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Adiponectin in the maintenance and protection of glomerular endothelial cells and the glycocalyx in diabetes

Adiponectin in the maintenance and protection of glomerular endothelial cells and the glycocalyx in diabetes

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Abstract:

Introduction: Adiponectin (Ad) is a hormone secreted primarily by adipocytes and is known to have anti-inflammatory and protective effects on vascular endothelial cells specifically via the 5' AMP-activated protein kinase (AMPK) signaling pathway. It is also known to protect against the development of albuminuria including in diabetes. Albuminuria is indicative of dysfunction of the glomerular filtration barrier of the kidney and therefore of the cells which make up the barrier: glomerular endothelial cells (GEnC) and podocytes. In particular, damage to the endothelial glycocalyx leads to an increase in albuminuria in disease states such as diabetes.

Aim: The aim of this work is to determine whether Ad acts directly on GEnC and whether as a result it can protect the glycocalyx against inflammatory mediators implicated in diabetes.

Methods: *in vitro* studies were performed in well-characterized conditionally immortalised human GEnC line (CiGEnC) and in sieved glomeruli from diabetic mouse models to investigate the effect of adiponectin on cell signaling and TNF-α-induced disruption of the glycocalyx.

Results: The AMPK pathway was activated when CiGEnC were stimulated with globular adiponectin (gAd) through Adiponectin Receptor 1. High glucose and tumor necrosis factor-α (TNF-α) inactivated this pathway but it was restored by co-treatment with gAd. By quantitative PCR (qPCR) and Western blot, I showed that there was an upregulation of syndecan-4 (SDC4), a glycocalyx proteoglycan, at both the mRNA (2.8 fold) and protein level (1.7 fold) in response to 2-hour treatment with 10ng/ml TNF-α. Adiponectin prevented this increase. There was also a significant increase (* $p<0.05$) in the mRNA expression of the metalloproteinase MMP2, which is known to induce the shedding of glycocalyx components, in response to TNF-α. Again, gAd prevented this increase. However, gAd treatment of CiGEnC in which MMP2 had been knocked down did not completely prevent the increase of SDC4 in response to TNF-α, suggesting that other MMPs might be involved.

Conclusion: These findings show that adiponectin-induced signalling in CiGEnC protects the glycoclayx *in vitro* and ex vivo and therefore fully understanding these pathways has the potential to provide new therapeutic targets to decrease albuminuria in diabetes *in vivo*.

Dedication

This thesis is dedicated to my baby sister Shirin. You are a great addition to this family and you coming to this world during my PhD journey, made it a whole lot easier. I wish you a great life ahead of you full of success and accomplishments.

Through patience, great things are accomplished

~ Imam Ali (AS)~

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Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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Table of Contents

List of Figures:

List of Tables

List of Abbreviations

ACC	Acetyl CoA carboxylase
ACE	Angiotensin-converting enzyme
Acrp30	Adipocyte complement-related protein of 30 kDa
ADAM17	A Disintegrin and metalloproteinase domain-containing protein 17
ADIPOR1	Adiponectin Receptor 1
ADIPOR2	Adiponectin Receptor 2
AGE	Advanced glycated end products
AICAR	5-Aminoimidazole-4-carboxamide ribonucleotide
AMPKα	AMP-activated protein kinase alpha
ANG II	Angiotensin II
Ang-1	Angiopoiten-1
ApM1	Adipose Most Abundant Gene Transcript 1 Protein
APPL	Adaptor protein containing PH domain, PTB domain and leucine zipper
ARB	Angiotensin II receptor blockers
BSA	Bovine serum albumin
CAMKKβ	Calicum/calmodulin-dependent protein kinase kinase β
CiGEnC	Conditionally immortalized glomerular endothelial cells

CiPod	Conditionally immortalized podocytes
CKD	Chronic kidney disease
CO_2	Carbon dioxide
CS	Chondroitin sulphate
D	Days
DAPI	4’,6-diamidino-2-phenylindole
DHB	Dorothy Hodgkin Building
DM	Diabetes Mellitus
DMSO	Dimethyl sulfoxide.
DN	Diabetic Nephropathy
DS	Dermatan sulphate
ECIS	Electric cell-substrate impedance sensing
e-NOS	Endothelial nitric oxide synthase
ERK	Extracellular receptor kinase
ESRD	End Stage Renal Disease
FA	Fatty Acid
fAd	Full length adiponectin
FBS	Fetal bovine serum

FGF	Fibroblast growth factor
gAd	Globular adiponectin
GAG	Glycosaminoglycans
GBM	Glomerular Basement Membrane
GBP28	Gelatin-binding protein of 28 kDa
GEnC	Glomerular endothelial cells
GFB	Glomerular Filtration Barrier
GLUT4	Glucose transporter type 4
GPCR	G-protein-coupled receptors
h	Hours
HA	Hyaluronic acid
HAS	Hyaluronan synthases
hEGF	Human Epidermal Growth Factor
HG	High glucose
HMW	High molecular weight
HS	Heparan sulphate
hTERT	Human telomerase reverse transcriptase
HUVEC	Human umbilical vein endothelial cells

ICAM	Intercellular Adhesion Molecule 1
IF	Immunofluorescence
IGF-1	Insulin-like growth factor
IL-6	Interleukin 6
IR	Insulin resistance
IRS1	Insulin receptor substrate 1
KS	Keratin sulphate
LKB1	Liver kinase B1
LMW	Low molecular weight
LSS	Laminar sheer stress
MAPK	Mitogen-activated protein kinase
min	Minutes
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
MS	Metabolic syndrome
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
^{o}C	Degrees Celsius

PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-buffer saline
PECAM	Platelet endothelial cell adhesion molecule
PI-3K	Phosphoinositide 3-kinases
PKC	Protein Kinase C
POD-ATTAC	Podocytes apoptosis through targeted activation of caspase-8
PPARα	Peroxisome proliferator-activated receptors alpha
PTB	Phosphotyrosine binding
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SA	Sialic acid
SDC1	Syndecan 1
SDC4	Syndecan 4
SFM	Serum free media
shRNA	Small hairpin RNA
siRNA	Small interfering RNA

STZ	Streptozotocin
SV40LT	Simian virus 40 Large T antigen
T2D	Type 2 Diabetes
TEER	Transendothelial electrical resistance
TG	Triglycerides
TNF-α	Tumor necrosis factor apha
VCAM1	Vascular cell adhesion protein 1
VEGFA	Vascular endothelial growth factor type A
VEGFR	Vascular endothelial growth factor receptors
WGA	Wheat germ agglutinin
ZO-1	Zonula occludens-1

Chapter 1 Introduction

1.1 The kidney

1.1.1 Introduction to the kidney

The kidneys are two major retroperitoneal organs located towards the posterior muscular wall of the abdominal cavity. The principal function of the kidneys is the excretion of waste products, which result from muscle contraction and protein metabolism [1]. They have an integral role in blood filtration specifically, the excretion of metabolic waste products and water regulation. The rate of the filtration of the kidneys is 125ml/min/1.73m^2, equal to 180 litres in a day. The kidney is also responsible for regulation of blood pressure via renin production and sodium ion excretion [2] as well as secretion of hormones such as calcitriol and erythropoietin [3].

The basic structural and functional unit of the kidney is the nephron, approximately 1 million of which are found in each kidney. The nephron is a group of capillaries knotted together to form a ball-like structure named the glomerulus which acts as a biological sieve by retarding the passage of plasma proteins while allowing relatively free flow of water and small solutes [4].

The renal glomerulus consists of a group of specialized cells called podocytes, and these are separated from the glomerular endothelial cells (GEnC) by the glomerular basement membrane (GBM) (Figure 1.1). It is well-known that these three components (endothelial cells, GBM and podocytes) constitute the glomerular filtration barrier (GFB) [5]. These cells are considered important to renal function and often undergo pathophysiological changes with disease.

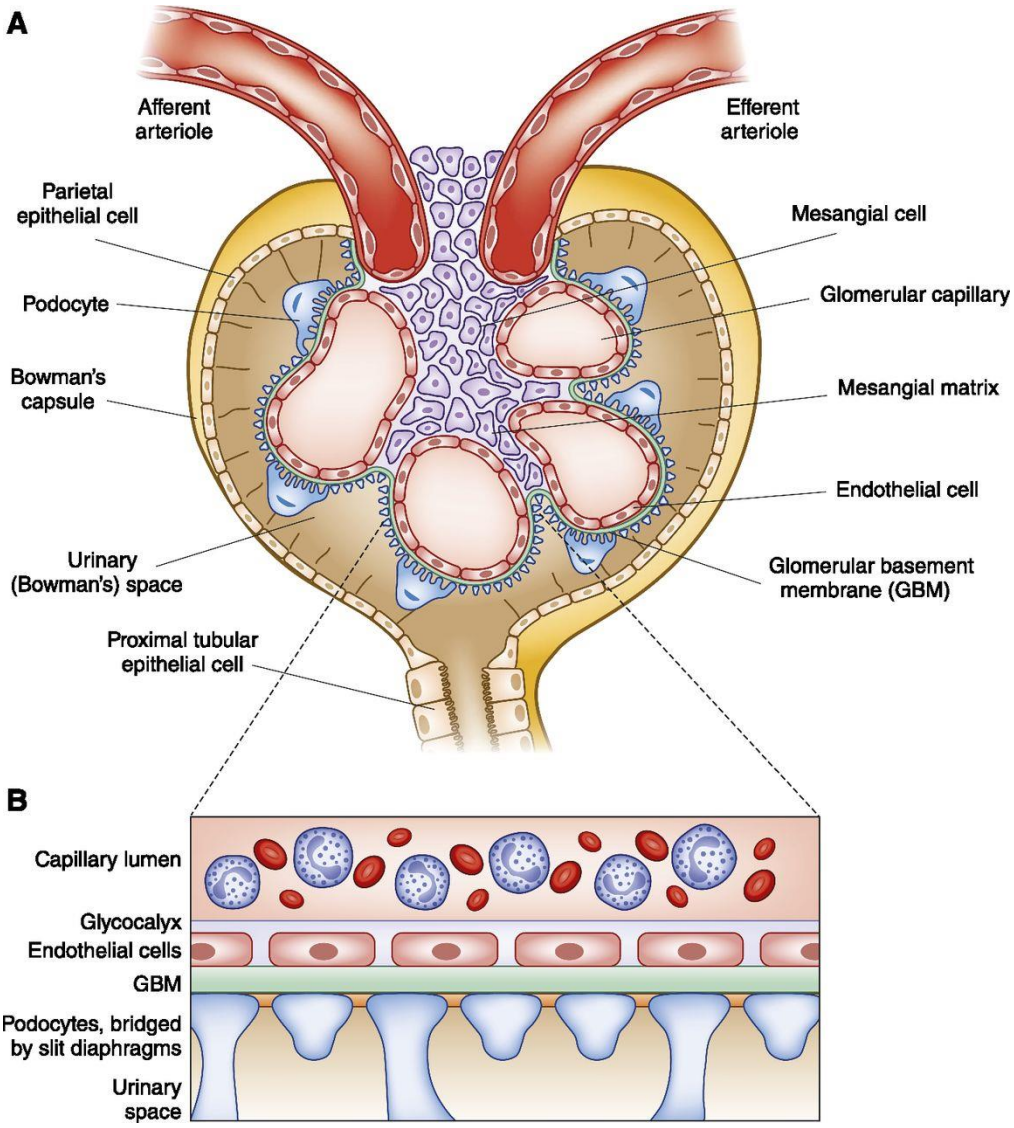

Figure 1.1 Basic structure of the kidney glomerulus and the GFB components

A: A section of the glomerulus that shows its different components. **B**: A detailed section of the GFB, a specialized molecular sieve, that is composed of 3 layers: the endothelial cells with its glycocalyx, the GBM and specialized podocytes [6].

