models following 2 months of diabetes and the effects were pronounced after 4 months (Figure 2.12F and G). Further, SA β-gal staining of mice retina with type 1 diabetes showed increased positivity in the blood vessels following 2 months of diabetes, along with reduced antioxidant levels in these tissues (Figure 2.12D and E).

2.4 Discussion

This study demonstrates that hyperglycemia accelerates aging both in vitro and in vivo. Although some studies have previously been performed [35] on
ECs investigating the effects of HG exposure for short term periods (24 to 72 hr), no study has yet been reported on chronic HG exposure of these cells, to simulate the process of chronic hyperglycemia in an *in vitro* setting. We simulated chronic hyperglycemia in the ECs with HG and propagated them in HG for weeks or even months until the cells stopped proliferation completely for more than 2 weeks. Such design allowed us to examine glucose induced accelerated aging process amongst ECs of different origins. We have seen signs of cellular senescence by SA β-gal positivity early in microvascular ECs and retinal ECs. However this process was slower in large vessel ECs. The differentiated ECs were more susceptible to hyperglycemia induced damage compared to HUVEC, which is more close to progenitor cells in terms of differentiation [36] and are a better survivor in hyperglycaemia. We have demonstrated that such process was associated with increased oxidative stress.

Molecular diversity in the genetic level in ECs of various origins has been studied before [37,38]. It has been shown that the gene expression profiles of ECs from large vessels are different from those of micro-vessels [39]. In keeping with these findings our study further demonstrated and elaborated some structural and functional significance of these type of changes.

We have shown an important role played by SIRTs in this study. Since the discovery of SIRTs, interests in these deacetylases have generated multiple lines of evidences indicating SIRTs as evolutionarily conserved regulators of lifespan [9, 18-21,40]. SIRTs regulate physiological response to metabolism and stress, the two key factors affecting the aging process. We found all SIRT1-7 mRNA
expressions were consistently reduced with the accelerated aging process in hyperglycemia. Although isolated SIRT alterations have been demonstrated in various studies [19, 20, 41-43], such comprehensive analysis of SIRTs have not been done. More interestingly, the level of SIRTs reduction paralleled that of β-gal positivity and such changes were prevented by SIRT activators indicating an important relationship between SIRTs and aging.

SIRT1, the leading enzyme in the SIRT family is found both in the cytoplasm and nucleus and has been found to have protective roles in stress resistance and cell survival in various diseases [41, 44, 45]. This study showed glucose induced alteration of downstream mediators of SIRT1, such as FOXO1 were corrected by SIRT1 activators further establishing the crucial role of SIRT1 in this process. The regulatory role of SIRT1 and p300 was further interesting. p300 and SIRT1 regulates each other as silencing one gene lead to increased expression of the other gene producing downstream affects. Other studies have shown, in a different model, p300 acetylates and activates PPAR-γ leading to its binding to SIRT1 promoter, thus decreasing SIRT1 expression [44]. Hence both direct and indirect relationship between these two molecules may exist in the ECs.

We have previously shown that p300 regulates multiple transcription factors and proteins [6, 16]. Other studies have also shown acetylation of FOXO1 by p300 facilitates its phosphorylation by Akt ultimately exporting it from the nucleus with chaperon 14-3-3 protein [24, 46]. In line with that, this study further showed HG induced upregulation of p300 leads to attenuation of FOXO1 DNA
binding activity in the ECs. This led to a reduction in MnSOD level, a known target gene of FOXO1 [27] and increased oxidative stress in the ECs. Furthermore, silencing p300 with siRNA corrected the MnSOD reduction and downstream changes in hyperglycemia, confirming such pathway.

On the other hand as FOXO1 deacetylation by SIRT1 is necessary for its retention in the nucleus [10, 29], we also found increased FOXO1 DNA binding with SIRT1 activators, which increased SIRT1 enzyme activity and reduced SA β-gal positivity. Additionally, both FOXO1 inhibitor and SIRT1 siRNA was able to induce early senescence in NG, and mimicked the HG effect in the ECs. Together, these results indicate an important mechanistic pathway of oxidative stress and aging in HG, mediated through FOXO1 and regulated by SIRT1 and p300.

In keeping with the *in vitro* studies, STZ induced diabetic mice showed increased β-gal positivity in the kidneys following 2 & 4 months of uncontrolled diabetes. The retina showed increased signs of aging following 2 months of diabetes. This was accompanied by significantly reduced antioxidant levels in the tissues. In addition, SIRT1 mRNA was found to be significantly reduced in kidney and retina of both type 1 and type 2 diabetic mice.

In summary, this study showed chronic hyperglycemia accelerated aging process through a novel SIRT1 and p300 regulated pathway. We demonstrated HG induced reduction in SIRT1 lead to increased oxidative stress mediated through FOXO1 which was prevented by SIRT1 activation. We further
demonstrated p300 negatively regulates such pathway, as silencing p300 mimicked resveratrol’s effect. Identification of such novel mechanisms will allow us to better understand the pathogenesis of diabetic complications as well as aging and eventually help to find potential therapeutic treatment.

2.5 References


Chapter 3

3 miR-195 regulates SIRT1 mediated changes in diabetic retinopathy

Rokhsana Mortuza, Biao Feng and Subrata Chakrabarti. Department of Pathology, Schulich School of Medicine & Dentistry, Western University, London ON Canada.

In this study our aim was to investigate SIRT1 regulation by miR-195 in diabetic animals. We investigated the role of miR-195 in glucose-induced SIRT1 mediated changes in the ECs and retinas of type 1 diabetic rats.

bA version of this chapter have been published: Mortuza R, Feng B, Chakrabarti S. miR-195 regulates SIRT1-mediated changes in diabetic retinopathy. Diabetologia. 2014;57(5):1037-46. doi: 10.1007/s00125-014-3197-9. Copyright © 2014, Springer-Verlag Berlin Heidelberg. The article is being reused with kind permission from Springer Science and Business Media.
3.1 Introduction

Diabetic retinopathy is a growing problem worldwide with no specific medical treatment. In diabetic retinopathy, ECs of the retinal microvessels undergo a series of changes in response to hyperglycemia causing activation of several transcription factors [1-5]. Such processes lead to alteration of various gene expressions resulting in structural and functional damages [6]. Glucose-induced oxidative stress mediated increased production of extracellular matrix (ECM) proteins such as fibronectin (FN) is a major characteristic feature of diabetic retinopathy. Glucose-induced increased FN synthesis in ECs and diabetes-induced augmented FN productions in the retina have been demonstrated previously [7-14]. It is of interest to know that FN upregulation is a common finding in endothelial and tissue aging [15-19].

Researchers have found SIRT1, the longevity gene, play crucial role in regulating cell cycle, survival, metabolism and development across species since its identification [20-25]. We have demonstrated glucose-induced increased oxidative stress causes rapid aging in ECs and retinas in diabetes [26]. Such processes are mediated through alteration of Sirtuins (SIRTs), a family of NAD$^+$ dependent class III histone deacetylases. Furthermore, transcriptional co-activator p300 regulates such pathway by modifying both SIRT1 (leading enzyme in the SIRT family) and FOXO1 (forkhead O group of transcription factor) and altering the antioxidant levels in these ECs [26]. However, other post transcriptional ways of SIRT1 regulations also holds tremendous potential.
With the discovery of miRNAs, research on SIRT1 regulation has progressed and several miRNAs have been found to regulate SIRT1 in various tissues and disease conditions [27]. miRNAs are highly conserved small (19-24 nucleotides), non-coding RNAs that regulate gene expression at the post transcriptional level, by binding with 3'UTRs (untranslated regions) of the specific mRNAs, causing their degradation or translational repression [27-29]. Over one third of genes are estimated to be regulated by miRNAs. miRNAs may play roles in many biological processes, including development of metabolic diseases such as diabetes [27-35].

Although several researches have been conducted on miRNA mediated regulation of SIRT1 in various physiological and pathological conditions [25], no studies have been conducted on such regulation in diabetic retinopathy. In this study we investigated the role of a specific miRNA, miR-195 in regulating SIRT1 in diabetic retinopathy. miR-195 is upregulated in circulation in patients with hypertension, metabolic syndrome and glucose intolerance [36]. Similar increases were seen in kidney and liver tissues of diabetic animals [37, 38]. In cardiomyocytes, miR-195 was found to inhibit SIRT1 and thus promote palmitate-induced apoptosis [39]. miR-195 deregulation has been associated with several other diseases, with upregulation in multiple cancers, hypertrophic cardiomyopathy and pre-eclampsia [40, 41]. In line with these research, our previous microarray analysis [42] showed miR-195 upregulation in glucose exposed ECs and in the retinas of diabetic rats. As SIRT1 is a possible target of miR-195 we explored this relationship further in diabetic retinopathy.
Since SIRT1 is a possible target of miR-195 (www.mirbase.org, www.microrna.org), we investigated whether miR-195 alteration occurs in diabetic retinopathy, and if such alterations causes EC aging in hyperglycemia. Microvascular ECs are the major target of glucose mediated damages in the retina, hence we examined human retinal and dermal microvascular ECs (HRECs and HMECs), to characterize glucose-induced miR-195 mediated regulation of SIRT1 and downstream cellular changes. We further expanded the study to investigate retinas of streptozotocin (STZ) induced type 1 diabetic animals [42, 43].

3.2 Materials and methods

3.2.1 Cell culture

HRECs were obtained from Olaf Pharmaceuticals (Worchester, MA) and HMECs were obtained from Lonza, Inc. (Walkersville, MD). Cells were grown in EC basal medium 2 (EBM-2, Lonza, Walkersville, MD) and trypan blue dye exclusion test was used to examine cell viability as described [42-44]. The cells were passaged in 6 well plates (Corning, Acton, MA) and treated with normal glucose (NG, 5 mmol/l D-glucose) or high glucose (HG, 25 mmol/l D-glucose) or osmotic control (OSM, 25 mmol/l L-glucose) for 72 hours. Human embryonic kidney (HEK293A) cells were obtained from ATCC (Manassas, VA) and were used as previously described [42-44]. Reagents were obtained from Sigma
Chemicals (Sigma, Oakville, ON) unless specified otherwise. All experiments were conducted with 6-10 replicates.

3.2.2 mRNA extraction and quantitative analysis

TRIZOL™ (Invitrogen, Burlington, ON) was used to isolate RNA [42-44]. Real Time RT-PCR was performed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA), Qiagen One Step RT-PCR kit with custom made SIRT1 and FN primers and LightCycler™ (Roche Diagnostics Canada, Quebec) as described [26, 43]. The data were normalized to 18sRNA [26, 43].

3.2.3 miRNA extraction and analysis

As described [42, 43], mirVana miRNA isolation kit (Ambion, Inc., Austin, TX) was used to extract miRNAs from cells and tissues. Real time RT-PCR was conducted with a reaction volume of 20 µL as described [42, 43].

3.2.4 miRNA mimic or antagomir transfection

ECs were transfected with miRIDIAN microRNA-195 mimic or antagomir (20 nmol/l) (Dharmacon Inc., Chicago, IL) using Lipofectamine2000 (Invitrogen, Burlington, ON). Scrambled controls were used in parallel [42, 43].
3.2.5 Adenoviral forced-expression of SIRT1 and SIRT1 gene silencing

SIRT1 adenovirus (devoid of 3'UTR) and null vector was obtained from ABM (Richmond, BC, Canada) and amplified in HEK293A cells. ECs were transduced with the adenovirus (with or without glucose) and following 72 hours of treatment samples collected. To silence SIRT1, transfection of small interfering RNA (siRNA) was performed as described by us earlier [26].

3.2.6 Luciferase Reporter Assay for Targeting SIRT1-3'UTR

The luciferase vector including 3'UTR of SIRT1 (1080 bp) containing the SIRT1-miR-195 response elements (wt-Luc-SIRT1) and the mutant (mu-Luc-SIRT1) were purchased from Norclone Biotech Laboratories (London, ON, Canada). Plasmid DNA (wt-Luc-SIRT1 or mu-Luc-SIRT1, β-galactosidase control vector) and miR-195 mimic or scrambled oligonucleotide were co-transfected in HRECs for 48 hours. Luciferase activity was measured using the Dual-Light Chemiluminescent Reporter Gene Assay System (Applied Biosystems, Burlington, ON) and SpectraMax M5 (Molecular Devices, Sunnyvale, CA) and normalized by measuring β-galactosidase activity.

3.2.7 MnSOD, FN ELISA and SIRT1 enzyme activity assay

MnSOD (manganese superoxide dismutase) and FN ELISA were performed using commercially available kits [26, 43]. As described earlier, [26]
SIRT1 enzyme activity was measured as per the manufacturer instructions (Sigma, Oakville, ON).

3.2.8 Animal Experiments

Animal experiments were conducted following guidelines specified by the Canadian Council of Animal Care. Protocols were approved by the Western University Animal Care and Veterinary Service. The investigations were in accordance with NIH publ. no. 85-23, revised 1996.

Sprague-Dawley rats (~175g, 6 weeks, male) were obtained (Charles River, Wilmington, MA). Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg, in citrate buffer, pH 5.6). Control animals received equal volume of citrate buffer. Diabetes was determined as a blood glucose level >20 mmol/l on 3 successive days (Abbott Diabetes Care, Alameda, CA). The animals were fed on a standard diet with water ad libitum and monitored daily [42, 43]. A group of diabetic rats (n=7/group) received weekly intravitreal injections of 1.5 µg miR-195 mimic or antagonim in lipofectamine reagent in one eye. The other eye received scrambled control. They were sacrificed one week after the fourth injection. Retinal tissues were collected, snap frozen and were stored at −80°C until further analysis.