1.1.2 Diabetic nephropathy

Many chronic diseases are now considered a major cause of morbidity and mortality worldwide. Diabetes mellitus (DM), especially type 2 (also known as non-insulin dependent diabetes) (T2DM) along with its complications is an emerging epidemic as well as a major public health issue [7]. Diabetic nephropathy (DN) is one of the major

microvascular complications of diabetes and now the commonest cause of renal failure known as end-stage renal disease (ESRD) requiring renal replacement therapy [8]. DN is a clinical syndrome characterized by persistent albuminuria (>300mg/day) [9]. The pathophysiological mechanisms in the development of DN are multifactorial. Hyperglycemia is a necessary prerequisite for the structural and functional changes such as glomerular hyperfiltration, renal hypertrophy and microalbuminuria, followed by the development of GBM thickening, mesangial expansion, overt proteinuria and finally ESRD [10]. The filtration system can be damaged by excess glucose in the bloodstream thereby allowing the kidneys to filter more blood. This will put extra load on the nephrons causing them to lose their filtration capacity. A hallmark of DN is nodular glomerulosclerosis, which was described by Kimmelstiel and Wilson [4]. DM causes injury of all renal compartments, such as mesangial expansion, thickening of GBM and podocyte loss as shown in Figure 1.2. Altogether, these result in progressive albuminuria, reduction in glomerular filtration barrier (GFR), elevation of blood pressure and fluid retention. Advanced glycosylation end products (AGE) , activation of protein kinase C (PKC) and acceleration of the polyol pathway along with the hemodynamic alterations were considered as the main cause of renal injury in diabetes [8]. However, evidence has been provided that not only these factors are part of the pathophysiology of DN, but also the immune-mediated inflammatory processes [11]. Different cell types, such as leukocytes, monocytes, macrophages and adipocytes release different inflammatory molecules known as cytokines, adipokines or chemokines that are also implicated in the pathogenesis of DN [11]. The focus of this thesis is to discover a new marker that can define DN and help find ways in the treatment of DN through alleviating albuminuria specifically while looking at GEnC.

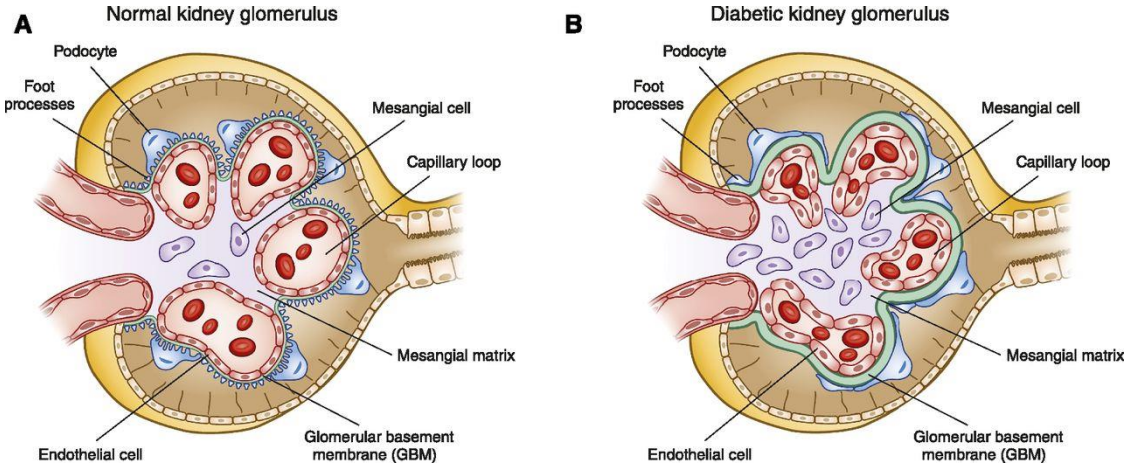

Figure 1.2 Normal kidney morphology and structural changes in diabetes mellitus

A: A normal kidney glomerulus that shows the different components in a healthy state. **B**: A diabetic kidney glomerulus that shows the structural changes in the GFB such as the thickening of the glomerular basement membrane, foot processes effacement known as flattening, loss of podocytes with denuding of the glomerular basement membrane and mesangial matrix expansion. Adapted from [12].

1.1.3 The glomerular filtration barrier (GFB)

1.1.3.1 Introduction of GFB

The GFB is a part of the glomerulus that acts as a selectively permeable sieve by preventing large proteins and macromolecules from leaving the blood whilst allowing unrestricted movement of water and small solutes. As stated earlier, the GFB has a unique structure of three components as shown in Figure 1.3; a fenestrated GEnC, a central GBM and podocytes [13]. Every layer is highly specialised and is responsible for its own role in filtration specificity. In the next part, the function of each layer will be introduced, before highlighting the crosstalk between each layer [13].

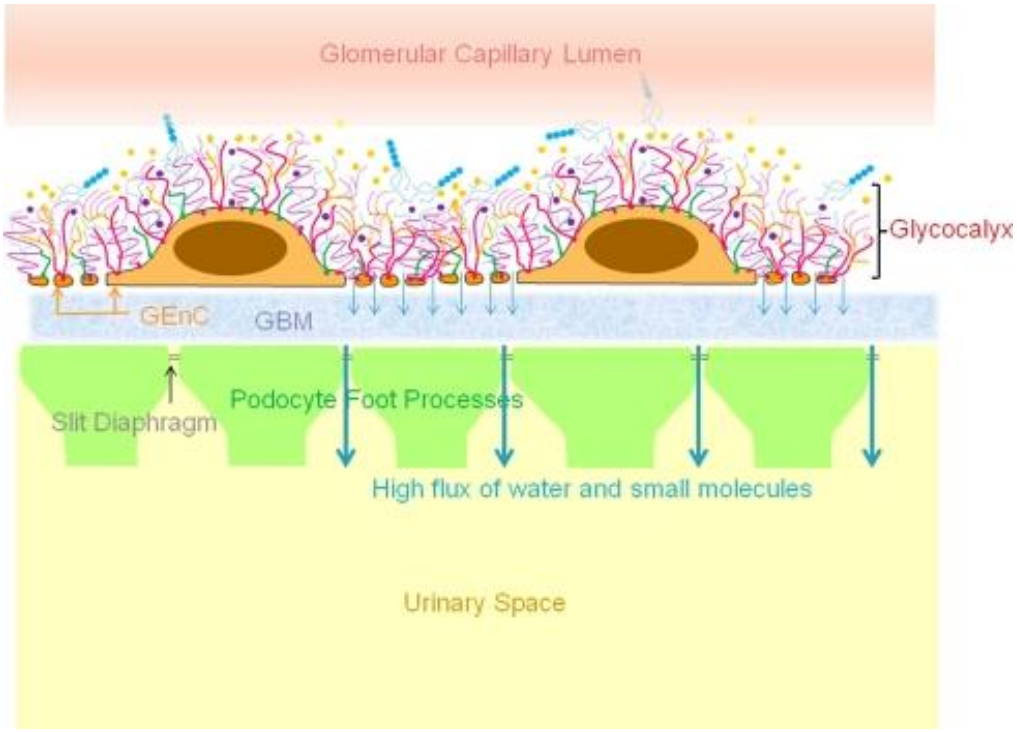

Figure 1.3 Schematic drawing showing the different components of the GFB

Schematic drawing of components of the glomerular filtration barrier (GFB). Fenestrated glomerular endothelial cells (GEnC) form the luminal side of the sieve and facilitate the high flux of water and small molecules (*blue arrows*); glomerular basement membrane (GBM) in the middle, and the podocyte foot processes and slit diaphragms on the urinary side. The GEnC (including the fenestrae) are covered by a mesh-like, anionic layer of glycocalyx composed of sialic acid-rich glycoproteins and proteoglycans consisting of core proteins and attached branching glycosaminoglycan chains (mainly heparan sulphate and chondroitin sulphate) Adapted from [14].

1.1.3.2 Glomerular endothelial cells

GEnC are highly specialized cells, which form a continuous inner layer of glomerular capillaries. The glomerulus begins filtration within the layer of fenestrated GEnC. They are specifically adapted to allow rapid filtration, with high hydraulic conductivity. The most distinctive physical feature of the endothelial layer is its perforated appearance, due to the presence of a 60-80nm fenestrae that cover it. The size of these pores allow for the passage of low molecular weight proteins such as albumin, with a size of around 80nm [15].

It is important to study human GEnC to enable complete understanding of glomerular diseases, glomerular filtration and response to glomerular injury. Hence, the first step which allow careful analysis of this individual cell type would be through *in vitro* cell culture. It had been difficult to grow human cells in culture, but our group has successfully rectified this problem by conditional immortalization technology. This was done as described by Satchell *et al* [16]. In brief, a primary culture human GEnC is generated and as tissue culture studies are limited with these due to early senescence, they were transformed into conditionally immortalized GEnC (CiGEnC) (see Chapter 2 Materials and Methods for more details).

1.1.3.3 Glomerular Basement Membrane (GBM)

The GBM is an extracellular matrix that separates the GEnC and podocytes. It is largely composed of four macromolecules commonly found in all basement membranes: laminin, collagen (type IV), nidogen, and heparan sulphate (HS) proteoglycan [1]. The GBM's highly negative charge has long been considered a key player in GBM permselectivity. The most ubiquitous HS proteoglycan in the GBM is agrin, which imparts significant negative charge due to the presence of its sulphated glycosaminoglycans (GAG) side chains [17].

1.1.3.4 Podocytes

Podocytes are highly differentiated, specialised epithelial cells that wrap around the outside of glomerular capillaries. They consist of three segments: the cell body, major processes and foot processes [18]. The latter can be branched further into minor foot processes. The foot processes of multiple neighbouring podocytes interdigitate, forming specialised cell-to-cell junctions called slit diaphragms [19]. The diaphragm itself is created by a variety of different proteins including nephrin, zona-occludens-1 (ZO-1) and podocin

[20-22] which are expressed in podocytes and strengthen the filtration properties of the slit diaphragm. These proteins, along with those in the podocyte actin cytoskeleton, are key to maintaining the structure and function of podocytes. A characteristic feature of many glomerular diseases is podocyte foot process effacement, a flattening of podocytes caused by disorganisation within the cytoskeleton [23]. Podocytes are rarely lost in healthy glomeruli and podocyte injury with reduced density is one of the features of diabetic kidney disease [7].

1.1.3.5 Cross Talk between podocytes, GBM and GEnC

It is now believed that the GFB is not just a selectively permeable sieve, but it is more of a dynamic system where the several cells types affect each other. Hence, endothelial cells communicate with podocytes across the GBM. Altered paracrine communication between GEnC and neighbouring cells is thought to contribute towards the development of albuminuria.

This was demonstrated in an *in vivo* model of diabetic mice deficient in endothelial nitric oxide (NO) synthase (e-NOS). It is an enzyme required for normal endothelial function that is present in the endothelium of the arterioles and capillaries of the glomerulus; podocytes do not express this enzyme [24]. It was shown that there was a podocyte injury and albuminuria in these e-NOS knockout diabetic mice [25]. Furthermore, laminar shear stress (LSS) is the result of the constant mechanical loading caused by blood pressure and flow. It has emerged that each component of the GFB is interdependent and in continual communication with its neighbours. Signalling crosstalk between the two cell types is vital for normal barrier function. One mode of crosstalk arises from the presence of vascular endothelial growth factor receptors (VEGF-Rs) and angiopoietin receptors on endothelial cells. The secretion and paracrine action of vascular endothelial growth factor A (VEGF-A)

and angiopoietin-1 (Ang-1) that are released by the podocyte are required for GEnC development and survival [26, 27]. Changes in the levels of these and other signalling proteins have been shown to cause changes in glomerular permeability [28].

Taken together these functions demonstrate the importance of the glomerular endothelium in the GFB. However, these are not the only functions and most of the other functions of the glomerular endothelium coincide with podocytes.

1.1.4 The endothelial glycocalyx

1.1.4.1 Components and Structure

The GEnC are covered with a filamentous surface layer known as the glycocalyx [29, 30]. The glycocalyx is best described as a heterogeneous structure consisting of core proteins such as syndecans or glypicans and decorated with GAG side chains forming a hydrated poly-anionic gel [31]. GAG are heteropolysaccharide chains consisting of repeating disaccharide units. There are five types of (GAG): HS, chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronic acid (HA), but the most predominant GAGs in the endothelial glycocalyx are HS and CS [31]. HA is synthesised by hyaluronan synthases (HAS) such as HAS1, HAS2 or HAS3 and is composed of repeating disaccharide units of glucuronic acid and N-acetylglucosamine. It is the only non-sulfated GAG and does not bind covalently to any of the core proteins to form proteoglycan. It is mainly anchored to the cell surface by specific hyaluranan receptors [32]. Proteoglycans are proteins that contain specific sites where sulphated GAGs are covalently attached and then are transferred from endoplasmic reticulum to the Golgi apparatus [33]. Upon transfer, a series of highly regulated enzymatic reactions, beginning with chain elongation, and proceeding with a multitude of modifications, through monosaccharide epimerization

and sulphation, determine the final form of a GAG [33]. For example, HS mainly attaches to syndecan 1 and 4 (SDC1/SDC4). Synthesis of HS onto the proteoglycan-attached tetrasaccharide is initiated by the addition of N-acetylglucosamine by N-acetylglucosaminyltransferase I [34]. The GAG chain then extends, containing repeating units of glucuronic or iduronic acid and glucosamine with modified regions being segmentally interspersed between larger unmodified units. SDC4 is a major HS proteoglycan and its removal accounts for a 38% reduction in HS and a 37% increase in albumin passage across cultured endothelial monolayers [35]. Chronic loss of SDC4 from the endothelial cell surface has also been linked to failure of microvascular angiogenesis in diabetes [36].

Along with the proteoglycans, glycoproteins bearing acidic oligosaccharides and terminal sialic acids (SAs) are also prominent within the glycocalyx. The major glycoproteins are the endothelial cell adhesion molecules that consist of three families; the selectin family, the integrin family and the immunoglobulin family. Common examples of these are E-selectin, P-selectin, integrin αVβ3, intercellular adhesion molecules 1 &2 (ICAM-1&2), vascular cell adhesion protein 1 (VCAM-1) and platelet endothelial cell adhesion molecule (PECAM-1) [31]. Soluble molecules are also embedded within the layers of proteoglycans and glycoprotein of the glycocalyx and are essential for the normal function of the layer. They are either derived from the endothelium or from the bloodstream, such as albumin which is essential for normal function of the layer in preserving the charge and selectivity of the permeability barrier [37].

1.1.4.2 Visualisation

Rambourg *et al.* were the first group to provide evidence that cells are covered with a glycocalyx [38]. They used a silver methenamine labelling technique for detection of

glycoproteins to stain a variety of rat tissues. Afterwards, under the electron microscope, it was shown that nearly all cells are coated with a thin layer of the stained material [38]. Correspondingly, Luft et *al.* used the cationic dye ruthenium red that binds to acidic mucopolysaccharides and generates electron density in the presence of osmium tetroxide [39]. Whilst finding methods in studying the function of the glycocalyx is not easy, there are still ways one can quantify the endothelial glycocalyx components. For example, Alcian blue binding assays have been used *in vivo* on tissues to assess the negative charge generated by the glycocalyx [40]. However, this assay only quantifies the charge components remaining on the cell surface and does not directly measure the glycocalyx structure. Moreover, combining Alcian blue binding with electron microscopy has become a standard method in glycocalyx assessment. There are precautions that need to be considered while using Alcian blue as polysaccharides react poorly with standard electron microscopy post-fixation stains. Finally, when this label is introduced with a fixative such as glutaraldehyde, the glycocalyx can be reliably imaged [41-43]. Another technique being developed within our group is lectin staining. Lectins are specific carbohydrate-binding proteins that recognize highly specific sugar units and their 3D conformations. For example, wheat germ agglutinin (WGA) has been used to identify the glycocalyx [41, 44]. This lectin binds to sialic acid residues throughout the glycocalyx structure. Lectin staining appears to be more specific as new individual lectins are being identified and thus more likely to become a valuable tool with which to examine the glycocalyx [41]. Although technically difficult, there are now a few methods available with which to study the structure of the glycocalyx. This will allow us to further develop our knowledge and understanding of the glycocalyx as we discover more details about this unique structure.

1.1.4.3 Functions of the endothelial glycocalyx

One of the major physiological functions of the endothelial glycocalyx is that it is an important determinant of vascular permeability [45]. It has the ability to limit access of certain molecules to the endothelial cell membrane, as has been demonstrated in small rat mesenteric arteries with the use of fluorescently labelled dextrans of various molecular weights, showing increasing permeability for smaller molecules [45]. It also functions as a mechanotransducer; endothelial cells line the inner surface of blood vessels and are continually exposed to different mechanical factors. The glycocalyx itself translates these mechanical forces to biochemical signals that lead to changes in metabolism, morphology and gene expression [46]. For example, an *in vitro* study using bovine aortic endothelial cells (BAECs) showed that the enzyme chondroitinase, employed to selectively degrade CS, did not inhibit the characteristic shear-induced NO production, but treatment with hyaluronidase did [47]. Also, endothelial cells exposed to shear stress alter their production of NO, which is a notable vasodilator that modulates vascular tone. Any damage to the glycocalyx impairs these mechanisms and the endothelial response to shear stress [48]. The negatively charged mesh of the glycocalyx acts as a macromolecular sieve; it repels like-charged molecules, as well as white and red blood cells and platelets and excludes macromolecules larger than 70kDa [49].

1.1.4.4 Mechanisms of glycocalyx damage

Endothelial dysfunction plays a major role in the development of nephropathy in diabetic subjects and it was shown to be common in DN subjects [50]. High glucose (HG) induces intracellular reactive oxygen species (ROS) via glucose metabolism and is considered a causal factor of endothelial dysfunction in diabetic patients [51]. However, because the

glycocalyx location is the interface between the endothelium and blood and as it is an important component in permeability, any direct damage to the glomerular endothelial glycocalyx would lead to albuminuria. For example, Salmon *et al.* suggested that loss or dysfunction of the endothelial glycocalyx contributes to increased microvascular permeability leading to albuminuria [44]. Glycocalyx damage is seen when tissues are ischaemic or hypoxic or under stress in diabetes or renal disease [52]. Figure 1.4 shows compounds or agents that can disrupt the glycocalyx.

One of the earliest findings suggested the presence of a glycocalyx in culture, and the ability to disrupt it by enzymatic degradation. *In vitro* experiments on GEnC demonstrated that enzymatic removal of the glycocalyx increases albumin passage across the GEnC monolayer [30]. Hence, changes in the level of these enzymes in GEnC would affect the structure and function of the endothelial glycocalyx and therefore could have significant physiological effects. This has been shown in a study using tumor-necrosis factor (TNF-α) which induced the shedding of SDC1 and HS through the release of proteases and heparanases causing an increase in vascular permeability [53].

Heparanase is a glucuronidase that is widely expressed in health and can be further induced during inflammatory responses [54]. It specifically cleaves the carbohydrate chains of HS to release smaller HS fragments. In *in vitro* podocytes and GEnC, heparanase secretion is increased in response to aldosterone and angiotensin II (AngII) which could be inhibited by spironolactone [55]. It has also been shown that heparanase is upregulated in several renal pathologies such as DN, IgA nephropathy and membranous nephropathy as well as in patients suffering from glomerular diseases [56].

Matrix Metalloproteinases (MMPs) are a family that contains up to 24 functionally and structurally related enzymes [57]. They all share a highly conserved catalytic domain

containing a zinc binding region. MMPs are generally expressed at a low level in health, however inflammatory cytokines such as TNF-α growth factors and hormones can all lead to rapid increase of the proteases [58]. They have been found to cleave syndecan ectodomains in human embryonic kidney cells [59, 60] and in CiGEnC [35]. The latter study demonstrated the effects of disruption of the glycocalyx by TNF-α showing an increase in SDC4 and HS mRNA levels in the culture medium, suggesting a TNF-α-induced shedding. Therefore, this highlights the fact that SDC4 proteoglycan can be targeted in future studies with the aim of restoring the glycocalyx by specific drugs [35]. Butler *et al.* have also shown that salt and aldosterone act together to damage the glycocalyx *in vitro* by reducing HS and SDC4 shedding [61]. While *in vivo*, MMP2 activity was increased with salt and aldosterone and this caused albuminuria. Finally, MMP-inhibitors preserved the glycocalyx and prevented albuminuria [61].