To assess intravitreal miRNA penetration efficiency DY547 (red fluorescent dye) tagged miRNA scramble transfection control (Dharmacon Inc., Chicago, IL) was injected intravitreally and retinal tissues were collected from
sacrificed animals at 2 hr, 4hr, 8hr and 24hr following injection and snap frozen. DY547 tagged RNA has previously been shown to penetrate in the retina following intravitreal injection [45]. Slides from the same sections were counterstained with H&E for orientation.

3.2.9 In situ Hybridization

As described earlier [43, 46] 5’ and 3’ double DIG-labelled custom-made mercury LNA™ miRNA detection probes (Exiqon, Vedbaek, Denmark) were used to detect miR-195 expression using in situ hybridization (ISH) Kit (Biochain Institute, Hayward, CA) in retinal tissue sections. Scrambled probe and no-probe were used as controls.

3.2.10 Immunohistochemistry

IgG stainings were performed on frozen retinal sections using anti-rat IgG (ebioscience, Sandiego, CA) with hematoxylin counterstain as described [42, 43]. Quantification was done in a masked fashion using an arbitrary scale (0 = no extravascular stain of retina, 1 = mild extravascular stain, 2 = moderate extravascular stain, 3 = marked extravascular stain).

3.2.11 SA β-GAL staining, phase contrast & fluorescent microscopy

Cells or frozen retinal sections were stained with SA β-GAL staining kit (abcam, Cambridge, MA). Retinal slides were counterstained with H&E for orientation. From each specimen, positive (blue) cells were photographed with
phase contrast inverted microscope [26]. They were counted per 10 microscopic fields at 20X magnification and presented as % of total number of cells [26]. Tissue slides were quantified based on staining intensity at 60X magnification as described [47]. Fluorescent microscopies of frozen retinal sections were performed with TRITC filter at 20X magnification.

3.2.12 Western Blot

Western blot analysis was conducted according to the standard protocol [10] using SIRT1 & β-Actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

3.2.13 Statistical analysis

Data are expressed as mean ± SEM, normalized to controls (n≥6). The statistical significances were analyzed by one or two way ANOVA followed by Tukey’s HSD post hoc correction and the two tailed Students t test as appropriate (PASW Statistics 18, IBM, Canada). A p value < 0.05 was considered statistically significant.

3.3 Results

In the initial screening of SIRT1 targeting microRNAs using our previously published array data of retinal tissues of normal and diabetic animals [42], we found miR-195 expression was increased almost 3 fold in the retinal tissues of
the diabetic animals (Table 3.1). Other SIRT1 targeting miRNAs such as miR-34a, miR-138, miR-9 were reduced, and miR-217, miR-199a, miR-200a and miR-132 expression levels were unchanged (Table 3.1). On the basis of our initial findings we further investigated the role of miR-195.

Table 3.1 Fold changes of SIRT1 targeting miRNAs.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold change (compared to control, p ≤ 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-195</td>
<td>2.8</td>
</tr>
<tr>
<td>miR-34a</td>
<td>0.53</td>
</tr>
<tr>
<td>miR-138</td>
<td>0.51</td>
</tr>
<tr>
<td>miR-9</td>
<td>0.43</td>
</tr>
<tr>
<td>miR-217</td>
<td>1.07</td>
</tr>
<tr>
<td>miR-199a</td>
<td>1.04</td>
</tr>
<tr>
<td>miR-200a</td>
<td>0.93</td>
</tr>
<tr>
<td>miR-132</td>
<td>1.02</td>
</tr>
</tbody>
</table>

3.3.1 High glucose causes upregulation of miR-195 and SIRT1 downregulation in the ECs.

Based on the hypothesis that glucose-induced SIRT1 downregulation is mediated by miR195, we investigated HRECs exposed to 5 (NG) and 25 (HG)
88 mmol/l D-glucose for 72 hrs. These concentrations are based on our previous dose dependent analysis of SIRT1 mRNA and protein expression [26]. We confirmed reduction of SIRT1 mRNA and enzyme activity following incubation with HG but not by 25 mmol/l L-glucose (osmotic control, OSM, Figure 3.1a and 3.1c) in HRECs. We then extracted miRNA from these cells. Quantitative Real Time RT-PCR analysis of the ECs in HG confirmed significant upregulation of miR-195 compared to 5 mmol/l glucose. No effects were seen following incubation with 25 mmol/l of L-glucose (Figure 3.1b).

Figure 3.1 miR-195 regulates SIRT1 in ECs in hyperglycemia. (a) SIRT1 mRNA and (b) miR-195 expressions in HRECs following exposure to HG and NG with miR-195 mimic and antagonir transfections. (c) SIRT1 enzyme activity and (d) protein levels with miR-195 mimic and antagonir transfections. (b) Efficiency of
miR-195 antagonir and mimic transfection in HRECs compared to scrambled controls. [HRECs= Human retinal microvascular endothelial cells, HG= 25 mmol/l glucose, NG= 5 mmol/l glucose, OSM= 25 mmol/l L-glucose. Scramble= scrambled miRNAs, 195= miR-195 mimics, 195(A)= miR-195 antagonirs, *= significantly different from NG, †= significantly different from HG. miRNA levels are expressed as a ratio to RNU6B (U6); mRNA levels are expressed as a ratio to 18s. All data are normalized to 5 mmol/l glucose. n=6] (e) Alignment of SIRT1-3'UTR (and mutated SIRT1-3'UTR) sequence with mature miR-195 based on bioinformatics predictions (www.targetscan.org, www.microrna.org, www.mirbase.org). The 5' end of the mature miR-195 is the seed sequence and has perfect complementarity with six nucleotides of the 3'UTR of SIRT1. In the mutated sequence (small caps) such complementarity was lost. (f) Luciferase reporter assay in HRECs, showing dose dependent binding of SIRT1-3'UTR with miR-195. Mutated (mut) SIRT1-3'UTR abrogated the inhibitory effects of miR-195. [Relative luciferase activities were expressed as luminescence units, normalized for β-galactosidase expression, *= significantly different from vector or mut. Vector= SIRT1-3'UTR luciferase plasmids+β-galactosidase plasmids, SIRT1 mut= mutated SIRT1-3'UTR luciferase plasmids+β-galactosidase plasmids. n=6]. (g) shows cellular MnSOD levels with miR-195 antagonir transfection in HG, n=6.
3.3.2 Glucose-induced SIRT1 reduction in the ECs is mediated by miR-195.

We then proceeded to explore functional significance of such glucose-induced miR-195 upregulation with respect to SIRT1 expression. To find a cause-effect relationship, we transfected cells in 25 mmol/l glucose with miR-195 antagonir. Transfection efficiencies, assessed by measuring miR-195 expression showed substantial reduction in intracellular miR-195 expression compared to scrambled miRNA transfection (Figure 3.1b). Such reduction could be attributed by degradation of the miRNA following binding with antagonir [48]. Additionally irreversible binding of antagonirs with the miRNAs may further prevent amplification.

Such transfection effectively rescued glucose-induced SIRT1 downregulation. No such effects were seen with scrambled miRNAs (Figure 3.1a, c). On the other hand, transfection of the ECs in 5 mmol/l glucose with miR-195 mimic downregulated SIRT1 mRNA production and enzyme activity mimicking the effects of 25 mmol/l glucose (Figure 3.1a-c). Furthermore, SIRT1 protein and enzyme analysis showed glucose-induced reduced SIRT1 enzyme activity in the ECs is prevented by miR-195 antagonir transfection (Figure 3.1c, d). On the other hand, transfection of HRECs in NG with miR-195 mimic caused effects similar to HG by reducing SIRT1 levels in these ECs (Figure 3.1a-d).

In parallel, we conducted similar experiments in HMECs (to examine whether the changes are unique to HRECs or universal to other microvascular
ECs) which showed similar effects confirming the changes observed in HRECs (Figure 3.2a-d).

Figure 3.2 miR-195 regulates glucose-induced SIRT1 mediated aging changes in HMECs. Human dermal microvascular endothelial cells (HMECs) showed glucose-induced (a) miR-195 upregulation (n=10) and (b) SIRT1 downregulation. (b-d) Transfection of HMECs with miR-195 antagonirs normalized HG induced downregulation of SIRT1 mRNA and enzyme activity; (c) shows efficiency of miR-195 antagonir and mimic transfection. n=6. (e) Senescence associated SA-βGAL staining of HMECs showed increased positivity with 25 mmol/l glucose (HG) treatment compared to 5 mmol/l glucose (NG) and 25 mmol/l L-glucose (OSM). MiR-195 antagonir transfection successfully prevented such signs of
cellular aging. Such changes were not seen in cells transfected with scrambled miRNA. Arrow indicates positive cell (blue). (f) Quantitation of SA-βGAL positivity, n=10 image/treatment. (g) shows HG induced induced reduction of cellular MnSOD levels were normalized with miR-195 antagonir transfection, n=6. [Scramble= scrambled miRNAs, 195= miR-195 mimics, 195(A)= miR-195 antagonirs, *= significantly different from NG, †= significantly different from HG. MiRNA levels are expressed as a ratio to RNU6B (U6); mRNA levels are expressed as a ratio to 18s. All data are normalized to 5 mmol/l glucose. MnSOD= manganese superoxide dismutase. Scale bar represent 100 μm for all micrographs. Inset = magnified image showing cytoplasmic SA-βGAL positivity.]

3.3.3 miR-195 binds to 3'UTR of SIRT1 mRNA.

To examine direct binding of miR-195 with 3'UTR of the SIRT1 mRNA, we did Luciferase assays in HRECs. We co-transfected miR-195 mimic with Luciferase reporter plasmids having cloned miR-195 binding sites for SIRT1-3'UTR or mutated SIRT1-3'UTR. SIRT1-3'UTR luciferase activity was significantly repressed with such miR-195 overexpression, which was however not seen with mutated SIRT1-3'UTR (Figure 3.1e, f and Figure 3.3a). These results confirmed binding of miR-195 with 3'UTR of SIRT1 mRNA.
3.3.4 miR-195 antagomir prevents HG-induced reduction of SIRT1 regulated antioxidant levels.

To investigate glucose-induced downstream functional alterations of such miR-195 increase in ECs, we measured intracellular antioxidant levels. In previous studies we have established such SIRT1 mediated changes in the ECs
[26]. SIRT1 regulated antioxidant MnSOD analysis showed upregulation of this enzyme with miR-195 inhibition (Figure 3.1g). Likewise, HMECs showed similar effects of miR-195 inhibition in 25 mmol/l glucose (Figure 3.2g).

3.3.5 miR-195 antagomir averts FN upregulation in ECs.

Increased production of ECM protein FN is a characteristic feature of diabetic retinopathy [7-14]. Hence we examined FN mRNA and protein level in these ECs. miR-195 antagomir transfection (not scrambled controls) attenuated HG-induced increased FN mRNA and protein levels in the ECs (Figure 3.4a, b). Moreover, knockdown of SIRT1 with siRNA in NG showed glucose-like effect in which there was an increase in FN mRNA and protein levels confirming such regulation (Figure 3.4c and Figure 3.5a, b). Such effect was not seen with scrambled siRNA treatment (Figure 3.4c and Figure 3.5a, b).
Figure 3.4 SIRT1 forced-expression prevents glucose-induced FN upregulation in ECs. (a) FN mRNA and (b) FN protein levels in HRECs with miR-195 transfection in HG. (c) FN mRNA levels following SIRT1 knockdown with siRNA in NG in the ECs. (d) SIRT1 enzyme activity with adenoviral overexpression in the ECs confirming transfection efficiency at the functional level. (e) FN mRNA levels in HG with Ad-SIRT1 transfection. (f) FN mRNA levels in NG with miR-195 mimic and Ad-SIRT1 transfection. [HG= 25 mmol/l glucose, NG= 5 mmol/l glucose, 195= miR-195 mimics, 195(A)= miR-195 antagonirs, *= significantly different from NG, †= significantly different from HG; mRNA levels are expressed as a ratio to 18s normalized to NG. HRECs= human retinal microvascular endothelial cells. All data normalized to controls. n=6]
Figure 3.5 SIRT1 knockdown in NG increases FN protein levels in HRECs. (a) SIRT1 knockdown efficiency with siRNA in NG shows significant reduction of SIRT1 mRNA levels in the ECs and such reduction caused an increase in (b) FN protein levels. Such increase is absent in scramble siRNA treated cells. (c, d) Shows effect of miR-195 antagonir treatment in HRECs in passage 3 has preventative effect against HG induced accelerated aging changes in these cells, n=10 image/treatment. (e) Shows MnSOD level in HREC passage 3 following miR-195 antagonir transfection. HG induced reduction of MnSOD level is efficiently increased with such treatment in these ECs. [Scramble = scrambled miRNAs. 195(A)= miR-195 antagonirs, HRECs = human retinal microvascular
endothelial cells. * = significantly different from NG, † = significantly different from HG. P3 = passage 3, MnSOD = manganese superoxide dismutase. Data normalized to NG. Scale bar represent 100 µm for all micrographs. Inset = magnified image showing cytoplasmic SA-βGAL positivity. n=6]

3.3.6 SIRT1 forced-expression prevents FN upregulation in ECs.

We further wanted to see if forced-expression of SIRT1 can prevent FN upregulation in ECs in hyperglycemia. We transfected HRECs with SIRT1 adenovirus and analysed the cells with Real Time RT-PCR (data not presented). SIRT1 enzyme activity was increased following the transfection in HG treated ECs compared to empty vector controls (Figure 3.4d). Such increase activity normalized the HG induced FN upregulation in these cells (Figure 3.4e). In parallel, we conducted similar experiments in HMECs which were in line with the findings observed in HRECs (Figure 3.6a-d).
Figure 3.6 SIRT1 forced-expression showed preventative effect against glucose-induced damage in HMECs. Transfection of miR-195 antagonir prevented glucose-induced upregulation of (a) FN mRNA and (b) protein levels in the HMECs. (c) Adenoviral forced-expression of SIRT1 in the ECs increased the enzyme’s activity both in NG (5 mmol/l glucose) and HG (25 mmol/l glucose) confirming transfection efficiency at the functional level. Such increase in activity were absent in null vector transfected ECs. Ad-SIRT1 transfected ECs showed (d) attenuated upregulation of HG induced FN mRNA levels and (e) reduced signs of aging with SA β-GAL stain. Arrow indicates positive cell (blue). (f) Quantitation of SA β-GAL positivity, n=10 image/treatment. [195(A)]= miR-195 antagonirs, * = significantly different from NG, † = significantly different from HG; mRNA levels are expressed as a ratio to 18s normalized to NG. HMECs= human dermal microvascular endothelial cells. All data normalized to controls. Scale bar
represent 100 µm for all micrographs. Inset = magnified image showing cytoplasmic SA β-GAL positivity. n=6]

We further conducted an additional experiment to see if the effects of miR-195 are reversible. We observed adenoviral overexpression of SIRT1 (plasmid lacking the binding site of miR-195), reverses the miR-195 mimic induced upregulation of FN mRNA levels in NG (Figure 3.4f). Such finding shows the detrimental effects of miR-195 is reversible, and confirms miR-195 mediated regulation of SIRT1 and downstream changes in these ECs.

3.3.7 miR-195 is overexpressed in the retina in diabetes.

We then moved to a well-established animal model to examine, if the mechanisms seen in ECs are indeed involved in the development of retinal microangiopathy. We used STZ- induced diabetic rats demonstrating hyperglycemia (serum glucose of diabetics 20.2±1.9 mmol/l vs controls 5.3±0.7 mmol/l, p<0.001) and reduced body weight (diabetics 299.0±59.2 g vs controls 460.8±13.2 g, p<0.001). Real Time RT-PCR analysis of retinal tissues of these animals following 1 month of uncontrolled diabetes showed significant upregulation of miR-195 (Figure 3.7a). It is of interest to note that, we have previously shown in this model, diabetes causes increased ECM protein and vasoactive factor expression at this time point [42, 43]. Such notion was further confirmed by using *in situ* hybridization and LNA probes. In the retinas of diabetic animals, miR-195 was overexpressed in the cells of inner and outer nuclear layers, ganglion cell layers and ECs of the microvessels (Figure 3.7b).
Figure 3.7 miR-195 is overexpressed in the retina in diabetes. (a) Rat retinal miR-195 expressions in control and diabetic animals. (b) LNA-ISH analysis of retinal tissues of control and diabetic animals. Blue chromogen shows miR-195 in the cells of the inner nuclear layers (thin arrow), outer nuclear layers (thick arrow), ganglion cell layers (arrowhead) and endothelial cells of the microvessels (arrow, inset). Diabetic rat retina stained with scrambled miRNA probes lacking such stain confirmed specificity of the miR-195 probe. Insets show enlarged view of the retinal capillaries. (Scale bar = 50 µm for all micrographs). [* = significantly different from control. miRNA levels are expressed as a ratio of RNU6B (U6)
normalized to control. ALK Phos was used as chromogen (blue) with no counter stain in LNA-ISH. n=7 eye/treatment]

3.3.8 Diabetes induced SIRT1 downregulation in the retina and its downstream effects are mediated by miR-195.

To establish the functional consequence of miR-195 upregulation in the retina, we investigated retinal SIRT1 mRNA and enzyme levels which is a target for miR-195. Diabetes-induced miR-195 upregulation was associated with SIRT1 mRNA downregulation (Figure 3.8c). Simultaneously SIRT1 protein and enzyme level was also reduced in the retina in diabetic rats (Figure 3.8d, e). To find a cause-effect relationship, we performed intravitreal miR-195 antagonir injection. Penetration efficiency of such drug delivery was confirmed with DY547 tagged miRNA injection, which shows effective transfection of the miRNA in rat retinal layers and microvessels following 2 hours of the injection (Figure 3.8a). Intraretinal delivery efficiencies, assessed by measuring miR-195 expression in the retina, showed reduction in retinal miR-195 expression (Figure 3.8b). Such intravitreal miR-195 injection normalized diabetes-induced SIRT1 reduction in the retinas of the treated animals (Figure 3.8c-e). We further measured antioxidant level in the retinas. Diabetes-induced reduction of retinal MnSOD levels were prevented with miR-195 antagonir injection (Figure 3.8f). No such prevention was seen with injection of scrambled oligonucleotides or vehicles (Figure 3.8f). In addition, injection of miR-195 mimic in control animals caused effects similar to diabetes with reduced SIRT1 mRNA and MnSOD levels (Figure 3.8c, f).
Figure 3.8 miR-195 antagonir treatment can ameliorate diabetes induced reduction of antioxidant levels in retinal tissues. (a) Fluorescent microscopy images of rat retinas following intravitreal injection of DY547 tagged miRNA and vehicle demonstrating penetration. Arrows show various layers of rat retina. Inset shows enlarged view of retinal microvessels. (Scale bar= 100 µm for both micrographs). (b) Retinal miR-195 expression following intravitreal injection of miR-195 antagonir to assess efficiency of intravitreal delivery. (c) SIRT1 mRNA levels in the rat retinas following intravitreal miR-195 antagonir treatments. (d) SIRT1 protein level and (e) enzyme activity following such injections. (f) Shows MnSOD levels in the retinal tissues of the control and diabetic animals following miR-195 mimic and antagonir injections. [*= significantly different from control
(Co), †= significantly different from diabetic (Di) or Di+Scramble. 195= miR-195 mimics, 195(A)= miR-195 antagomirs. miRNA levels are expressed as a ratio of RNU6B (U6) normalized to control; mRNA levels are expressed as a ratio to 18s normalized to control. MnSOD= manganese superoxide dismutase. DY547= red fluorescent dye. n=7 eye/treatment]

We further expanded the study to see the downstream effects of miR-195 mediated increased oxidative stress and measured the level of FN protein in the rat retinal tissue. We observed diabetes-induced upregulated FN protein levels in the retina were reduced by intravitreal mir-195 antagomir injection. Retinal tissue with scrambled miRNA treated animals did not show such effects (Figure 3.9a).
Figure 3.9 miR-195 antagomir prevents diabetes-induced increased FN protein and vascular leakage in retina. (a) FN protein levels in rat retinal tissues with intravitreal miR-195 antagomir injections in the diabetic animals. (b) Immunohistochemical stains on the rat retinas following miR-195 antagomir treatment. Brown chromogen indicates IgG extravasation. (Hematoxylin counterstain). Arrow shows diffuse staining in the retina of the diabetic animal. [195(A)= miR-195 antagomirs, *= significantly different from control (Co), †= significantly different from diabetic (Di) or Di+Scramble; Scale bar represent 50 µm for all micrographs. Data normalized to controls. Inset = magnified images of retinal microvessels. n= 7 eye/treatment]

We then investigated permeability changes and measured IgG extravasation from the retinal vasculature using an IgG immunostaining. We found diabetes-induced increased vascular permeability was reduced by the miR-195 antagomir injection (Figure 3.9b). No extravascular IgG was seen in the control animals (score= 0). Diabetic animals demonstrated a score of 2 to 3, indicating increased extravasated IgG. Scores of 0 to 1 were noted in eyes injected with the miR-195 antagomir, indicating prevention of diabetes-induced increased vascular permeability by such treatment.

3.3.9 miR-195 antagomir prevents SIRT1 mediated EC senescence in hyperglycemia.

As SIRT1 is an aging-associated molecule and we have previously shown that the changes in diabetes are comparable to accelerated aging [26], we
investigated additional downstream alterations of miR-195 increase and SIRT1 downregulation in ECs and retina. We stained the cells with SA β-GAL (a lysosomal enzyme accumulated in senescent cell), an established marker for assessing cellular senescence [26, 47]. miR-195 antagonir transfection (not scrambled controls) prevented HG induced ECs aging, as evidenced by reduced β-GAL positivity (Figure 3.10a, b and Figure 3.2e, f). Furthermore, HRECs transfected with miR-195 mimic in NG caused cellular changes similar to HG (Figure 3.10a, b). On the contrary, adenoviral overexpression of SIRT1 in HG ameliorated such effects in the ECs (Figure 3.10a, b and Figure 3.6e, f).
Figure 3.10 miR-195 inhibition halts glucose-induced SIRT1 mediated cellular senescence in ECs. (a) Senescence associated SA β-GAL staining of HRECs in HG and NG following miR-195 mimic, antagonir and with Ad-SIRT1 transfection. Arrow indicates positive cell (blue). (b) Quantitation of SA β-GAL positivity, n=10 image/treatment. [Scale bar represent 100 µm for all micrographs. Inset = magnified image showing cytoplasmic SA β-GAL positivity. HRECs = human retinal microvascular endothelial cells. HG= 25 mmol/l glucose, NG= 5 mmol/l glucose, OSM= 25 mmol/l L-glucose. *= significantly different from NG, †= significantly different from HG.] (c) SA β-GAL staining of rat retinal sections following intravitreal miR-195 antagonir treatment, n=10 image. Arrow shows retinal inner nuclear layers. No counterstain. [Inset = magnified images of retinal microvessels. Scale bar represent 50 µm for all micrographs. 195= miR-195 mimics, 195(A)= miR-195 antagonirs. *= significantly different from control, †= significantly different from diabetic (Di) or Di+Scramble]

We expanded the study and investigated miR-195 mediated cellular changes at a later passage [26]. miR-195 antagonir transfection in HRECs at passage 3 showed reduction of cellular senescence along with increased MnSOD level with such treatment (Figure 3.5c-e). In parallel, retinal tissues stained with SA-βGAL showed increased positivity in the diabetic animals. Such positivity was significantly reduced with intravitreal miR-195 antagonir treatment in the retina, especially in ECs of the microvessels (Figure 3.10c). Scrambled miRNA treated diabetic animals lacked such improvements (Figure 3.10c).
3.4 Discussion

In this study we have shown a novel role of miR-195 in regulating hyperglycemia induced changes in microvessels in diabetic retinopathy. Using miR-195 antagonir transfection, we have demonstrated that diabetes-induced SIRT1 downregulation and subsequent functional consequences can be prevented by decreasing the availability of miR-195. We further verified the *in vitro* findings *in vivo*, by injecting miR-195 antagonir intravitreally in STZ induced type 1 diabetic rats, which showed such treatment prevented SIRT1 reduction in these tissues following 1 month of uncontrolled hyperglycemia, and normalized FN proteins levels in diabetes. Such role of miR-195 regulated, SIRT1 mediated FN regulation and aging-like changes, in glucose-induced ECs and retina of diabetic animals have not been reported earlier.

In diabetes, overproduction of superoxides by the mitochondrial electron transport chain in the ECs causes DNA damage, activating several transcription factors and altering multiple gene expressions [6, 9-11, 49]. We have demonstrated that glucose-induced SIRT1 mediated downregulation of MnSOD causes aging like changes in the ECs and retina in diabetes [26]. Such changes are also regulated by transcriptional coactivator and histone acetyl transferase p300 [26]. In this research, we have characterized another level of regulation of glucose-induced SIRT1 downregulation and its downstream effects through alteration of miR-195.
Several lines of evidence have recently been presented to demonstrate that miRNAs play a significant role in a large number of cellular processes [27-34]. Adding to such data, we have previously showed miR-200b & miR-146a plays an important role in diabetic retinopathy through VEGF and FN modulation respectively [42, 43]. Here we described the role of miR-195 causing SIRT1 downregulation in ECs, which also mediate aging-like changes, vascular permeability and FN upregulation in diabetes. We investigated the mechanisms at multiple levels of complexities. After the initial identification of miR-195 upregulation in the human retinal capillary ECs, we used miR-195 antagonir and mimic to identify in vitro biological significance. We further observed similar changes in human dermal microvascular ECs. We have also showed similar alterations and functional significance of this mechanism in an established animal model of diabetic retinopathy.

Several changes seen in chronic diabetic complications are similar to normal aging process [7-19]. However, such changes are accelerated in diabetes and we have demonstrated they are mediated by oxidative stress induced SIRT alterations [26]. Increased FN production has been demonstrated in aging [15-19]. Furthermore, augmented ECM protein such as FN production is a characteristic feature of diabetic retinopathy and is a result of EC dysfunction induced by hyperglycemia [7-14]. We and others have previously demonstrated glucose-induced increased FN synthesis in the ECs and in the retina in diabetes [7-14]. Such increased FN production can cause outside-in signaling and alteration of vasoactive factors [14, 44]. This is the first study to demonstrate
SIRT1 mediated FN regulations in diabetic retinopathy, which is modulated by specific miRNA. These findings were further confirmed by SIRT1 forced-expression in ECs, which showed reduced FN expression in high glucose. We have shown that miR-195 regulates SIRT1 mediated FN alteration, further establishing the importance of such mechanisms of tissue damage and potential therapeutic role in diabetic retinopathy.