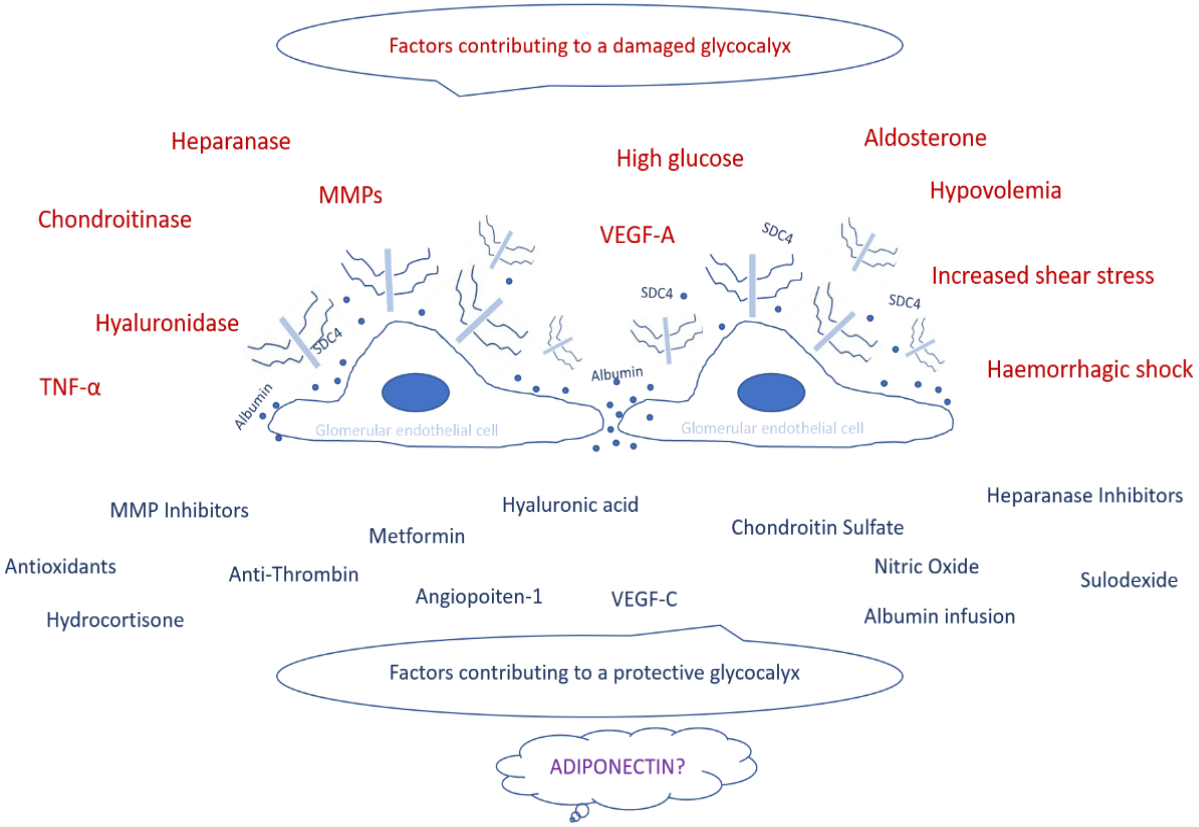

Figure 1.4 Factors damaging and protecting the endothelial glycocalyx

An illustrative drawing of GEnC with factors that damage the glycocalyx (upper part) layer and factors that help restoration of the glycocalyx (lower part). Damaging factors include VEGF-A (vascular endothelial growth factor A) and tumor necrosis factor A (TNF-α). Restoration factors include vascular endothelial growth factor C (VEGF-C)

1.1.4.5 Mechanisms of glycocalyx protection

A critical question for the field is "how can the endothelial glycocalyx be protected?" Some of the possible treatments are also listed in the above figure (Figure 1.4). Schött *et al.* in a review, listed a few of the possible treatments that were linked to protecting the glycocalyx [62]. Searching through the literature, a few drugs have been shown to have the ability to either increase the synthesis of the glycocalyx or prevent its enzymatic degradation. For example, polyethylene glycol, NO [63], and TNF-α inhibitors [64] were used as antioxidants in animal studies, and these agents reduced oxidative stress, thereby

reversing glycocalyx damage. Several drugs used in renal medicine have recently been found to modify the glycocalyx directly [53, 65]. For example, sulodexide, a mixture of low-molecular weight GAG (heparan (80%) and dermatan sulfate (20%)), has been used to treat microvascular complications in diabetic patients [77]. Sulodexide can be given orally and is thought to provide precursors for GAG synthesis. Even though sulodexide therapy has been shown in increase depth of glycocalyx after 2-month therapy in diabetics, however, no longer lasting renal protection was detected in another study [66]. Unfortunately, there are only a few clinical studies targeting specifically the glycocalyx under hyperglycaemia/diabetes. However, several drugs that have been used clinically could mediate some of their effects through protecting the glycocalyx. For example, hydrocortisone, a glucocorticoid secreted by the adrenal cortex, binds to the glucocorticoid receptor and triggers a variety of important cardiovascular, metabolic, immunologic and homeostatic effects [67]. It has been shown by Chappell and co-workers that it is able to preserve the glycocalyx in coronary capillaries, sustaining the vascular barrier and reducing interstitial edema [67]. The same group demonstrated that hydrocortisone is also able to prevent glycocalyx shedding induced by TNF-α [53].

Overall, targeting to restore the glycocalyx would be a very intriguing approach to use because, to date, there are no medicines that can effectively and definitively rescue patients from diabetic complications. Experimentally, restoration of the glycocalyx has been demonstrated to be achievable and effective in blood vessels outside of the kidney in the coronary [84], pulmonary [85] and mesenteric microcirculation [16]. We have also demonstrated that the glycocalyx can be restored in glomeruli [46, 66, 86] which is key in targeting albuminuria.

1.2 Adipokines

1.2.1 Adipokines history

Interest in adipose tissue has increased noticeably over the last 2 decades. Traditionally, this tissue was considered as just a storage organ for fat and energy. However, it has since been shown to be a metabolically active organ that has endocrine capabilities by synthesizing a wide range of hormones that regulate energy homeostasis along with other diverse biological functions [68]. There are two types of adipose tissue known as white and brown. The latter is mainly for thermogenesis and is almost absent in human adults. However, white adipose tissue functions are wider and much broader than brown adipose tissue [68]. The importance of white adipose tissue as an endocrine organ is underlined by the wide metabolic consequences of adipose tissue deficiency and excess [69]. A major step forward in the acknowledgment of the endocrine activity of adipose tissue occurred with the discovery of leptin in 1994 [70]. Indeed, the list of adipocyte-derived factors has been growing at an extraordinary pace. This unlocked a whole field of adipokines that have pro-inflammatory and anti-inflammatory factors [70]. Although different adipocytes, such as leptin, have been identified and characterized, further evaluation is required to define their physiological roles. Adipokines are diverse in the aspects of function and structure. They can be grouped according to primary function; classical cytokines such as TNF-α and interlukin-6 (IL-6), chemokines, proteins which form part of the fibrinolytic system (PAI-1), proteins for blood pressure regulation (angiotensin), glucose homeostasis and insulin sensitivity (adiponectin and omentin) and regulation of appetite and satiety (leptin and vaspin) (Figure1.5) [68]. Table 1 summarizes the main functions of the above listed adipokines [71].

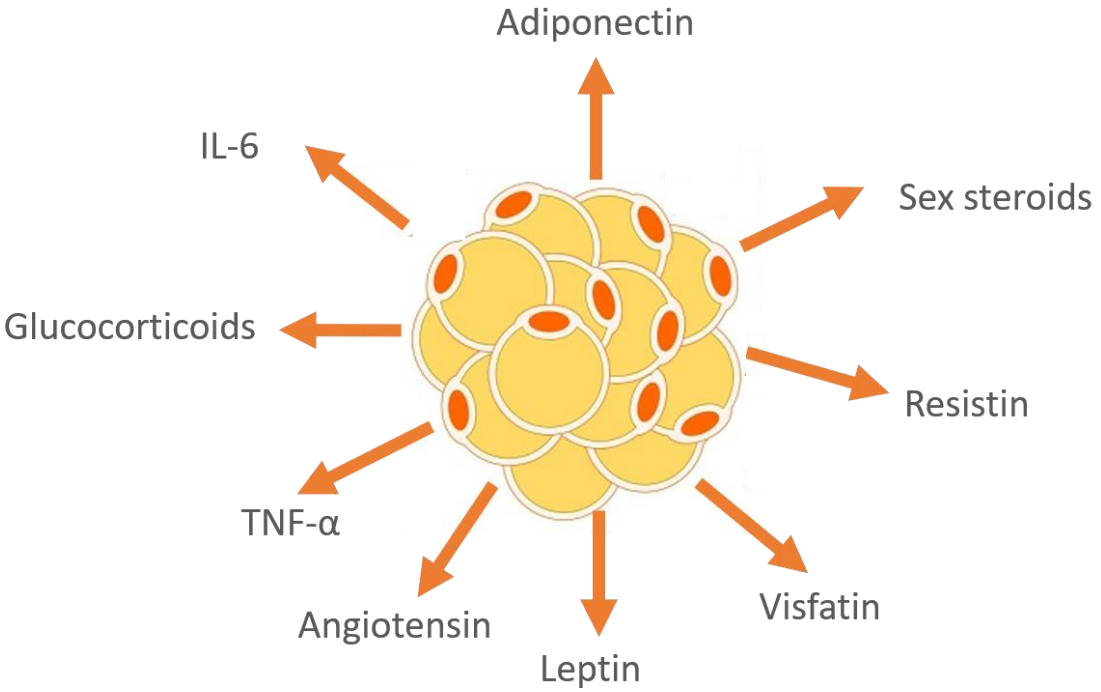

Figure 1.5 List of Adipokines

An illustrative figure that shows the different adipokines that the adipose tissue secretes such as adiponectin, IL-6, resistin, leptin, angiotensin and sex steroids. Adapted from [68].

A promising adipokine known as adiponectin, has an important role in metabolic processes such as carbohydrate and fatty acid metabolism [72]. Adiponectin also helps in the maintenance of normal function of blood vessels and protects against functional and structural disorders such as endothelial dysfunction and atherosclerosis. Clinical studies have demonstrated that hypoadiponectinemia is closely related to endothelial dysfunction, obesity and diabetes [73]. However, the link between adiponectin and DN has not been extensively studied. Specifically, the effect of adiponectin on GEnC and its glycocalyx in limiting albuminuria (thereby considering adiponectin as an endothelial glycocalyx therapeutic tool).

In cultured non-glomerular endothelial cells, adiponectin has been shown to exhibit various anti-inflammatory effects, in particular those that counter the adverse cellular

influences of increased oxidative stress or stimulation with cytokines such as TNF-α [74, 75]. Another adipokine, TNF-α, was used in our model of experimental diabetes *in vitro*. TNF-α is thought to disrupt the GEnC barrier through effects on components of the glycocalyx such as SDC4 [35]. Therefore, I am going to focus on two adipokines, adiponectin (anti-inflammatory) and TNF-α (pro-inflammatory).

1.2.2 Tumor necrosis factor-α (TNF-α)

TNF-α is a major pro-inflammatory cytokine that is produced by adipocytes, macrophages, mast cells and other immune cells and is capable of the induction of other inflammatory cytokines and chemokines. It is a 26kDa transmembrane protein that undergoes cleavage by a metalloproteinase known as a disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) to release a 17kDa biologically active protein [76]. TNF-α itself inhibits leptin release from adipocytes [77]. It was one of the first adipose derived proteins that was dysregulated by obesity, inflammation and diabetes [78]. Hence, studies have shown that the mRNA levels of TNF-α are increased in metabolic disorders and positively correlate to insulin resistance; this is because TNF-α can impair insulin signalling in adipocytes [79]. Other studies have shown that chronic exposure to TNF-α decreased insulin-stimulated glucose uptake [75]. However, neutralizing TNF-α receptors improved insulin sensitivity in mice but not humans [75, 80]. In hepatocytes, TNF-α reduces expression of genes involved in glucose uptake and metabolism through protein phosphatase 2C and increases the expression of genes involved in de novo synthesis of cholesterol [81]. TNF-α exerts several effects and it can contribute to the development of DN through several mechanisms. In the kidney, substances such as Ang II and AGEs can increase the synthesis of TNF-α [82]. Increased production of TNF-α can also produce oxidative stress, through the activation of nicotinamide adenine dinucleotide phosphate,

(NADPH) in mesangial cells. Finally, TNF-α appears to have a direct apoptotic and cytotoxic effect on glomerular cells [83, 84].

Adiponectin	Insulin-sensitizing, anti-inflammatory and anti-atherogenic
Adipsin	Involved in the alternative complement pathway
Angiotensinogen	Regulation of blood pressure
Apelin	Inhibits insulin secretion
Chemerin	Regulates adipogenesis
IL-6	Pro-inflammatory
Leptin	Regulates appetite and food intake; energy expenditure
Omentin	Anti-inflammatory
PAI-1	Inhibitor of fibrinolytic system
Resistin	Insulin resistance and inflammation
TNF-α	Pro-inflammatory
Vaspin	Decreases food intake; serine protease inhibitor
Visfatin	NAD synthesis through NAMPT; B cell function

Table 1 Functions of adipokines

A table showing the adipokines discovered and their main functions. IL-6: Interleukin-6, PAI-1: Plasminogen activator inhibitor-1. Adapted from [85].

1.2.3 Adiponectin

1.2.3.1 History of adiponectin discovery

After the discovery of leptin in 1994 and the fact that adipocytes do release specific hormones, Scherer et *al.* [86] identified a novel serum protein and named it Acrp30 (adipocyte complement related 30kDa protein) due to its similarity to complement factor

C1q, and stated that its exclusively made in adipocytes and its secretion is enhanced by insulin [86]. The discovery started with a northern blot analysis that showed over 100-fold induction during mice adipocyte differentiation at mRNA level. Mouse Acrp30 encodes a protein that contains 247 amino acids with a 28kDa molecular weight [86]. Adiponectin has also different names such as adipose most abundant gene transcript (apM1), AdipoQ [87] and gelatin binding protein of 28kDa (GBP28) [88]. The evolution of adiponectin and its widely known functions are depicted in Figure 1.6. As shown in the below timeline, it has been of great interest to study adiponectin in order to understand its relation to diabetes and diabetes-related diseases. Indeed, there are few adiponectin review papers that allowed me to extract some of adiponectin actions, especially ones related to DN [89, 90]. Hence the goal of this project is to decipher the role of adiponectin on GEnC and glycocalyx in health and disease as we understand further the mode of action of adiponectin via its receptors.

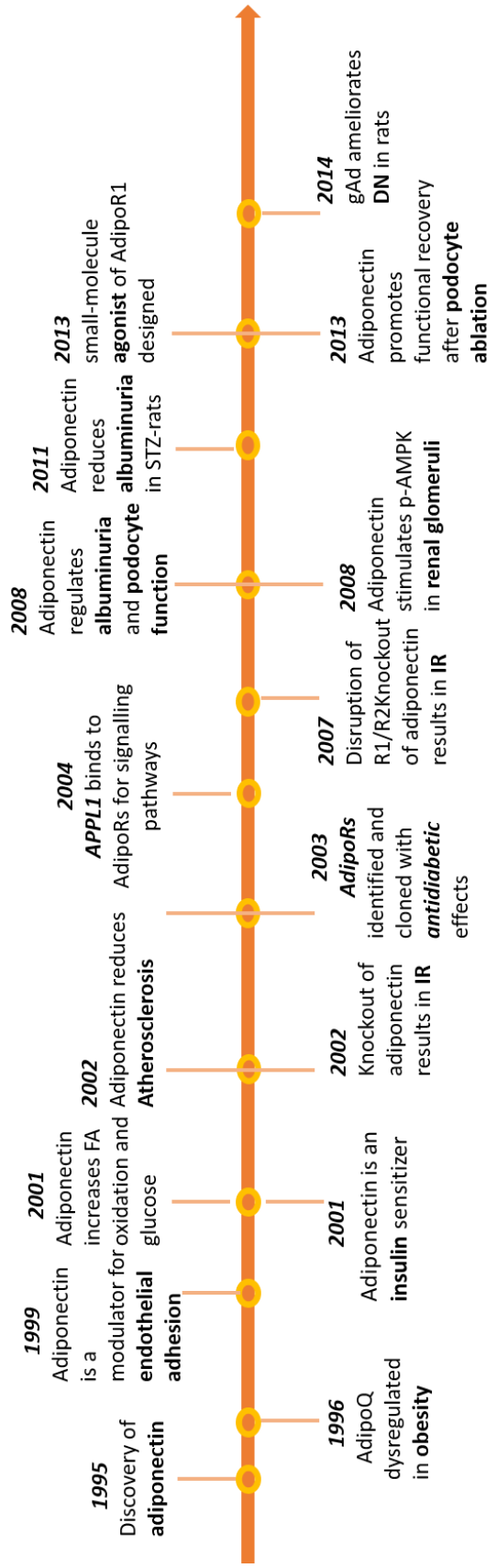

Figure 1.6 Timeline of the history of adiponectin and its main functions over the past 2 decades.

A timeline that shows the evolution of adiponectin functions from 1995 from its discovery to considering it to increasing FA oxidation and protecting against atherosclerosis in the 2000s. Finally, adiponectin's ability to reduce albuminuria in 2011 and protecting against DN in 2014.

1.2.3.2 Biological forms and structure

The human adiponectin gene is located on chromosome 3q27 and encodes a protein of 244-amino acids. It is composed of an amino-terminal collagenous domain signal sequence, a variable region and a carboxy-terminal globular domain. The basic form of the protein is a homotrimer of three 30kDa subunits (Figure 1.7) [91]. Post-translational modification plays a vital role for assembling adiponectin to form its functional oligomeric complexes. The first step of adiponectin multimerization is the formation of trimers through the non-collagenous globular domain. Then, disulphide bonds form between the collagenous domain of the trimers to become a low molecular weight (LMW) hexamers of 180kDa and a high molecular weight (HMW) of 16mers of >400kDa (Figure 1.7). Without the collagenous domain, the globular domain of adiponectin (gAd) can still trimerize but not into the higher order structures. The product of a proteolytic cleavage of a full length adiponectin (fAd) is gAd, that also circulates at physiological conditions and has biological activity [92]. All forms are found in the circulation but LMW and HMW are the predominant ones with the homotrimer nearly undetectable [89]. It circulates at high levels in human plasma accounting for approximately 0.01% (0.5-30μg/ml) of all plasma protein in normal individuals ~1000-fold higher than other hormones such as leptin and insulin [93]. Higher levels of adiponectin, specifically HMW, are found in females than males due to the amount of visceral fat [94].

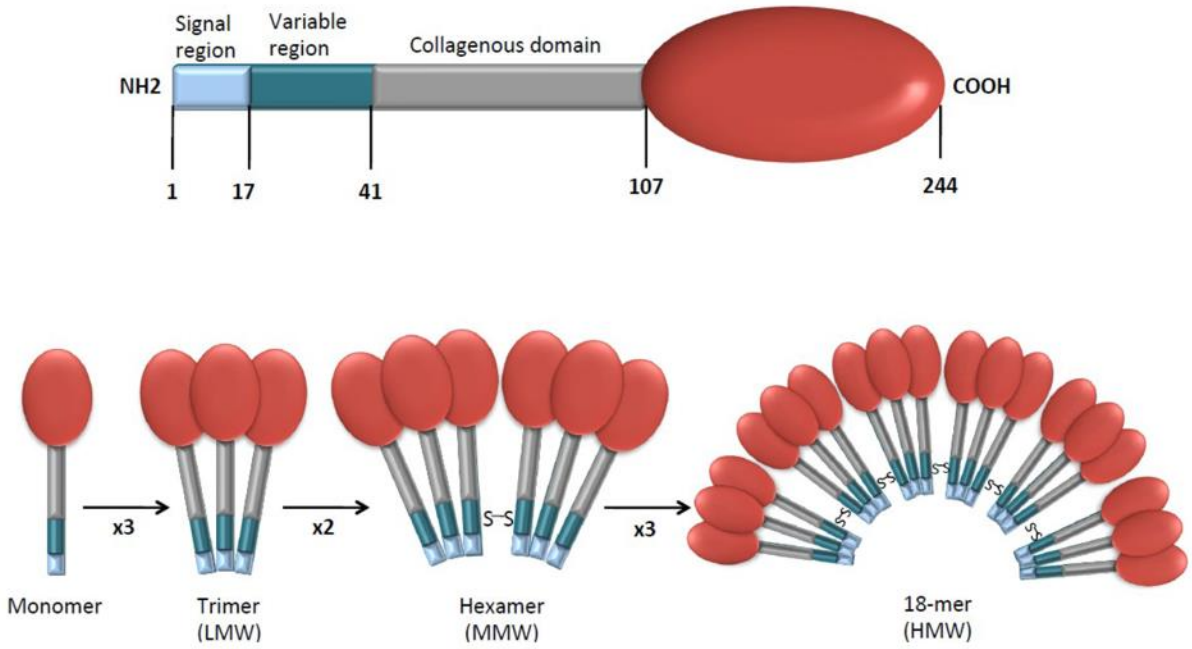

Figure 1.7 Adiponectin molecular structures and different isoforms

Monomeric adiponectin (upper panel) that consists of an amino-terminal collagenous domain and a carboxy terminal domain can trimerize to form low LMW adiponectin (lower panel). Two trimers can then combine to form middle molecular weight (MMW) hexamers. The trimers can form 12- or 18-mers with HMW. Figure adapted from [91].