To our knowledge, there is no previous work characterizing the role of miR-195 in diabetic retinopathy. Our findings are in keeping with previous reports regarding miR-195 where it was found to prevent palmitate-induced cardiomyocyte apoptosis and was elevated in liver and kidney of diabetic animals and in circulation of glucose intolerant patients [36-40]. It is to be noted that there are other SIRT1 targeting miRNAs which were not investigated. In keeping with such notion, in our array analysis, we noted that some SIRT1 targeting miRNAs were downregulated (Table 3.1). Exact significance of such changes is not clear and biological relevance remains to be established. Specific investigations are required to establish whether such alterations may represent counter-regulatory mechanism(s). Nevertheless, data from our study indicate that miR-195 may represent a major miRNA, modulating SIRT1 activity in the context of early diabetic retinopathy.

There are enormous evidences on the diverse role of miRNAs in various physiological and pathological conditions; and have been associated in many cellular processes including feedback loops for various signal transduction pathways [27-34]. Interestingly, in a model of tumour angiogenesis, miR-195 has
been shown to be decreased causing upregulation of VEGF [50]. Hence, it appears that molecular mechanisms regarding miRNAs regarding VEGF regulation may vary from tumoral to non-tumoral angiogenesis. It is also possible that in diabetic retinopathy, additional mechanisms such as miR-195 mediated reduced SIRT1 causing increased p300 and histone acetylation may lead to increased VEGF production [10, 26]. Hence a complex interaction of miRNAs and other epigenetic mechanism, such as histone acetylation may be involved in specific protein production. Other miRNAs, eg. miR200b has also been shown to regulate VEGF in diabetic retinopathy [42]. Nevertheless, as deregulation of miRNAs lead to a variety of diseases, miRNAs have the potential to be used as drugs. RNA based therapies are potentially advantageous due to their specificity. Therefore, targeting few miRNAs in combinational therapy holds the potential to prevent multiple gene expressions in a multifaceted disease like diabetic retinopathy.

3.5 References


Chapter 4

4  SIRT1 causes renal and retinal injury in diabetes through endothelin 1 (ET-1) and transforming growth factor beta 1 (TGF-β1)

Rokhsana Mortuza, Biao Feng and Subrata Chakrabarti. Department of Pathology, Schulich School of Medicine & Dentistry, University of Western Ontario, London ON Canada.

In this study our aim was to investigate the effect of SIRT1 overexpression in the organ damage in diabetic animals. We examined SIRT1 mediated regulation of ET-1 and TGF-β1 in the renal and retinal tissues of the transgenic mice with diabetes.
4.1 Introduction

Diabetes and its complications are a growing concern worldwide [1]. Number of people being diagnosed with diabetes in North America is increasing in an alarming rate [1]. Dysfunction of vascular endothelium caused by hyperglycemia in diabetes is the key initiating factor in the development of all chronic diabetic complications including diabetic nephropathy and retinopathy [2, 3]. A structural hallmark of chronic diabetic complication is vascular basement membrane thickening and renal mesangial expansion, due to an increased production of extracellular matrix (ECM) proteins such as fibronectin (FN) and collagen. Although other cells may take part in such process, endothelial cells (ECs) lining the blood vessels play a major role [2-5].

In diabetes glucose-induced increased oxidative stress activates several signaling pathways altering crucial gene expressions in the ECs [6, 7]. We and others have previously shown that endothelin-1 (ET-1) and transforming growth factor β1 (TGF-β1) are increased in circulation of patients and in organs of the animals with diabetes [2, 8-14]. ET-1 is a potent mitogen and vasoconstrictor that plays a major pathogenic role in diabetic complications by altering the blood flow, increasing vascular permeability and ECM protein production [8-13]. TGF-β1 is a cytokine belonging to a large family of regulatory proteins and in recent years has been evolved as one vital regulator of ECM protein production in diabetes [2, 8-
Increased TGF-β1 alters ECM protein synthesis and degradation, a delicate balance that is lost in diabetes causing fibrosis in the affected tissues [2, 14]. We have previously shown that diabetes-induced upregulated ET-1 and TGF-β1 levels causes increase production of ECM proteins in ECs and tissues of diabetic animals [2, 8-14]. Moreover, we have demonstrated transcriptional co-activator and histone acetyl transferase (HAT) p300 interacts with transcription factors NFκB and AP-1 to regulate the expression of these genes [15-19].

SIRTs (silent information regulator proteins) are a group of NAD dependent class III histone deacetylases (HDACs) that regulate epigenetic gene silencing. SIRTs deacetylase not only histones but also many proteins and transcription factors. SIRT1 is the leading enzyme in the SIRT family residing both in the cytoplasm and nucleus. SIRT1 has been found to play crucial role in cellular oxidative stress response, metabolism, differentiation, longevity and various diseases including diabetes [20-23]. SIRT1 being a deacetylase also has a regulatory relationship with p300 [20].

We investigated the role of SIRT1 and its relationship with p300 in regulating ET-1 and TGF-β1 and their possible downstream consequences in ECs and tissues of animals affected by diabetes. To this extent, we used human microvascular ECs (HMECs) for in vitro inspections of glucose-induced endothelial alterations. We further expanded the study using SIRT1 overexpressing transgenic mice in which we induced diabetes with streptozotocin (STZ) and examined their renal and retinal tissues.
4.2 Materials and methods

4.2.1 Cell culture

Dermal-derived human microvascular endothelial cell (HMEC) was obtained (Lonza, Walkersville, MD) and grown in endothelial cell basal medium 2 (EBM-2, Lonza, Walkersville, MD). Human embryonic kidney (HEK293A) cells were obtained from ATCC (Manassas, VA). We have previously described the culture conditions of these cells [24-27]. The glucose concentration in the growth medium was 5 mmol/L. No insulin was present in any media. All cells were maintained in a humidified atmosphere containing 5% CO₂ and at 37°C incubation. Cells were treated with normal glucose (NG, 5 mmol/L) or high glucose (HG, 25 mmol/L D-glucose) or osmotic control (OSM, 25 mmol/L L-glucose). All reagents were obtained from Sigma Chemicals (Sigma, Oakville, ON) unless specified otherwise. Experiments were conducted with 6-10 biological replicates.

4.2.2 Animal experiments

SIRT1 transgenic mice (C57BL/6-Actb\textsuperscript{tm3.1(Sirt1)Npa/J}), male, 8 weeks old were obtained (Jackson Laboratory, Bar Harbor, ME) and genotyped with Real Time RT-PCR. Diabetes was induced by single intra-peritoneal injection of streptozotocin (STZ, 65 mg/kg in citrate buffer). Age and sex matched mice were
used as controls and received equal volume of citrate buffer. Diabetes was defined in the animals with blood glucose level >20 mmol/L on two consecutive days (Abbott Diabetes Care, Saint-Laurent, QC). The animals were fed on a standard rodent diet with water ad libitum and sacrificed after 2 months following the development of diabetes ($n = 10$ /group). Urinary micro-albumin (Exocell, Philadelphia, PA) was measured as per the kit instructions. Retina and kidney tissues were collected and snap frozen in liquid nitrogen for histology. All tissues were stored at −80°C until further analysis.

All animal experiments were performed in accordance with regulations specified by the Canadian Council of Animal Care. All protocols were approved by the University of Western Ontario Animal Care and Veterinary Service. The investigation was in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publ. no. 85-23, revised 1996).

4.2.3 SIRT1 enzyme activity assay

Whole cell lysate were collected following treatment with RIPA buffer (Millipore, MA) with addition of protease inhibitor (Roche, Laval, QC). The total protein concentrations of the samples measured using a commercially available BCA protein assay kit (Pierce, Rockford, IL). Enzyme assay for SIRT1 activity was performed as per the manufacturer instructions (Sigma, Oakville, ON). The assay procedure is based on a two-step enzymatic reaction. In the first step SIRT1 deacetylates the substrate that contains an acetylated lysine side chain. In
the following step the deacetylated substrate is cleaved by the developing solution and a highly fluorescent group is released. This measured fluorescence is directly proportional to the deacetylation activity of the enzyme of the sample. In short 35 µl of assay buffer was added to each well following which 10 µl of sample and 5 µl of NAD$^+$ was added. Adding to that 10 µl of SIRT1 substrate solution was added and incubated at 37°C for 30 minute. Following this 5 µl of developing solution was added and plate was incubated at 37°C for 10 minute. The plates were read with a fluorescent spectrophotometer (Biotek, Winooski, VT) at excitation 340 nm and emission 430 nm.

4.2.4 mRNA extraction and cDNA synthesis

RNA from cells was isolated using TRIZOL™ (Invitrogen, Burlington, ON) reagent as established at our laboratory. Briefly RNA was extracted with chloroform followed by centrifugation to separate the sample into aqueous and organic phases. The RNA was recovered from the aqueous phase by isopropyl alcohol precipitation and suspended in DEPC water. RNA concentration was assessed on a spectrophotometer (Gene Quant-Pharmacia Biotech, Cambridge, MA). First-strand cDNA was made by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer instruction. The resulting products were stored at −20°C until further analysis by Real Time RT-PCR.
4.2.5 mRNA analysis with Real Time RT-PCR

Real time RT-PCR was performed in LightCycler™ (Roche Diagnostics, Laval, QC) to quantify the mRNA expression of SIRT1 and using the Qiagen One Step RTPCR kit (detection platform SYBR Green I). All of the primers were either ordered or custom made from Sigma (Table 4.1). For a final reaction volume of 20 µl, the following reagents were added: 4.4 µl of H₂O, 10 µl of SYBR (Sigma, Oakville, ON), 1.6 µl of MgCl₂, 1 µl forward/reverse primer and 2 µl of cDNA. To optimize the amplification of the genes, melting curve analysis (MCA) was used to determine the melting temperature (Tₘ) of specific products and primer dimers. According to the Tₘ value of specific products for respective genes, an additional step (signal acquisition step, 2–3 °C below Tₘ) was added following the elongation phase of RT-PCR. The data were normalized to housekeeping gene 18sRNA to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures.

Table 4.1 Oligonucleotide sequences for Real Time RT-PCR.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen Iα(I) (human)</td>
<td>GAGGGCCAAGACGAAGACATC CAGATCACGTCATCGCACAAC</td>
</tr>
<tr>
<td>ET-1 (human)</td>
<td>AAGCCCTCCAGAGAGCGTTAT CGAAGGTCTGTCACCAATGT 6FAM-TGACC CAAACCGAG-GBNFQ</td>
</tr>
<tr>
<td>ET-1 (mice)</td>
<td>TTAGCAAGACCATCTGTGTG GAGGGCTCATTCGCAATG</td>
</tr>
<tr>
<td>SIRT1 (human/mice)</td>
<td>GCAGATTAAGTAGCCGCTTG TCTGGCATGTCCACTATCA</td>
</tr>
<tr>
<td>P300 (human)</td>
<td>GGAGCTAATGGGCTGGT GTATTGGGAGAAGTCAAGCTC</td>
</tr>
<tr>
<td>P300 (mice)</td>
<td>AGGCAGAGTAGACAGTGAA</td>
</tr>
</tbody>
</table>
4.2.6 Adenoviral overexpression of SIRT1 and SIRT1 gene silencing

SIRT1 adenovirus was obtained (ABM, Richmond, BC) and amplified in HEK293A cells. HMECs were transfected with the adenovirus as described earlier [24]. To silence SIRT1, transfection of small interfering RNA (siRNA) was performed using N-TER nanoparticle siRNA transfection system (Sigma, Oakville, ON) as described previously [20, 27]. Transfection efficiency was assessed by Real Time RT-PCR.

4.2.7 p300 gene silencing and p300 overexpression

siRNA was used to specifically silence the p300 expression in ECs as described by us previously [20]. P300 overexpression was achieved with expression vectors (generously provided by Dr. Joan Boyes, The Institute of Cancer Research, London, UK) containing the wild-type p300 (pCI-p300) and its HAT-deletion mutant (pCI-p300 HATΔ1472–1522) as described earlier [15]. Transfection efficiency was assessed by measuring p300 mRNA expression by Real Time RT-PCR.
4.2.8 ET-1, TGF-β1 & FN ELISA

ET-1 ELISA (Enzo, Farmingdale, NY) and TGF-β1 ELISA (eBioscience, San Diego, CA) was done on cell and tissue lysate as per the manufacturer instructions. FN ELISA (abcam, Toronto, ON) was done on tissue lysate as per the manufacturer instructions. The total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL). The plate was read at 450 nm using a plate reader (Multiskan, Thermofisher, Toronto, ON).

4.2.9 Total ROS/RNS assay

Total ROS/RNS (reactive oxygen species/reactive nitrogen species) assay (Cell Biolab, San Diego, CA) on tissue lysate was measured as per the manufacturer instructions. The plates were read with a fluorescent plate reader (Biotek, Winooski, VT) at excitation 480 nm and emission 530 nm.

4.2.10 Endothelial permeability assay

HMECs were seeded onto inserts (1-mm pores) in 24-well plates, with or without incubation with specific reagents for 24 h, and were tested for vascular permeability using the In Vitro Vascular Permeability Assay Kit (Millipore, Billerica, MA) according to the manufacturer’s instructions.

4.2.11 Western blotting
The western blot analysis was conducted according to the standard protocol established at our lab [15] using acetylated H3 lysine (Epigentek, Farmingdale, NY) & β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

4.2.12 Statistical analysis

Data are expressed as mean ± SEM, normalized to controls. The statistical significance of the results was analyzed by one way or two way ANOVA followed by Tukey’s HSD post hoc correction and the two tailed Students t test as appropriate (PASW Statistics 18, IBM, Canada). A p value < 0.05 was considered statistically significant.

4.3 Results

4.3.1 SIRT1 regulates ET-1 and TGF-β1 expressions in the ECs in high glucose.

As microvascular ECs are the major target of glucose-induced organ damage, we investigated HMECs exposed to 5 (NG) and 25 (HG) mmol/L glucose for 72 hrs. These concentrations are based on our previous dose dependent analysis of SIRT1 levels [20, 24]. Quantitative Real Time RT-PCR analysis of the ECs showed significant upregulation of ET-1 and TGF-β1 mRNA
levels (Figure 4.1A,C) and downregulation of SIRT1 mRNA levels (Figure 4.1E) with HG compared to NG. No effects were seen following incubation with osmotic control (25mmol/L of L-glucose, OSM) (Figure 4.1A,C,E). These findings were further reflected in ET-1 and TGF-β1 protein analysis and SIRT1 enzyme activity analysis, which showed a marked increase of ET-1 and TGF-β1 protein levels (Figure 4.1B,D) and reduced SIRT1 enzyme activity levels (Figure 4.1F) in these cells following HG treatment (compared to NG).

Figure 4.1 SIRT1 regulates ET-1 and TGF-β1 expression in ECs. Human Microvascular Endothelial cells (HMECs) exposed to 25 mmol/L (HG) glucose compared to 5 mmol/L glucose (NG) showed increased (A) ET-1 mRNA, (C)
TGF-β1 mRNA and reduced (E) SIRT1 mRNA expressions. Such changes were not seen when the cells were incubated with 25 mmol L-glucose (osmotic control, OSM). Transfection of endothelial cells with Ad-SIRT1 (but not the null vector) increased (F) the enzyme activity in treated cells and normalized glucose-induced upregulation of (A, C) ET-1 and TGF-β1 mRNA and (B, D) protein levels. Glucose-like effects (ET-1, TGF-β1 upregulation) were further seen when cells in NG were transfected with SIRT1 siRNA (A, C, E) but not with scramble siRNA. Such knockdown of SIRT1 significantly reduced (F) the enzyme activity in NG and increased the (B) ET-1 and (D) TGF-β1 protein levels in these cells. (*= significantly different from NG, ‡= significantly different from HG, mRNA levels are expressed as a ratio of 18s. All data were normalized to NG).

We then proceeded to explore any relationship, between the observed glucose-induced downregulation of SIRT1 and upregulation of ET-1 and TGF-β1 expressions in these ECs. For this purpose, we analysed the ECs following transfection with SIRT1 adenovirus (Ad-SIRT1). Transfection efficiency was measured with Real Time RT-PCR and confirmed with SIRT1 enzyme analysis (Figure 4.1E,F). We noted the Ad-SIRT1 transfection significantly increased the SIRT1 mRNA and enzyme levels in the ECs in HG (Figure 4.1E,F). Most importantly, this transfection caused a significant reduction in ET-1 and TGF-β1 mRNA and protein levels in these ECs in HG (Figure 4.1A-D). ECs transfected with null vector did not show such change (Figure 4.1A,C).
In addition, SIRT1 knockdown with siRNA (efficiency in Figure 4.1E,F) in NG mimicked the effects of HG, causing an increase in ET-1 and TGF-β1 levels in these ECs (Figure 4.1A-D). Conversely, ECs transduced with scramble siRNA did not show these changes (Figure 4.1A-D). These results demonstrated a SIRT1 mediated regulation of ET-1 and TGF-β1 in the ECs in hyperglycemia.

4.3.2 SIRT1 regulates ET-1 and TGF-β1 expressions via p300.

In order to investigate the mechanism of such SIRT1 mediated ET-1 and TGF-β1 regulation we looked at transcriptional co-activator p300, as we previously have found SIRT1 and p300 balance each other [20]. In keeping with the earlier findings we noted SIRT1 overexpression with adenovirus in 25 mmol/L glucose normalizes glucose-induced augmented p300 mRNA levels (Figure 4.2A). On the contrary, SIRT1 knockdown in NG with siRNA caused an increase in p300 mRNA levels (Figure 4.2A). Furthermore, p300 overexpression in these cells in NG reduced the SIRT1 mRNA levels (Figure 4.2B,C). These changes were not seen with null vector, scramble siRNA and p300 mutant transfections. These results re-established a regulatory and balancing role of SIRT1 and p300 on each other in ECs.
Figure 4.2 SIRT1 regulates ET-1 and TGF-β1 via p300. HG caused increased (A) p300 mRNA and reduced (B) SIRT1 mRNA levels in ECs. (A) Ad-SIRT1 transfection in HG prevented p300 upregulation and SIRT1 knockdown with siRNA in NG upregulated p300 mRNA levels. (B) p300 siRNA transfection in HG (efficiency in A) normalized SIRT1 expression. Furthermore p300 overexpression in NG (efficiency in A) reduced SIRT1 mRNA levels. (C, D) HG induced ET-1 and TGF-β1 upregulation was abrogated with p300 siRNA (not with scramble) transfection. p300 overexpression in NG upregulated the expressions of these transcripts. (E, F) Rescue experiment showing upregulation of ET-1 and TGF-β1 mRNA levels in NG with SIRT1 knockdown can be resolved with subsequent p300 knockdown in the ECs. (NG= 5 mmol/L glucose, HG= 25 mmol/L glucose,
OSM = osmotic control, *= significantly different from NG, ‡= significantly different from HG, mRNA levels are expressed as a ratio of 18s. All data were normalized to NG).

To confirm the downstream effects of such changes we examined the cells following knockdown of p300 with siRNA (efficiency in Figure 4.2B) in HG. Such treatment not only normalized the SIRT1 mRNA levels (Figure 4.2C) but also reduced the ET-1 and TGF-β1 mRNA levels in HG (Figure 4.2D,E) compared to scrambled controls. In addition, p300 overexpression in NG lead to an increased mRNA levels for both ET-1 and TGF-β1 mimicking the HG treatments (Figure 4.2D,E). P300 mutant transfected cells did not show such change. These results further establish that SIRT1 regulates ET-1 and TGF-β1 through p300.

We further conducted a rescue experiment to confirm such pathway. We first knocked down SIRT1 with siRNA in NG in these ECs, following which we transfected the cells with p300 siRNA. Transfection efficiency assessed with Real Time RT-PCR showed reduction of both SIRT1 (88%) and p300 (61%) mRNA levels in the treated cells. We noted such transfection prevented the ET-1 and TGF-β1 mRNA upregulation in the treated cells (Figure 4.2F).
4.3.4 SIRT1 overexpression prevent glucose-induced increased endothelial permeability and collagen Iα(I) expression.

To investigate the functional consequences of SIRT1 mediated ET-1 and TGF-β1 regulation we conducted trans-endothelial permeability assay as increased endothelial permeability is a characteristic alteration in early diabetic microangiopathy. Data from the In vitro permeability test showed HG significantly increases ECs permeability and Ad-SIRT1 transfection prevented such leakage in these cells (Figure 4.3A,B). P300 siRNA transfection in HG also had similar effect showing reduction in glucose-induced leakage whereas; SIRT1 knockdown or p300 overexpression in NG both significantly increased the ECs permeability mimicking the HG (Figure 4.3A,B).
Figure 4.3 SIRT1 overexpression prevents glucose-induced increased endothelial permeability and collagen Iα(I) expression. A) Duration dependent endothelial permeability and (B) end point analysis showed, HG induced increased endothelial permeability was prevented by Ad-SIRT1 transfection or p300 siRNA treatment. SIRT1 siRNA or p300 overexpression in NG caused increased permeability by these cells mimicking the effects of HG. (C) HG caused an increase in Collagen Iα(I) mRNA expression in the ECs which was prevented by Ad-SIRT1 or p300 siRNA transfection. Opposingly, SIRT1 knockdown or p300 overexpression both lead to an upregulation of Collagen Iα(I) mRNA levels in NG. (D) Western blot analysis of acetylated histone (Ac-H3K9/14) shows Ad-SIRT1 or p300 siRNA transfection reduced HG-induced
increased histone acetylation in HMECs. On the other hand SIRT1 siRNA or p300 overexpression increased such acetylation in NG in these ECs. (NG= 5 mmol/L glucose, HG= 25 mmol/L glucose, * = significantly different from NG, †= significantly different from HG. HMECs= human microvascular endothelial cells. mRNA levels are expressed as a ratio of 18s. All data were normalized to NG).

Previously we have shown SIRT1 overexpression prevented glucose-induced FN upregulation in the ECs [27]. We further examined collagen Iα(l) expression levels following HG exposure. With Ad-SIRT1 transfection, we observed a normalization of glucose-induced upregulation of collagen Iα(l) mRNA levels (Figure 4.3C). HG induced increased collagen Iα(l) mRNA expression was further prevented by p300 siRNA transfection (Figure 4.3C). In parallel, SIRT1 knockdown or p300 forced expression both caused an upregulation of collagen Iα(l) mRNA levels in NG (Figure 4.3C). Moreover, we conducted western blot analysis of acetylated histone (Ac-H3K9/14) to see the functional consequences of p300 and SIRT1 alteration. Such analysis showed HG-induced increased Ac-H3K9/14 was reduced with Ad-SIRT1 or p300 siRNA transfection (Figure 4.3D). Opposingly, SIRT1 siRNA or p300 forced expression in NG increased such acetylation in the ECs. These experiments together provide further evidence that SIRT1 regulate ET-1 and TGF-β1 through p300.
4.3.5 SIRT1 regulate ET-1 and TGF-β1 expressions in the kidney and retina of diabetic mice.

Following the establishment that SIRT1 regulated ET-1 and TGF-β1 in ECs, we expanded our study to investigate whether the mechanisms seen in these cells was important in the development of renal and retinal microangiopathy in a well-established animal model. STZ-induced diabetic mice showed hyperglycemia (diabetics 21.91±4.58 mmol/L vs controls 7.38±0.91 mmol/L, P<0.001) and reduced body weight (diabetics 22.80±1.40 g. vs controls 30.25±2.36 g., P<0.001). Initially we performed Real Time RT-PCR analysis of the renal and retinal tissue from these mice following 2 months of uncontrolled diabetes. We have previously shown that diabetes induced increased ECM protein and vasoactive factor expression is established at this time point [19, 20, 24-27]. mRNA analysis showed diabetic mice had significant downregulation of SIRT1 expressions in the kidneys and retinas (Figure 4.4A,B). This finding was further reflected in the SIRT1 enzyme activity levels (Figure 4.4C,D). Moreover, these mice showed significant upregulation of p300 mRNA levels in the renal and retinal tissues which were abrogated in SIRT1 overexpressing diabetic mice (Figure 4.4E,F). Western blot analysis further showed SIRT1 overexpression caused reduction in histone acetylation (Ac-H3K9/14) in these tissues demonstrating the downstream functional consequences of p300 alteration in these tissues (Figure 4.4G).
Figure 4.4 SIRT1 overexpressing diabetic mice shows reduced p300 expressions in the kidney and retina. Transgenic mice with SIRT1 overexpression (SIRT1 Tg) showed increased expressions of (A, B) SIRT1 mRNA levels and (C, D) enzyme activity in the kidneys and retinas of diabetic and control animals. Such overexpression averted diabetes-induced increased (E, F) p300 mRNA levels and (G) histone acetylation (Ac-H3K9/14) in these tissues. (Co= Control, Di=Diabetic, *= significantly different from control, †= significantly different from Diabetic. mRNA levels are expressed as a ratio of 18s. All data were normalized to Co).
We further looked at ET-1 and TGF-β1 levels in these mice. SIRT1 overexpressing diabetic mice showed significantly reduced level of ET-1 and TGF-β1 mRNA levels compared to diabetic mice in both tissues (Figure 4.5A-D). We further confirmed these results using ELISA which showed diabetes-induced increased ET-1 and TGF-β1 protein levels in the renal and retinal tissues are significantly reduced with SIRT1 overexpression in these tissues (Figure 4.5E-H).

Figure 4.5 SIRT1 regulates ET-1 and TGF-β1 expressions in kidney and retina of diabetic mice. SIRT1 overexpression was protective against diabetes-induced (A, B) ET-1 and (B, C) TGF-β1 mRNA upregulation in the kidneys and retinas of mice. In keeping with the mRNA expressions SIRT1 transgenic mice (SIRT1 Tg)
with diabetes showed reduced (E, F) ET-1 and (G, H) TGF-β1 protein levels in these tissues compared to the wild type diabetic mice. (Co= Control, Di= Diabetic, *= significantly different from control, ‡= significantly different from Diabetic. mRNA levels are expressed as a ratio of 18s. All data were normalized to Co).