Surprisingly, the 3-dimensional structure of gAd has a striking homology to TNF-α and thus adiponectin has been subsequently named as a TNF superfamily member [86]. Despite the lack of homology at the primary amino acid sequence level, the structural features between TNF-α and adiponectin are highly conserved [86]. Both proteins form bell-shaped homotrimeric oligomers, and the evolutionary relationship between adiponectin and TNF family proteins suggests that the human adiponectin receptor may also be a member of the TNF receptor superfamily. Different forms of adiponectin have relatively short half-life. The half-life of circulating adiponectin monomer is 75 minutes as reported in a study in mice, while HMW 16mers had the half-life at 83 minutes [95]. The concentration of adiponectin is between 2-20μg/ml in healthy individuals and is usually measured by ELISA. It is cleared from the circulation primarily by the liver and

secondarily by the kidneys. Urinary adiponectin levels are measured by the low complex forms that can pass through the normal functioning GFB such as the monomers and dimers (30kDa subunits) [96].

1.2.3.3 Physiological role of adiponectin

Adiponectin has important roles in anti-inflammatory responses and metabolic regulation. Nowadays, adiponectin is considered as a hormone by acting on peripheral target tissues through its receptors [89]. Adiponectin and insulin concentrations have been studied and established in different *in vitro* and *in vivo* models (mice, humans and other animals). Notably, adiponectin is known as an insulin-sensitizer, with anti-inflammatory and anti-diabetic properties [97]. Adiponectin administration in wild type mice significantly decreased serum glucose levels after 4 hours. Furthermore, in obese mice, adiponectin mRNA levels are significantly lower than in lean mice [87]. This was also seen in between lean and obese human fat samples [87]. Adiponectin also lowers hepatic glucose production in cultured rat hepatocytes through inhibition of the major enzymes in gluconeogenesis, thereby making the liver a major target tissue for adiponectin [97]. Serum and mRNA levels of adiponectin were also reduced in mice with hyperglycemia and hyperinsulinemia. Finally, administration of adiponectin in lipoatrophic diabetic mice ameliorated hyperglycemia and hyperinsulinemia [72]. One specific study in rhesus monkeys showed that adiponectin was lowered in an obesity model that develops T2DM. It was also noted that the decrease preceded the onset of diabetes in the obese monkeys [98].

The gAd can be purified from recombinant fAd by enzymatic cleavage using acetylated trypsin V from bovine pancreas [99]. A study was done to evaluate the effects of gAd and fAd in mice fed a high fat/sucrose diet. Administration of gAd decreased the levels of

glucose, free fatty acids (FFA) and triglycerides (TG) in mice fed a high fat/sucrose diet [99]. In contrast, fAd administration showed only transient effects on glucose, plasma FFA and TG. This suggested that purified gAd exhibited novel pharmacological properties for the regulation of glucose and lipid metabolism and that it is more potent than fAd [99]. Correspondingly, Yamauchi et *al,* also analysed which domain mediates the biological effects attributed to adiponectin [100]. They showed as well that gAd ameliorated hyperglycemia and hyperinsulinemia much more potently than fAd. Interestingly, they also recognized that gAd was present in serum in very low doses, suggesting that adiponectin undergoes cleavage to exert its effects [72]. Furthermore, chronic effects of adiponectin in *in vivo* models were generated by overexpressing adiponectin in transgenic obese mice (ob/ob). Globular adiponectin showed promising results including at least a partial amelioration of insulin resistance (IR) and diabetes [100].

1.3 Adiponectin receptors 1 and 2

1.3.1 Introduction of adiponectin receptors

Kadowaki *et al.* established a cDNA library of human skeletal muscle cells and identified a clone that had a strong affinity to gAd [92]. It was termed AdipoR1 for adiponectin receptor 1 and showed specific expression in skeletal muscle and liver. Human and mouse AdipoR1 share 96.8% identity. Another similar sequence was discovered and was termed AdipoR2 for adiponectin receptor 2 and it was exclusively found in the liver. Human and mouse AdipoR2 share 95.2% identity [92]. The two transmembrane receptors AdipoR1 and AdipoR2 are located integrally in the cell membrane with seven transmembrane domains. They differ from any other G protein-coupled receptors (GPCR) in that they have their C-terminal located extracellularly while their N-terminal located intracellularly. This

opposite topology suggests that the AdipoRs represent a novel class of receptor structure [92].

AdipoR1 is highly expressed in skeletal muscle and has high affinity for gAd while AdipoR2 is highly expressed in the liver and has intermediate affinity for fAd and gAd [101]. As the isoforms have distinct distribution patterns in various tissues, it is likely that the biological effects of adiponectin will be tissue-specific [102]. AdipoR1 is shown to also be expressed in the different cell types of the kidney; GEnC, mesangial cells, epithelial cells, proximal tubular cells and podocytes [103, 104]. AdipoR2 is expressed in all kidney cell types but to a lesser extent than AdipoR1.

An adaptor protein containing pleckstrin homology domain, phosphotyrosine binding (PTB) domain and leucine zipper motif (APPL) is considered one of the first identified adiponectin receptor binding proteins [105]. It has the ability to associate with both receptors through its PTB domain and therefore activates several downstream signalling pathways that exert metabolic effects in liver, muscle and endothelial cells [105]. APPL1 expression was decreased in obese subjects and any deficiency of it will lead to systematic insulin resistance. Interestingly, a whole-body knockout of APPL1 impaired adiponectin signalling and resulted in insulin resistance [106]. Therefore, it is important to note that APPL1 is a critical mediator of adiponectin action.

1.3.2 Physiological and pathophysiological role of AdipoRs

One of the earliest findings about the actions mediated by adiponectin receptors was shown in 2 different cell lines, 293T cells (human embryonic kidney cells) and C2C12 myocytes (mice myoblast cells). Both cell lines expressed AdipoR1 and AdipoR2 on the

cell surface that enhanced binding of adiponectin [107]. Treating C2C12 myocytes with either gAd or fAd for 7h stimulated fatty-acid oxidation and glucose uptake. However, when AdipoR1 was silenced by specific small interfering siRNA, it greatly reversed the above mentioned actions only when gAd but not fAd was administered suggesting that gAd's actions are mediated through AdipoR1 [107]. Another study by Yamauchi and colleagues suggested that adiponectin receptors are decreased significantly in the liver of diabetic (db/db) mice compared to wild type mice [108]. Moreover, restoring AdipoR1 by an adenovirus by either 1.5 or 5-fold significantly improved insulin resistance and ameliorated diabetes in a dose-dependent manner. However, in adiponectin deficient db/db mice in which there was a disruption of the coding region of adiponectin, the restoration effect of AdipoR1 by either 1.5 or 5-fold was lost suggesting that the effect of the adiponectin receptors was due to increased adiponectin signalling. Furthermore, AdipoR1-knockout mice showed significantly impaired glucose tolerance and higher insulin levels which are signs of insulin resistance [108]. A double knockout of AdipoR1 and AdipoR2 did not show reduced plasma glucose level when adiponectin was administered suggesting the importance of the receptors for the physiological properties of adiponectin *in vivo.* This study suggested that a downregulation of both receptors in obesity are involved in the development of insulin resistance and diabetes [108].

Therefore, it is important to understand the signalling pathways that are activated by the different forms of adiponectin via its receptors. While adiponectin actions are tissue-specific, skeletal muscles and liver are the main adiponectin targets. These actions, that were explained in detail in the previous part, are summarized as 1) increase in fatty acid oxidation and glucose utilization in skeletal muscle [109], 2) reduced TG content in the liver and muscle and improved in vivo insulin sensitivity. Particularly, adiponectin has anti-

apoptotic effects in cardiac myocytes [110] and pancreatic β-cells [111], and alleviates oxidative stress in endothelial cells [112] and podocytes [113].

1.4 Adiponectin signalling pathways

1.4.1 The AMPK pathway

A major signalling pathway of adiponectin is through the stimulation of 5' AMP-activated protein kinase (AMPK). AMPK is a heterotrimeric kinase that has a catalytic subunit alpha and 2 regulatory subunits Beta β and Gamma γ [114]. AMPK is a serine/threonine kinase known also as a stress kinase and is regulated by the ratio of AMP:ATP [109]. It is considered as a cellular sensor and a major metabolic switch to initiate catabolic processes by activating ATP-producing pathways such as FA oxidation and shutting down anabolic processes such as hepatic gluconeogenesis. Other than AMP, AMPK is activated by several factors such as vigorous exercise, heat shock, starvation and hypoxia [115]. The catalytic alpha subunits contain 2 isoforms (α1 and α2) and they are expressed in different tissues. The AMPKα1 isoform is mostly predominant in skeletal muscle and adipose tissue, whereas the α2 isoform is expressed in higher levels in cardiomyocytes [116]. Remarkably, endothelial cells exhibit both α subunits with α2 being the predominant one.

The AMPK pathway can be activated by its two major upstream kinases, the liver kinase B (LKB1) and Ca^{+2}/calmodulin-dependent protein kinase kinase (CaMKKB). It is translocated from the cytosol to cell membrane to be activated. LKB1 is a serine/threonine and tumour suppressor kinase that has been shown to activate AMPK in response to stress by translocating it to cytosol [117]. LKB1 not only regulates AMPK but also 12 other kinases. In cells missing LKB1, such as HeLa cells, there is still some basal AMPK activity [118]. However, this can be increased by addition of a calcium and

therefore, the CaMKKB kinase can be responsible for AMPK activation. The anti-diabetic drug metformin has shown to increase and lead to the phosphorylation of LKB1 in endothelial cells [119].

The main role of AMPK is to regulate energy homeostasis by balancing between glucose and lipid metabolism [114]. To date, there are few known activators of AMPK. Most importantly, a commercial AMPK agonist known as 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) mimics the effects of AMP as permeable precursor to ZMP (AICAR monophosphate) and binds to the γ subunit of AMPK [120]. Also, metformin is known to activate AMPK to increase insulin sensitivity and glucose production. Other naturally occurring compounds include resveratrol which has also been shown to lead to AMPK activation [121]. One of the downstream effects of AMPK is the phosphorylation of acetyl CoA carboxylase (ACC) and hence inactivation of a metabolic rate-limiting enzyme involved in FA synthesis in which acetyl CoA is converted to malonyl CoA. Another downstream effect of AMPK is the phosphorylation of e-NOS thus stimulating NO production in endothelial cells, leading to beneficial vasoprotective effects [112].

Recent studies indicate that adiponectin stimulates the phosphorylation and activation of AMPK in skeletal muscle liver, endothelial cells and adipocytes leading to the regulation of glucose and fatty acid metabolism [120].

One of the major complications of diabetes is cardiovascular diseases and studies have shown that over two thirds of diabetic patients develop heart disease. Therefore, it is not surprising that adiponectin is involved in the regulation of cardiac function under diabetic conditions. One of the early studies by Walsh and colleagues found that adiponectin deficiency leads to worsening of cardiac hypertrophy [122]. AMPK activity is also stimulated in the heart by adiponectin while suppressing extracellular signal-regulated

kinases (ERK) activation. Overproduction of adiponectin by adenovirus ameliorates cardiac hypertrophy and cardiac function [122]. *In vitro* studies have also shown that adiponectin reduces cardiac cell growth thereby highlighting the role of adiponectin in cardiomyocytes [122]. HUVEC are human umbilical vein endothelium cells that are widely used in *in vitro* studies. Treatment of gAd in HUVEC induced AMPK phosphorylation at the α1 subunit and it was maximum after 15 minutes [123].