### 4.3.6 SIRT1 overexpression prevents diabetes-induced micro-albuminuria and FN upregulation in tissues.

To examine the downstream consequence of SIRT1 mediated alteration of ET-1 and TGF-β1 in diabetes, we investigated urinary micro-albumin levels to assess renal function in these mice. Diabetic mice showed increased micro-albuminuria compared to controls indicating poor kidney function in these animals (Figure 4.6A). SIRT1 overexpression significantly reduced micro-albuminuria and diabetes-induced increased collagen Iα(I) mRNA expression by the transgenic diabetic animals (Figure 4.6A,B). Furthermore in the renal and retinal tissues of the diabetic animals such overexpression reduced diabetes-induced increased FN protein and total ROS/RNS levels (Figure 4.6C,D). These results suggest that SIRT1 has a protective role against diabetes-induced renal and retinal damages.
Figure 4.6 SIRT1 transgenic mice shows improved renal function and reduced oxidative stress and FN upregulation with diabetes. (A) SIRT1 overexpression prevented diabetes-induced micro-albuminuria in mice. Furthermore such overexpression averted diabetes-induced increased (B) collagen Iα(I) mRNA expression (C) FN protein and (D) total ROS/RNS levels in the renal and retinal tissues. (SIRT1 Tg= SIRT1 transgenic mice, Co= Control, Di= Diabetic, *= significantly different from control, †= significantly different from Diabetic. mRNA levels are expressed as a ratio of 18s. All data were normalized to Co).
4.4 Discussion

In this study we have shown that SIRT1 regulates glucose induced overexpression of ET-1 and TGF-β1 in the ECs. We have also shown that SIRT1 regulates ET-1 and TGF-β1 levels in the kidneys and retinas of diabetic animals. In addition we have demonstrated such processes are regulated through transcriptional co-activator p300. Using adenoviral overexpression of SIRT1 and knockdown of SIRT1 with siRNA, we have directly demonstrated that glucose-induced ET-1 and TGF-β1 upregulation can be markedly reduced by increasing the availability of SIRT1. In the kidneys and retinas of SIRT1 overexpressing transgenic mice we have further established the existence of such regulation in a STZ-induced type 1 model of diabetes. To be best of our knowledge, such SIRT1 mediated regulation of ET-1 and TGF-β1 in diabetic complications have not been shown earlier.

In diabetes, high oxidative stress due to hyperglycemia causes DNA damage and activates several transcription and vasoactive factors altering crucial gene expressions [2, 6, 7]. Here we describe the role of SIRT1 causing protection against ET-1 and TGF-β1 induced endothelial damage in hyperglycemia. We initially demonstrated SIRT1 downregulation in parallel to ET-1 and TGF-β1 upregulation in glucose exposed ECs. Then, we directly demonstrated its functional significance in terms of endothelial permeability and collagen Iα(I) expression and the relationship with p300 in these cells. In keeping with our earlier findings here we have shown that acting in opposing manner, p300 and SIRT1 regulate each other in hyperglycemia. We further showed the
functional significance of this mechanism in an animal model of diabetic microangiopathy.

Although there is no previous work showing SIRT1 mediated regulation of ET-1 or TGF-β1 expressions, SIRT1 has been found to regulate downstream effectors of TGF-β1 via deacetylation of SMAD7 [28]. This study demonstrates a SIRT1 mediated regulation of TGF-β1 expressions which signals through SMADs. One of the major glucose-induced endothelial dysfunctions includes augmented ECM protein production [2-14]. Increased ECM proteins are deposited in the tissue which is manifested as structural changes such as basement membrane thickening, mesangial expansion etc. We and others have demonstrated glucose-induced increased collagen and FN synthesis in the endothelial cells and in the retina, kidney and heart in diabetes [2-19]. We have also demonstrated that glucose induced ECM protein upregulation is mediated through p300-dependent histone acetylation and p300 binds to the promoter regions of ET-1 and FN genes [15-19]. In this study we have shown one further step of p300 mediated regulation of ET-1 and TGF-β1 in ECs and tissues of diabetic animals. We have shown that by modulating SIRT1 we can prevent such glucose induced damages.

ET-1 and TGF-β1 are two important cytokines playing key roles in tissue damage in diabetic complications. It is of interest to note that such SIRT1 induced regulation is mediated through p300. P300 as a transcriptional co-activator is potentially capable of altering multiple transcripts [15, 18, 19].
study shows it is possible to target either SIRT1 or p300 to prevent diabetes-induced alterations. We have previously shown that p300 is regulated by miR-200b and it regulates miR-146a [24, 26]. Additionally we have shown that miR-195 regulates SIRT1 expression [27]. In this assumption we showed a ying-yang relationship between p300 and SIRT1. Hence a complex web connecting multiple such epigenetic processes involving acetylators, deacetylators, miRNAs and other undetermined epigenetic process may ultimately dictate expression of specific transcripts. It is also possible that SIRT1 may influence several other inflammatory cytokines which are of importance in the context of nephropathy and retinopathy and other chronic diabetic complications. However, such roles of SIRT1 in these processes need to be established in future through specific experiments.

The pathogenic mechanisms leading to chronic diabetic complications are complex. Several vasoactive and growth factors are simultaneously activated in response to hyperglycemia and an intricate interplay occurs among such factors [2-20, 24-26]. The findings of this study indicate that SIRT1 is a protective molecule in diabetic nephropathy and retinopathy. Loss of SIRT1 production represents a key event in the pathogenesis of chronic diabetic complications. Hence SIRT1 holds the potential to be used as a drug target for the treatment of chronic diabetic complications.
4.5 References


Chapter 5

5  Thesis summary and future directions

Some content of this chapter have been published in the following papers:


As per the journal policies, authors retain ownership of the copyright for their content. No permission is required from the publishers.
5.1 Overall findings

The major contributor of vascular diabetic complications is damage to the ECs [1-22]. Hyperglycemia induced increased oxidative stress causes ECs to secrete large amount of vasoactive factors, growth factors and cytokines [1-22]. These factors lead to structural and functional changes in the ECs such as increased permeability and basement membrane thickening [1-22]. We and others have previously shown that glucose-induced increased oxidative stress alters expression of several genes ultimately causing increased production of ECM proteins by the ECs such as collagen and FN [1-22]. Interestingly some of these above mentioned changes are also seen in normal vascular aging [23-37]. Hence in this study we asked whether hyperglycemia in diabetes accelerates aging-like process in ECs and tissues of diabetic animals (chapter 2).

To create an aging-like *in vitro* process, we continuously propagated ECs of three different origins (HMEC, BREC and HUVEC) in high and normal glucose and examined for signs of cellular senescence and oxidative stress. We found oxidative stress in hyperglycemia accelerated aging-like changes in ECs. However such processes were variable among the various ECs. Microvascular ECs were more susceptible to glucose induced rapid aging compared to large vessel ECs. As we looked into the mechanism behind such aging process we
found SIRT enzymes were reduced in the aged cells. Glucose-induced increased oxidative stress caused reduction of mitochondrial antioxidant enzymes in a SIRT1 and FOXO1 mediated pathway. Moreover level of SIRT1 was negatively regulated by p300, a HAT and transcriptional co-activator. Using activators of SIRT1 we were able to effectively prevent such aging-like changes in the ECs. In addition, examination of kidneys and retinas of diabetic mice showed that diabetes causes rapid aging in these tissues. Such changes were associated with increased oxidative stress and reduced SIRT1 enzyme levels. Overall we have found an important role played by SIRTs in such process. We showed an important mechanistic pathway of oxidative stress and aging in HG, mediated through FOXO1 and regulated by SIRT1 and p300 (chapter 2).

Next we investigated regulation of SIRT1, specially by miRNAs (chapter 3). miRNAs are post transcriptional regulators of genes. miRNAs are conserved non-coding RNAs and are highly specific in nature, hence holds tremendous potential to be used as therapeutic targets. Several miRNAs have been found to regulate SIRT1 in various physiological and pathological conditions. In this study we examined alteration of SIRT1 targeting miRNAs in the retinal tissues of animals with diabetes.

Our initial microarray analysis showed miR-195, a SIRT1 targeting miRNA was significantly overexpressed in the retinas of diabetic animals. We then validated such findings in two different type of microvascular ECs. This was associated with reduced SIRT1 levels in these cells. We then examined the
possible miR-195 mediated regulation of SIRT1 at multiple levels of complexities in the context of diabetic retinopathy.

We used miR-195 antagonim, mimic and adenovirus with SIRT1 overexpression to identify in vitro biological significance. We found miR-195 antagonim transfection significantly upregulated SIRT1 levels in the ECs. Such transfection further prevented SIRT1 mediated aging changes and reduced glucose-induced increased FN levels in the ECs. SIRT1 overexpression in high glucose showed similar effect whereas miR-195 mimic transfection in NG mimicked the high glucose effect. We also showed similar alterations and its functional significance in an animal model of diabetic retinopathy. By injecting miR-195 antagonim intravitreally in STZ induced type 1 diabetic rats, we showed such treatment prevented SIRT1 reduction in these tissues following 1 month of uncontrolled hyperglycemia. Such treatment also reduced diabetes induced increased vascular permeability and normalized FN proteins levels. Overall in this study we have shown a novel role of miR-195 regulating hyperglycemia induced changes through SIRT1 in microvessels in diabetes. Data from this study indicate that miR-195 may represent a major miRNA, modulating SIRT1 activity in the context of early diabetic microangiopathy.

In order to explore the role of SIRT1 further and to examine whether such mechanisms are universal in other organs affected by diabetic complications, we investigated transgenic mice with SIRT1 overexpression (chapter 4). We induced diabetes in these mice with STZ injection and examined their kidney and retinal
tissues, for possible alteration of genes which are major players of diabetic microangiopathy. We found these transgenic mice had significantly reduced levels of ET-1 and TGF-β1, two major factors of vascular pathogenesis, in the renal and retinal tissues. This finding intrigued us to investigate a possible SIRT1 mediated regulation of ET-1 and TGF-β1 in diabetes.

Using adenoviral overexpression of SIRT1, we found that glucose-induced ET-1 and TGF-β1 upregulation can be markedly reduced by increasing the availability of SIRT1. Such treatment also corrected glucose-induced increased endothelial permeability and increased FN, collagen expressions. Moreover we found such SIRT1 mediated ET-1 and TGF-β1 regulation is mediated through transcriptional co-activator p300. We further found similar relationships in the SIRT1 transgenic mice with diabetes. These findings established that SIRT1 can provide protection against ET-1 and TGF-β1 induced endothelial damage in hyperglycemia. In keeping with our earlier findings we found again that p300 and SIRT1 regulate each other in hyperglycemic conditions and by modulating any of these we can prevent some glucose induced SIRT1 mediated damages.

Together the findings of this research show a novel role played by SIRT1 in diabetic microangiopathy. Data from this study indicate that SIRT1 is a protective molecule in diabetic vascular complications and holds the potential to be used as a therapeutic target for the treatment of diabetes.
5.2 Limitations and future directions

There is no study without limitations. In this study we have mainly focused on SIRT1 mediated alterations in ECs and renal and retinal tissues in diabetes. Whether similar changes occur in other organs affected by diabetes such as brain, liver, heart etc were not examined and needs further investigation. Furthermore, we have not looked in detail the role of other SIRTs (2-7) in endothelial dysfunction. As mitochondrial oxidative stress is the root cause of endothelial damage in diabetes, mitochondria specific SIRTs such as SIRT 3, 4, 5 may play key role in such process and needs further investigation.

The present study was performed on animals with type 1 diabetes. Future studies need to be done to see the effect of SIRT1 alteration in type 2 and gestational diabetes. In chapter 3 we examined the regulation of SIRT1 with miR-195 in the context of diabetic retinopathy. We have seen miR-195 inhibitor has some protective benefit in such disease process mediated through SIRT1. More work is needed to examine if such beneficial effects are present in other target organs of diabetic complications.

Although we have done several animal studies, a lot of our work is based on cultured ECs. ECs in cultured in vitro conditions is much simpler an environment than in vivo, which has more interaction between ECs, ECM proteins and other cells (smooth muscle cells, immune cells, fibroblasts etc). Furthermore though rodent models share a large percentage of their DNA with
humans significant differences exist. These factors should be considered before translating the current study to human.

Several SIRTs share molecular targets and affect common pathogenic pathways. To what extent SIRTs possess unique versus redundant functions and the degree to which crosstalk exists between these enzymes are some of the key questions that need to be systematically investigated. Questions such as to what extent activation of all SIRTs occur as a consequence of increased cellular NAD+ levels or whether different SIRTS have opposing roles in a disease condition (such as cancer) need to be investigated. We further need to examine possible consequences of long term increase in SIRT activity in vivo, before considering it to be a viable therapy. Although overexpression of SIRTs in some specific contexts have been found to confer health benefits and increase longevity, in general it is unknown whether long-term sustained increase of SIRT activity may have any deleterious consequences or not.

Finally although recently some studies investigating posttranslational modifications of SIRTs are emerging, further mechanistic studies on physiological regulation of SIRT needs to be done to gain insights into its function and features so that it may be used as a specific drug target.
5.3 References


Appendix-Experimental methods and materials

Cell culture

Bovine retinal microvascular endothelial cells (BRECs) were obtained from VEC Technologies (Rensselaer, NY) and were grown in a defined endothelial cell growth medium (MCDB-131 complete). The cells were passaged in 12 well plate (Corning, Acton, MA) coated with fibronectin (Sigma, St. Louis, MO). Dermal-derived human microvascular endothelial cell (HMEC) was obtained from Lonza, Inc. (Walkersville, MD) and human retinal microvascular endothelial cells (HRECs) were obtained from Olaf Pharmaceuticals (Worcester, MA). HRECs and HMECs were grown in endothelial cell basal medium 2 (EBM-2, Lonza, Walkersville, MD). Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Rockville, MD, USA), were cultured in endothelial cell growth medium (EGM). Human embryonic kidney (HEK293A) cells were obtained from ATCC (Manassas, VA) and cultured as per the manufactures protocol. The glucose concentration in the growth medium was 5 mmol/l. No insulin was present in any media. All cells were maintained in a humidified atmosphere containing 5% CO2 and at 37°C incubation [1-7]. Reagents were obtained from Sigma Chemicals (Sigma, Oakville, ON) unless specified otherwise.

For the long term continuous exposure to glucose, ECs were cultured in 12 well plates (Corning, Acton, MA) and treated with 5mM glucose (NG) or 25mM
glucose (HG, D-glucose) or osmotic control (OSM, 25 mM L-glucose). Upon confluence cells were propagated & maintained in the same treatment condition until they stopped proliferating completely. During each passage subculture cells from each treatment group were stained for SA β-gal and collected for RNA analysis. Cells were monitored daily and images taken for morphological and growth analysis. To test the effect of SIRT activation on accelerated aging in diabetes, cells were treated with 10 μM resveratrol (Sigma, Oakville, ON) dissolved in ethanol or with 25 μM BML278 (ENZO, Farmingdale, NY) in DMSO for 72 hr. To investigate the effect of FOXO1 inhibition, cells were treated with 0.1 μM FOXO1 inhibitor AS1842856 (Millipore, Billerica, MA) in DMSO similarly [3].

Cell lysates were collected with RIPA (Millipore, Billerica, MA) buffer with protease inhibitor (Roche, Laval, QC) for total ROS/RNS, MnSOD, ET-1, TGF-β1, LDH, SIRT1 analysis. Total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL). All experiments were conducted with 6-10 biological replicates [3].

**Extraction of mRNA and cDNA synthesis**

RNA from cells was isolated using TRIZOL™ (Invitrogen, Burlington, ON). Then RNA was further extracted with chloroform followed by centrifugation to separate the sample into aqueous and organic phases. The RNA was recovered from the aqueous phase by isopropyl alcohol precipitation and suspended in DEPC water. RNA concentration was assessed on a spectrophotometer (Gene
First-strand cDNA was made by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) as per the manufacturer instruction [1-7]. The resulting products were stored at −20°C until further analysis by Real Time RT-PCR.

**mRNA analysis with quantitative Real Time RT-PCR**

Real time RT-PCR was performed in LightCycler™ (Roche Diagnostics, Laval, QC) to quantify the mRNA expression of SIRT(1-7), hTERT, p300, FN, ET-1, TGF-β1 and CollagenIα(I) using the Qiagen One Step RT-PCR kit (detection platform SYBR Green I). All of the primers were either ordered or custom made from Sigma. For a final reaction volume of 20 µl, the following reagents were added: 4.4 µl of H$_2$O, 10 µl of SYBR (Sigma, Oakville, ON), 1.6 µl of MgCl$_2$, 1 µl forward/reverse primer and 2 µl of cDNA. To optimize the amplification of the genes, melting curve analysis (MCA) was used to determine the melting temperature ($T_m$) of specific products and primer dimers. According to the $T_m$ value of specific products for respective genes, an additional step (signal acquisition step, 2–3 °C below $T_m$) was added following the elongation phase of RT-PCR. The additional step in the PCR reactions is allowed for signal acquisition from specific target products. The data were normalized to housekeeping gene 18sRNA or β-Actin to account for differences in reverse transcription efficiencies and the amount of template in the reaction mixtures [1-7].
**SIRT1 enzyme activity assay**

Enzyme assay for SIRT1 activity was performed on cell lysate or tissue homogenate as per the manufacturer instructions (Sigma, Oakville, ON). The assay procedure is based on a two-step enzymatic reaction. In the first step SIRT1 deacetylates the substrate that contains an acetylated lysine side chain. In the following step the deacetylated substrate is cleaved by the developing solution and a highly fluorescent group is released. This measured fluorescence is directly proportional to the deacetylation activity of the enzyme of the sample. In short 35 µl of assay buffer was added to each well following which 10 µl of sample and 5 µl of NAD⁺ was added. Adding to that 10 µl of SIRT1 substrate solution was added and incubated at 37°C for 30 minute. Following this 5 µl of developing solution was added and plate was incubated at 37°C for 10 minute. The plates were read with a fluorescent spectrophotometer (Biotek, Winooski, VT) at excitation 340 nm and emission 430 nm [3, 8].

**SIRT1 gene silencing**

To silence SIRT1, transfection of small interfering RNA (siRNA) was performed using N-TER nanoparticle siRNA transfection system (Sigma, Oakville, ON) according to the manufacture’s protocol [1, 3, 9]. SIRT1 siRNA1 was purchased from Dharmaco Inc. (Lafayette, CO) and SIRT1 siRNA2 was purchased from Santa Cruz Biotecnology (Santa Cruz, CA). Cells were incubated with or without glucose after transfection for 24 hr and sample collected for mRNA analysis. To test the effect of SIRT1 siRNA on resveratrol, the siRNA was added 48 hr post
treatment with resveratrol in the cultured cells and samples collected 24 hr post transfection. The transfection efficiency was assessed by real-time RT-PCR.

**p300 gene silencing and p300 overexpression**

To silence the p300 expression in ECs, p300 siRNA1 (Silencer, Ambion, Austin, TX) and p300 siRNA2 (Santa Cruz Biotechnology, Santa Cruz, CA) was used with siPORT Lipid transfection reagent (Ambion, Carlbad, CA). ECs were transfected with p300 siRNAs (100 nM/l) with the siRNA transfection reagent (1 µl/500 µl transfection volume). Cells were incubated with or without glucose for 24 hr following the transfection and collected for mRNA analysis [1, 3]. Some transfected cells were further treated with glucose upto passage 1 and sample collected for SA-βgal staining, nuclear fraction isolation and protein analysis. siRNA transfection efficiency was indirectly assessed by measuring p300 mRNA expression by real-time RT-PCR.

P300 overexpression was achieved with expression vectors (generously provided by Dr. Joan Boyes, The Institute of Cancer Research, London, UK) containing the wild-type p300 (pCI-p300) and its HAT-deletion mutant (pCI-p300 HATΔ1472–1522). The plasmids were transfected and amplified in DH5a *Escherichia coli* bacteria following which extracted with the QIApre Spin Miniprep Kit (Qiagen, Toronto, ON). HMECs were transfected with 2 µg/flask (T25) of plasmids with Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s instructions [1, 3]. Cells were incubated with or without glucose for 24 hr following the transfection and collected for mRNA
analysis. Some transfected cells were further treated with glucose upto passage 1 and sample collected for mRNA and protein analysis. Transfection efficiency was assessed by measuring p300 mRNA expression by Real Time RT-PCR.

**ET-1, TGF-β1 ELISA and Telomerase activity**

ET-1 ELISA (Enzo, Farmingdale, NY) and TGF-β1 ELISA (eBioscience, San Diego, CA) was done on cell and tissue lysate as per the manufacturer instructions [2, 6, 10-13]. The total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL). The plate was read at 450 nm using a plate reader (Multiskan, Thermofisher, Toronto, ON). Telomerase activities of the samples were measured using a commercially available kit following instructions provided by the manufacturer (Allied Biotech Inc., Germantown, MD).

**Endothelial permeability assay**

To examine endothelial permeability, the In Vitro Vascular Permeability Assay Kit (Millipore, Billerica, MA) was used according to the manufacturer’s instructions [14]. HMECs were seeded onto collagen coated inserts (1mm pores) in 24-well plates. Following the formation of cell monolayer (2-3 days), cells were treated with specific reagents (Ad-SIRT1, p300 siRNA, p300 plasmid, SIRT1 siRNA) with or without glucose for 72 h. After treatment FITC-Dextran is added to the cells and permeability of this fluorescent molecule is measured at several intervals (30 minute, 1 hour, 1.5 hour and 2 hour). The plates were read with a fluorescent spectrophotometer (Biotek, Winooski, VT) at excitation 485 nm and emission 535 nm.
**Western blot for protein analysis**

Western blot analysis was conducted according to the standard protocol established at our lab [1, 3] using SIRT1, p300, Ac-FOXO1, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) and acetylated H3 lysine antibody (Epigentek, Farmingdale, NY). About 200 µg of protein was extracted from each well/tissue. 20 µg per lane of cellular or nuclear protein was resolved by 5–10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Signals were detected with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and developed with the chemiluminescent substrate (Amersham Pharmacia Biotechnology, Piscataway, NJ) as per the manufacturer instructions.

**miRNA extraction and analysis**

miRNAs were extracted from the cells and tissues using the mirVana miRNA isolation kit (Ambion, Inc., Austin, TX). Briefly, the cells were collected and washed 2 times by using PBS. The tissues were homogenized in the Lysis/Binding solution and miRNA additive(1:10) was added to the samples on ice for 15 minutes. Equal volume acid-phenol: chloroform was added to cell suspension and the tissue lysate, and mixed for 30 seconds by vortex. Following centrifugation and removal of the aqueous phase, the mixture was added 1.25 fold 100% ethanol. The mixture was passed through the filter cartridge and eluted with elution solution. The real-time PCR was used with a final reaction volume of 20 µL containing 10 µL TaqMan 2X Universal PCR Master Mix (No
AmpErase UNG), 1 µL TaqMan microRNA assay (Applied Biosystems Inc, CA, USA), 8 µL Nuclease-free water and 1 µL RT-product. The data was normalized to RNU6B snRNA to account for differences in reverse-transcription efficiencies and amount of template in the reaction mixtures [14-16].

miRNA mimic or antagonir transfection

The HMECs and HRECs were transfected with miRIDIAN microRNA-195 mimic or antagonir (20 nM) (Dharmacon Inc., Chicago, IL) using transfection reagent Lipofectamine2000 (Invitrogen, Burlington, ON). miRIDIAN microRNA mimic or antagonir scrambled control were used in parallel. Transfection efficiency of miRNA was determined by real time RT-PCR [14-16].

Adenoviral forced-expression of SIRT1

SIRT1 adenovirus (devoid of 3’UTR) and null vector was obtained from ABM (Richmond, BC, Canada) and amplified in HEK293A cells [16, 17]. HRECs & HMECs were grown in standard 6-well plates to 90% confluence. Next, cells were transfected with 1 ml Ad-SIRT1 or the empty vector. Subsequently, 3 mL of additional media was added 4 h later and the transfected cells were incubated with or without glucose for 72 h. Cells were either collected with RIPA buffer for protein analysis or TRIZOL™ (Invitrogen, Burlington, ON) for RNA analysis.

Luciferase Reporter Assay for Targeting SIRT1-3’UTR

The luciferase vector including 3’UTR of SIRT1 (1080 bp) containing the SIRT1-miR-195 response elements (wt-Luc-SIRT1) and the mutant (mu-Luc-SIRT1)
were purchased from Norclone Biotech Laboratories (London, ON, Canada). Plasmid DNA (wt-Luc-SIRT1 or mu-Luc-SIRT1, β-galactosidase control vector) and miR-195 mimic or scrambled oligonucleotide were co-transfected in HRECs for 48 hours. The luciferase activity was measured using the Dual-Light Chemiluminescent Reporter Gene Assay System (Applied Biosystems, Burlington, ON) and SpectraMax M5 (Molecular Devices, Sunnyvale, CA). The luciferase activity was normalized for transfection efficiency by measuring the β-galactosidase activity according to the manufacturer’s instructions [14-16].

**Animal Experiments**

All animal experiments were conducted following guidelines specified by the Canadian Council of Animal Care. Protocols were approved by the Western University Animal Care and Veterinary Service. The investigations were in accordance with NIH publ. no. 85-23, revised 1996.

Male C57BL/6 mice (20-25g), were obtained (Charles River, Wilmington, MA) and diabetes was induced by 1-2 intraperitoneal injection of streptozotocin (STZ) (65 mg/kg, in citrate buffer, pH 5.6). Age- and sex-matched mice were used as controls and given equal volumes of citrate buffer. db/db (Leprdb, DBA/J) mice (8 weeks, Jackson Laboratory, CA) were used as type 2 model of Diabetes. The animals were monitored daily [3, 18-23]. The animals were killed at 2 & 4 months following the development of diabetes (n = 10/group). Retinal and renal cortical tissues were dissected out and snap frozen in liquid nitrogen. All tissues were stored at −80°C until further analysis. Urinary albumin (Exocell,
Philadelphia, PA) and serum creatinine (Arbor assays, Ann Arbor, MI) were measured as per the instructions.

Sprague-Dawley rats (~175g, 6 weeks, male) were obtained (Charles River, Wilmington, MA) and diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (65 mg/kg, in citrate buffer, pH 5.6). Control animals received equal volume of citrate buffer. Diabetes was determined as a blood glucose level >20 mmol/l on 3 successive days (Abbott Diabetes Care, Alameda, CA). Animals were fed on a standard diet with water ad libitum and monitored daily [16, 18-23]. A group of diabetic rats (n=7/group) received weekly intravitreal injections of 1.5 μg miR-195 mimic or antagonim in lipofectamine reagent in one eye. The other eye received scrambled control. They were sacrificed one week after the fourth injection. Retinal tissues were collected, snap frozen and were stored at −80°C until further analysis. To assess intravitreal miRNA penetration efficiency DY547 (red fluorescent dye) tagged miRNA scramble transfection control (Dharmacon Inc., Chicago, IL) was injected intravitreally and retinal tissues were collected from sacrificed animals at 2 hr, 4hr, 8hr and 24hr following injection and snap frozen [24]. Slides from the same sections were counterstained with H&E for orientation.