In the kidney, the pattern of AMPK catalytic subunit expression shows that α1 is the predominant α isoform [115]. Although the regulatory β2 is predominant in rat kidneys, the β1 is the dominant form in mice [124, 125]. Specifically, it was shown that the α1 subunit of AMPK is ubiquitously expressed throughout the kidney. Activation of the AMPK pathway appeared to be of importance in the maintenance of normal renal physiology [126]. Many factors are involved in the regulation of renal AMPK including salt and water, adiponectin, diabetes, ischemia, inflammation and endothelial function. Our focus here is adiponectin and its relation to renal AMPK [115]. The AMPK catalytic subunits α1 and α2 subunits are expressed on mesangial, glomerular endothelial cells and podocytes [103].

Sharma *et al.* studied the AMPK pathway in conditionally immortalized mouse podocytes [113]; gAd treatment increased phosphorylation of AMPK in normal glucose conditions. Glucose concentration of 25mM (considered as HG) reduced AMPK activity, and this was reversed by treatment with gAd [113]. *Ex vivo* treatment of isolated rat glomeruli was also carried out to assess the role of gAd on AMPK activity. Indeed, the phosphorylation of AMPK was effectively increased by gAd as well as AICAR [103]. The effect of adiponectin on AMPK activity was also evaluated on human mesangial cells that were treated with adiponectin, and the phosphorylation of AMPK peaked at 15min [127]. These data show

that adiponectin can switch anabolic processes and control oxidative stress in the glomerulus through the AMPK pathways in order to contribute to normal renal function.

Since there is little work on the role of adiponectin on GEnC, one of the main goals of this thesis is to determine the effect of adiponectin on different signalling pathways including the AMPK pathway in the CiGEnC and isolated glomeruli. Figure 1.8 shows an illustrated diagram of pathways of how adiponectin mediates a cascade of signalling pathways, and how it is related to the insulin signalling pathway.

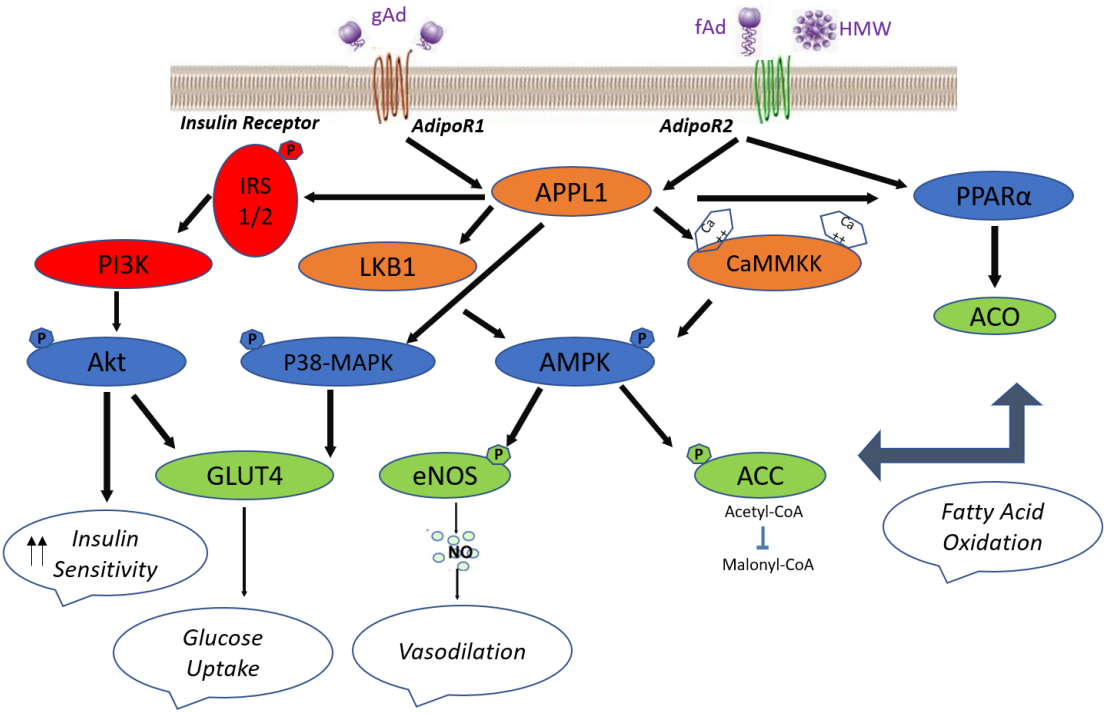

Figure 1.8 Schematic representation of adiponectin signalling pathways.

A Schematic diagram of adiponectin signal transduction associating a cross talk with the insulin signaling pathway: Adiponectin and insulin interact with their respective receptors, which prompt a cascade of signaling events. Metabolic actions of insulin are largely carried out by PI3K/AKT pathway, resulting in increased lipogenesis and glucose uptake via GLUT4. The interaction of adiponectin with its receptors AdipoR1/2 starts with binding with APPL then its upstream kinases (LKB1 and CaMMKK) that results in the activation of multiple signaling pathways including IRS1/2, AMPK and p38 MAPK. This increases FA oxidation through ACC, vasodilation through e-NOS activation and glucose uptake via p38 and Akt. AdipoR2 also activates the peroxisome proliferator-activator alpha (PPARα) to further activate acyl CoA oxidase (ACO). Adapted from [128]

1.4.2 The PPARα pathway

Another key regulator in lipid metabolism is the peroxisome proliferator-activator alpha (PPARα). It is a nuclear receptor protein that is mostly expressed in tissues that derive most of their energy from fatty acid oxidation such as liver, skeletal muscle, heart and kidney [114]. AdipoR2 appears to be related more closely with the activation of PPARα pathways that encourage energy dissipation and the inhibition of oxidative stress. AdipoR2 is expressed at higher levels in liver and adipose tissue. Adiponectin can drastically increase the expression and activity of PPARα thereby promoting fatty acid oxidation in skeletal muscles [107]. Similarly, in the liver, adiponectin also upregulates different PPAR-α target genes including CD36, which mainly modulates fatty acid uptake and metabolism [107]. Moreover, AdipoR2-induced activation of PPARα promotes fatty acid catabolism by upregulating genes involved in fatty acid transport, binding and activation, and peroxisomal and mitochondrial fatty acid oxidation. PPARα-mediated gene transcription enhances mitochondrial oxidative capacity to reduce oxidative stress and further contributes to decreased lipid accumulation in the target organ [44]. However, in this project, the PPARα pathway will not be investigated due to its limited information of PPARα signalling in the kidney.

1.4.3 The p38 mitogen activated protein kinase (MAPK) pathway

The p38 mitogen activated protein kinase (MAPK) is a major kinase in the MAPK family and studies suggest that this pathway also plays a role in adiponectin signalling [129]. The p38 MAPK pathway is activated by inflammatory cytokines and plays a vital role in activating immune responses [129]. Hypoxia and metabolic stress such as muscle

contraction are known activators for p38 MAPK activity. Similarly, AMPK is also activated during most of these physiological conditions, thereby suggesting a crosstalk between AMPK and p38 MAPK pathways. Yamauchi *et al*. showed that gAd increased the phosphorylation of AMPK and ACC and p38 MAPK in C2C12 myocytes [107]. Consistently in an independent study, it was also shown that in C2C12 myocytes, adiponectin stimulated not only the phosphorylation of AMPK but also p38 MAPK, thereby promoting glucose uptake and fatty acid oxidation in muscle cells [105]. The p38 MAPK pathway has not been studied thoroughly in the kidney in relation to adiponectin, so in this thesis I will investigate whether gAd mediates p38 MAPK activation in CiGEnC.

1.4.4 The Akt pathway

Insulin binds to its main membrane receptors and exerts its biological actions via the phosphatidylinositol 3-kinase (PI-3 kinase) and MAPK signalling pathways. This leads to the membrane translocation of glucose transporter 4 (GLUT4) and glucose uptake in skeletal muscles [24]. Activation of the PI3K/Akt pathway controls several downstream functions, which may be dependent on cell type [130, 131]. The anti-apoptotic responses mediated by increased Akt signalling may also involve the regulation of mitochondrial processes [132].

The effects of adiponectin on Akt phosphorylation has also been shown to be tissue specific. In cultured cardiomyocytes, adiponectin pre-treatment protected the cells from a palmitate-induced apoptosis. Hence, this shows that adiponectin can activate Akt pathway in cardiomyocytes to prevent apoptosis [133]. Furthermore, in cultured pancreatic β-cells, adiponectin also stimulates Akt phosphorylation as well as insulin, used as a positive control [134]. Finally, in skeletal muscle, adiponectin induces tyrosine

phosphorylation of insulin receptor substrate 1 (IRS1) and Akt, thereby increasing insulin sensitivity [135]. To date, the effect of gAd on phosphorylation of Akt has not been integrated in renal studies and specifically albuminuria. Therefore, the effects on the Akt pathway will be examined in response to gAd in CiGEnC.

1.5 The role of adiponectin in the kidney

It has been well-established the relationship between adiponectin and metabolic diseases such as obesity, type 2 diabetes and cardiovascular diseases [136]. The main question here is how adiponectin is related to albuminuria and the development or progression of diabetes into DN? Since albuminuria is considered as an early sign of progressive renal disease with or without diabetes, the influence of adiponectin on the initiation of albuminuria is of high clinical significance. Throughout the literature, there are only a few studies deciphering the role of adiponectin in renal physiology [137], and a few examples and studies that have tried to link adiponectin and renal diseases will be discussed.

The low molecular weight of adiponectin monomers and dimers is small enough to cross a functioning GFB, thereby adiponectin can be quantified in urine of healthy subjects [96]. However, patients with albuminuria also have adiponectin trimers in their urine due to increased GFB permeability, and it was reported that also HMW adiponectin is detected in proteinuria subjects [104]. Adiponectin has been implicated in exerting beneficial renal effects against the development and progression of albuminuria in diabetic and non-diabetic renal diseases [113, 138, 139]. More precisely, the effects were through the activation of AMPK in the kidney. Low adiponectin levels prior to a kidney transplant is a major risk factor to developing new-onset diabetes. Obesity and low adiponectin

predisposed the patients in developing diabetes. Hence, this also shows the protective effect of adiponectin in patients receiving renal transplantation [140].