SIRT1 transgenic mice (C57BL/6-ActbIm3.1(Sirt1)Npa/J), male, 8 weeks old were obtained (Jackson Laboratory, Bar Harbor, ME) and genotyped with Real Time RT-PCR. Diabetes was induced by 1-2 dose of intra-peritoneal injection of streptozotocin (STZ, 65 mg/kg in citrate buffer). Age and sex matched mice were used as controls and received equal volume of citrate buffer [3, 16, 18-23].
Diabetes was defined in the animals with blood glucose level >20mmol/L on two consecutive days (Abbott Diabetes Care, Saint-Laurent, QC). The animals were fed on a standard rodent diet with water *ad libitum* and sacrificed after 2 months following the development of diabetes (n = 10/group). Urinary micro-albumin (Exocell, Philadelphia, PA) was measured as per the kit instructions. Retina and kidney tissues were collected and snap frozen in liquid nitrogen for histology. All tissues were stored at −80°C until further analysis.

**In situ Hybridization & Immunohistochemistry**

A 5' and 3' double DIG-labelled custom-made mercury LNA™ miRNA detection probes (Exiqon, Vedbaek, Denmark) were used to detect miR-195 expression using in situ hybridization(ISH) Kit (Biochain Institute, Hayward, CA) in retinal tissue sections. Scrambled probe and no-probe were used as controls [14-16, 25]. IgG stainings were performed on frozen retinal sections using anti-rat IgG (ebioscience, Sandiego, CA) with hematoxylin counterstain [14-16]. Quantification was done in a masked fashion using an arbitrary scale (0 = no extravascular stain of retina, 1 = mild extravascular stain, 2 = moderate extravascular stain, 3 = marked extravascular stain).

**SA β-gal staining for senescence**

Cells or frozen tissue sections were fixed and stained with SA β-gal staining according to the manufacturer’s instructions (abcam, Cambridge, MA). In short, cells or frozen tissue sections were washed with PBS and fixed with fixative solution for 10-15 minutes at room temperature. Following two washes with PBS
cells/slides were covered in staining solution and incubated overnight at 37°C. For long-term storage of the stained plates/slides, the staining solution was removed and overlaid with 70% glycerol and stored at 4°C. Tissue slides were counterstained with H&E staining for orientation purpose [26].

**Phase contrast and fluorescent microscopy**

From each specimen, positive (blue) cells were photographed with phase contrast inverted microscope using SPOT Basic software (Meiji Techno, TC5400, Santa Clara, CA). Morphometrical analysis of the images was done by ImageJ software (NIH, Bethesda, MD). They were counted per 10 microscopic fields at 20X magnification and presented as % of total number of cells.

Images of the tissue slides were recorded by an Olympus BX51 microscope (Olympus, Center Valley, PA) with Northern Eclipse software (Empix Inc, Cheektowaga, NY). Tissue slides were quantified based on staining intensity [27] at 60X magnification. Quantification of SA-β-Gal intensity was done in a masked fashion using an arbitrary scale (0 = no stain, 1 = focal weak stain, 2 = multifocal moderate stain, 3 = multifocal intense stain). Fluorescent microscopies of frozen retinal sections were performed with TRITC filter at 20X magnification.

**Total ROS/RNS and Intracellular LDH analysis**

Total ROS/RNS level in the EC lysates/tissue homogenates were measured as per the manufacturer instructions using a commercially available kit (Cell Biolabs
Inc., San Diego, CA). The plates were read with a fluorescent plate reader (Biotek, Winooski, VT) at excitation 480 nm and emission 530 nm. Total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL). Quantification of intracellular lactate dehydrogenase (LDH) in the cell lysates was used as an additional measure of cell growth. The assay was conducted as per the manufacturer instruction (Caymen Chemical, Ann Arbor, MI).

**MnSOD & FN ELISA**

MnSOD ELISA was done [3] on cell lysate and tissue homogenate as per the manufacturer instruction from a commercially available kit (abcam, Cambridge, MA). Fifty µl sample is added to each pre antibody coated wells and sealed and incubated for 2 hours at RT. Following 3 wash 50 µl detection antibody is added to each well and incubated for 1 hour. After 3 more washes 50 µl of HRP conjugate is added to each well and incubated another hour. Following 5 wash 100 µl developing solution is added and upon colour development 100 µl of stop solution is added to each well. The plate is read at 450 nm using a plate reader (Multiskan, Thermo Scientific, Burlington, ON). Total protein concentrations were measured by BCA protein assay kit (Pierce, Rockford, IL). ELISA for FN was performed using commercially available kits for human FN (Millipore, Billerica, MA), rat FN (Kamiya Biomedical, Seattle, WA), mice FN (abcam, Toronto, ON) according to the manufacturers’ instructions [15, 16]. The plates were read at 450 nm using a plate reader (Multiskan, Thermo Fisher, Canada).

**Nuclear fraction isolation**
Nuclear fractions were isolated from ECs and tissues as per the kit instruction (Active Motif, Carlsbad, USA). Briefly media was aspirated out of the flasks and washed with 5 ml ice cold PBS/Phosphatase Inhibitors. Another 3 ml of ice cold PBS/Phosphatase Inhibitor solution was added to the cells. Cells were gently scrapped and transferred to a pre chilled 15 ml conical tube and centrifuged for 5 minute at 500 rpm at 4°C. The cell pellet was resuspended in 500 µl 1X hypotonic buffer and incubated on ice for 15 minute following mixing. Following this 25 µl of detergent was added, vortexed and centrifuged at 14000 x g for 30 seconds at 4°C. The supernatant containing the cytoplasmic fraction was separated following which the nuclear pellet was resuspended in 50 µl of complete lysis buffer and vortexed for 10 seconds. Following 30 minute incubation on ice on a shaker the suspension was centrifuged for 10 minutes at 14000 x g in a microcentrifuge precooled at 4°C. The supernatant containing the nuclear fraction was collected [1, 3]. Fresh tissue sections were cut and weighed washed in 3 ml (per gram tissue) ice cold 1X hypotonic buffer supplemented with DTT and detergent and homogenized. Following incubation on ice for 15 minute sample was centrifuged for 10 minute at 850 x g and pellet collected. Nuclear extraction of the pellet was done as per the protocol of the cells described above. Protein concentrations in the samples were measured by BCA protein assay (Pierce, Rockford, IL).

FOXO1 DNA binding activity measurement

FOXO1 DNA binding ELISA was conducted [3] on the collected nuclear fraction as per the manufacturer’s instruction (Active Motif, Carlsbad, CA). In short 10 ul
diluted nuclear extract samples (2-20 ug protein concentration) were added to oligonucleotide coated plates. The 96 well plate was sealed and incubated for 1 hr at room temperature following which the plate was washed 5 times with wash buffer and 100 µl primary antibody (FOXO1) was added to each well. Following 1 hr of incubation at RT the plate was washed 5 times and 100 µl anti IgG HRP conjugate antibody was added to each well. After 1 hr incubation plate was washed 5 times and 100 µl developing solution was added to each well. As color developed stop solution was added. The plates were read at 450 nm using a plate reader (Multiskan, Thermo Fisher, Canada).

References


This is a License Agreement between rokbasa mortuza ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number 3439441124472
License date Jul 31, 2014
Licensed content publisher Springer
Licensed content publication Diabetesologia
Licensed content title miR-165 regulates SIRT1-mediated changes in diabetic retinopathy
Licensed content author rokbasa mortuza
Licensed content date Jan 1, 2014
Volume number 57
Issue number 5
Type of Use Thesis/Dissertation
Portion Full text
Number of copies 1
Author of this Springer article Yes and you are the sole author of the new work
Order reference number None
Title of your thesis / dissertation Role of SIRT1 in vascular complications of diabetes
Expected completion date Sep 2014
Estimated size(pages) 161
Total 0.00 CAD

Introduction
The publisher for this copyrighted material is Springer Science + Business Media. By
clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.rightslink.com/).

Limited License
With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry.

Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process.

This License includes use in an electronic form, provided its password protected or on the university’s intranet or repository, including UMI (according to the definition at the Sherpa website: http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com).

The material can only be used for the purpose of defending your thesis limited to university use only. If the thesis is going to be published, permission needs to be re-obtained (selecting "book/textbook" as the type of use).

Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, subject to a courtesy information to the author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).

Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material: Not Permitted
You may not alter or modify the material in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this
licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice/Disclaimer
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Springer and the original publisher/journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure number(s), original copyright notice) is given to the publication in which the material was originally published. By adding: with kind permission from Springer Science and Business Media"

Warranties: None

Example 1: Springer Science + Business Media makes no representations or warranties with respect to the licensed material.

Example 2: Springer Science + Business Media makes no representations or warranties with respect to the licensed material and adopts on its own behalf the limitations and disclaimers established by CCC on its behalf in its Billing and Payment terms and conditions for this licensing transaction.

Indemnity
You hereby indemnify and agree to hold harmless Springer Science + Business Media and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

No Transfer of License
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing
This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC’s Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and
conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science + Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC’s Billing and Payment terms and conditions, these terms and conditions shall control.

Jurisdiction

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in The Netherlands, in accordance with Dutch law, and to be conducted under the Rules of the ‘Netherlands Arbitration Institute’ (Netherlands Institute of Arbitration) OR:

All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.

Other terms and conditions:

v1.3

You will be invoiced within 18 hours of this transaction date. You may pay your invoice by credit card upon receipt of the invoice for this transaction. Please follow instructions provided at that time.

To pay for this transaction now, please remit a copy of this document along with your payment. Payment should be in the form of a check or money order referencing your account number and this invoice number BLN6501364344.

Make payments to “COPYRIGHT CLEARANCE CENTER” and send to:

Copyright Clearance Center

Dept 001
P.O. Box 843008

Boston, MA 02284-2008

Please disregard electronic and mailed copies if you remit payment in advance.

Questions? customer@copyright.com or +1-855-239-5415 (toll free in the US) or +1-978-646-2777.

Gratis licenses (referencing $0 in the Total field) are free. Please retain this printable license for your reference. No payment is required.
Curriculum Vitae

ROKHSANA MORTUZA

EDUCATION

PhD  2009-2014  **Western University**, London, ON

Field/Discipline: Pathology

Research focus: Genetic Changes in Diabetic Complications

HBSc  2000-2004  **University of Toronto**, St. George Campus, Toronto, ON

Field/Discipline: Pathobiology

PUBLICATIONS (Articles in Peer-Reviewed Journals & Abstracts in National/International Conferences)


INVITED LECTURE/PRESENTATION

2013-06  73rd Scientific Sessions, American Diabetes Association, Chicago, IL, USA. Title: miR-195 regulated accelerated endothelial aging in diabetes through SIRT1.

OTHER PROFESSIONAL PRESENTATIONS (Workshops, Symposia, Regional meetings, Research Days)

Mortuza R, Chakrabarti S (2012). Glucose induced SIRT mediated alterations in microvascular endothelial cells. *Diabetes research day*, Lawson Health Research Institute, London, ON


**TEACHING EXPERIENCES (Undergraduate)**

2013 **Teaching Assistant, Genetics 2581B**, Dept. of Biology, Western University, London, ON
2012 **Teaching Assistant, Biology for Science II 1002B**, Dept. of Biology, Western University, London, ON

2012 **Teaching Assistant, General Biology II 1202B**, Dept. of Biology, Western University, London, ON

**WORK EXPERIENCES**

2009-2014 **Graduate Research Assistant** - Department of Pathology, Western University, London, ON

2007-2010 **Lab Manager** - CRS Division,

KGK Synergize Inc., London, ON

2005-2007 **Toxicologist** - Biological Activity Department

Labstat International Inc, Kitchener, ON

2005 **Microbiologist (Co-op)**, Microbiology Department

Maxxam Analytics Inc, Mississauga, ON

2004 **Research Assistant**

Hospital for Sick Children, Toronto, ON

2003 **Research Student**

DEPARTMENT OF SURGERY, University of Toronto, ON

**DISTINCTIONS, HONOURS, SCHOLARSHIPS**

2011-2014 Western Graduate Research Scholarship, UWO, London, ON
2011, 2013  Dutkevich Memorial Foundation Award, Department of Pathology, UWO, London, ON

2012  Nominated, Queen Elizabeth II Graduate Scholarships in Science and Technology, Ministry of Training, Colleges, and Universities, ON

2004  Graduated with distinction from U of T, Toronto, ON

2003  Undergraduate Summer Student Research Award, Department of Laboratory Medicine & Pathobiology, U of T, Toronto, ON

2001  Millennium Scholarship, University of Toronto, Toronto, ON

COMMUNITY INVOLVEMENT, COMMITTEES, SOCIETIES & OTHER ACTIVITIES

2011-2012  Volunteered and served as a councillor (2011-2012) at the Society of Graduate Students (SOGS) on behalf of the department of pathology graduate students, Western University, London, ON

2009  Volunteered as a coordinator (2009) at the Toronto Man to Man Prostate Cancer Support Group on a research related to effect of Vit D on prostate cancer, Toronto, ON

2007-2011  Volunteered as a member (2007-2011) of the Safety Task Force at the South Street Hospital, London Health Sciences Centre, London, ON

2007  Volunteered as a member (2007) of the Health and Wellness Committee to organize various social activities for the betterment of employees at Labstat International ULC, Kitchener, ON
2006  Volunteered as a member (2006) of the Ergonomic Committee for helping the employees injured at work at Labstat International ULC, Kitchener, ON

2001  Volunteer summer student (2001) at the Respiratory Physiology Research lab at the University of Toronto, Toronto, ON

2002  Life time member of Golden Key International Honorary Society, Canada

2004  Member of Laboratory Medicine & Pathobiology Alumni 2004, University of Toronto, ON