Sharma *et al*. were one of the first to determine the importance of adiponectin on albuminuria [113]. Compared to wild type mice, adiponectin deficient mice demonstrated increased albuminuria, but this was normalised when adiponectin was administered. Specifically, they examined glomerular podocytes in the knockout mice model, and found that their foot processes were fused but that they regained their normal foot process architecture with adiponectin [113]. Also, through an *in vitro* differentiated podocyte monolayer, albumin permeability was greatly reduced by the addition of adiponectin. Secondly, Ohashi *et al.* studied partial nephrectomy (5/6) in which there is hypertrophy, podocyte injury, glomerular fibrosis and eventually proteinuria [138]. Adiponectin knockout mice with partial nephrectomy worsened these features but exogenous adiponectin attenuated the adverse changes in renal structure [138]. A different model of kidney damage in podocytes was induced by apoptosis through targeted activation of caspase-8 (POD-ATTAC) [141]. These POD-ATTAC mice exhibited aspects of human renal disease, such as foot process effacement, mesangial expansion, and glomerulosclerosis [141]. These mice were crossed with either mice lacking or overexpressing adiponectin. POD-ATTAC knockout adiponectin mice developed albuminuria and renal failure; conversely, the POD-ATTAC overexpressed adiponectin mice recovered more rapidly and exhibited less interstitial fibrosis. Finally, this shows that adiponectin is a renoprotective protein even after podocyte injury [141]. Table 2 shows other examples of the expression of adiponectin and receptors in different diseases, some of which were explained above [90]

	Location	Diseases	Expression
Adiponectin	Serum/plasma	Lipodystrophy patients	↓
		ASCVD patients	↓
		Metabolic syndrome	↓
		Obese mice	↓
		T2D patients	↓
		T2D patients with CAD	↓
		T1D patients	↑
		T1D patients with DN	↑
		T1D mice	↑
AdipoR1	Skeletal muscle	STZ-induced T1D mice	↑
		T2D mice	↓
	Heart	T1D mice	↓
		T2D rats	↓
	Renal tissues	T1D rats	↓
		T2D mice	↓
		Diabetic rat with DN	↓
		T2D mice with DN	↓
AdipoR2	Skeletal muscle	T2D patients ↓	↓
	Liver	STZ-induced T1D	No change
	Renal tissues	T1D rats	No change
		T2D mice with DN	NS
		T2D mice with DN	↓

Table 2 Changes in expression of adiponectin and receptors in patients or animal models [141]

The relationship between adiponectin and metabolic diseases such as obesity, T2D and cardiovascular diseases is well-established. A critical question here is how adiponectin

related to albuminuria and the development and progression of DN? Since albuminuria is considered as an early sign of progressive renal disease with or without diabetes, the influence of adiponectin on the initiation of albuminuria is of high clinical significance. Throughout the literature, there are only a few studies deciphering the role of adiponectin in renal physiology. It has been shown that hypoadiponectinemia in T2D patients will possibly predispose them to albuminuria because of the decreased renoprotective effects of adiponectin [142, 143]. That is, the lower the adiponectin levels, the higher the possibility of progression to albuminuria [98]. Of many cytokines, adiponectin, but no other inflammatory markers, has been shown to be significantly related to development of diabetes in Indians and Japanese subjects [144, 145]. These findings propose that hypoadiponectinemia directly contributes to the regulation of glucose homeostasis and decreased insulin sensitivity observed in diabetes. The table below (Table 3) [90] outlines the different prospective studies done in animal models, whether diabetic or obese and how adiponectin and/or receptors levels are correlated between these groups.

	Changes	Models	Biological activities
Adiponectin	Deficiency	Adiponectin-deficient mice	Insulin resistance with glucose intolerance
		Akita/ Adiponectin-deficient mice	Exaggerated inflammatory response; renal hypertrophy and fibrosis
		AdipoQ gene knockout	Increased albuminuria and fusion of podocyte foot processes; reduced oxidative stress
		Subtotal (5/6) nephrectomy in AdipoQ gene knockout	Glomerular hypertrophy; tubulointerstitial fibrosis
		Podocyte-ablation mice crossed with AdipoQ gene knockout	Irreversible albuminuria and renal failure
	Over-expression	Transgenic mice with overexpression of Adiponectin	Downregulation of proinflammatory factors
		Injected adenovirus adiponectin in db/db mice	Improved endothelial-dependent vasodilation; decreasing superoxide production
		Podocyte-ablation mice crossed with AdipoQ gene knockout	Recovery from kidney damage, foot process effacement, mesangial expansion, and glomerulosclerosis
AdipoRs	Deficiency	AdipoR1/2 double knockout mice in leptin deficient mice	Increased tissue triglyceride content; increased inflammation and oxidative stress; insulin resistance and marked glucose intolerance
	Functional activated	db/db mice stimulated by AdipoR agonist	Ameliorated reduction of insulin sensitivity and glucose tolerance; decreased inflammation; reduced oxidative stress

Table 3 Functions of adiponectin and receptors in diabetic animal models

Nakamaki *et al*. injected an adenovirus to overexpress adiponectin in streptozotocin (STZ) diabetic-induced rats to show that proteinuria was reduced in comparison to rats with no overexpression of adiponectin [139]. They also demonstrated that nephrin, a crucial podocyte protein for proper GFB function, was increased in the overexpressed mice. It is noteworthy to say that low nephrin contributes to the development of proteinuria in diabetic nephropathy. Therefore, the upregulation of nephrin mRNA appears to be redirecting adiponectin in a pathway to decrease albuminuria.

These above-mentioned studies are a starting point to demonstrate the beneficial effects of adiponectin regarding the reduction of albuminuria. Although these were carried out in rodent experimental settings, the relevance to the human situation should be interpreted with care. However, not all these studies were representative of a T2D model that develops nephropathy, our long-termed aim is to show adiponectin effects in a T2D model with progressive albuminuria. Hence, adiponectin will act directly on GEnC to protect the glycocalyx from further damage and alleviate albuminuria.

1.6 Rationale of the study

The burden caused by hyperglycemia and insulin resistance on T2D can result in immediate metabolic injuries such as tissue inflammation and oxidative stress in the kidney [146]. This burden can result in one of the major complications of diabetes. Thus, the prevention of an increased GFR and albuminuria is of great interest in providing for new therapeutic targets for DN. Despite massive efforts to discover the key mechanisms of DN, the clinical trials done to date have been unsuccessful. At present, angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are the only therapeutic agents approved for the treatment of DN [147]. However, it has been shown that the GEnC

glycocalyx, which forms part of a healthy GFB, is reduced in DN and that restoration of this structure may prevent further damage and leak of albumin [148]. Therefore, the present study focused on examining the potential of adiponectin as an agent capable of modifying the glycocalyx in DN models.

Adiponectin has recently gained publicity because of its antidiabetic and anti-atherogenic effects. Several studies have demonstrated that hypoadiponectinemia is associated with insulin resistance [142], endothelial dysfunction [73, 149], obesity [87], coronary heart disease [110] and hypertension [150]. It is also discussed that there is a downregulation of serum adiponectin in T2D patients that may predispose an individual to development of albuminuria [151, 152]. This suggests that replenishment with exogenous adiponectin may be a targeted and effective strategy for the prevention of progression of diabetes to DN.

Of adiponectin beneficial effects, its insulin sensitizing effect is the most studied. Adiponectin is known to increase glucose uptake, restore energy levels by increasing fatty acid oxidation and other major catabolic pathways [89]. Adiponectin actions are mediated through its major receptors AdipoR1 and AdipoR2. These receptors start a cascade of signalling especially through the AMPK signalling pathway which is also known to be modified in diabetic conditions. To date, the role of AMPK in the kidney acting through the GEnC has not been determined.

The fact that lower plasma adiponectin contributes to development of diabetic microvascular complications through endothelial dysfunction is still under investigation [153]. However, in the literature, different researchers have shown that adiponectin can exert beneficial renal effects in diabetes-induced models [72, 154]. Over expressing adiponectin resulted in amelioration of diabetes and albuminuria [126] whilst a knockdown of adiponectin worsened the symptoms and resulted in proteinuria [155] .

Protecting the endothelial glycocalyx specifically is an exciting novel approach to limit the extent of DN and hence albuminuria. It has been shown that several agents can protect against damage to the glycocalyx in GEnC (whether in culture or in db/db animal models) [148, 156]. Although it has never been shown that adiponectin can affect the glycocalyx directly, the aim of this work is to prove that the components of the glycocalyx that are altered in diabetic conditions or models, can be modified and hence protected with adiponectin.

In conclusion, this project will elucidate the role that adiponectin plays on GEnC and its glycocalyx in health and disease. This will be directly assessed through its receptors to understand the adiponectin signalling pathway in GEnC. Moreover, the endothelial glycocalyx will be assessed particularly to define the damage that occurs in diabetes and whether adiponectin is a valid candidate to restore this damage.

1.7 Hypothesis

These observations have led to the hypothesis that adiponectin contributes to the maintenance of the GFB through direct actions on GEnC and its glycocalyx in experimental diabetes.

Specific objectives:

1. To define expression of adiponectin system components (adiponectin and receptors) in different cultured GEnC conditions and isolated glomeruli of a db/db mice model (**Chapter 3**)

2. To understand how adiponectin orchestrates its effects on cultured GEnC and isolated glomeruli through its receptors, thereby activating signalling pathways under normal and experimental conditions (**Chapter 4**)

3. To define the protective effects of adiponectin on the glycocalyx against experimental diabetes (**Chapter 5**)

Chapter 2 Material and Methods

2.1 Materials and chemicals

All materials were purchased from Sigma Aldrich unless otherwise stated. Antibodies and primers and other related chemicals are listed in the Appendix. Materials and methods of more specialised techniques are detailed in their relevant chapters

2.2 In vitro work

2.2.1 Conditionally immortalised glomerular endothelial cells (CiGEnC)

Bristol Renal has previously generated CiGEnC lines which were isolated from non-diseased kidneys that were not suitable for transplants [16]. Briefly, primary GEnC were transduced with a temperature sensitive simian virus 40 large tumour antigen (SV40LT) construct and human telomerase (hTERT). This allows immature CiGEnC to proliferate at temperature of 33°C and then become quiescent and fully differentiate at 37°C, a non-permissive temperature for 3-5 days before using them. Monolayers of GEnC were cultured in EBM-2MV media which contains basal medium (Lonza, #3156) supplemented with 5% foetal bovine serum (FBS) and the EGM2-MV bullet kit (Lonza, #4147) (hydrocortisone, fibroblast growth factor (FGF), insulin-like growth factor-1 (IGF-1), human epidermal growth factor (hEGF), and ascorbic acid.

2.2.2 Conditionally immortalised podocytes (CiPod)

Conditionally immortalized human podocyte cell lines (CiPod) have been also developed in our lab in which kidneys were isolated from non-diseased kidneys [157]. Podocytes were cultured in RPMI-1640 media with 5mM L-glutamine (Thermo Fisher Scientific). RPMI-1640 media was further supplemented with a penicillin/streptomycin antimicrobial agent at 1% of the final media volume (Sigma P4333) [157]. FBS at 10% of media volume (Sigma F9665) was also added into the media. These cells proliferate at 33^{o}C. In contrast to the GEnC, CiPod need 9-12 days to fully differentiate at 37^{o}C, in which they enter growth arrest and express specific protein markers of differentiated *in vivo* podocytes, including nephrin and podocin.

2.2.3 Cell culture

Tissue culture was performed under sterile conditions in a Microflow Biological Safety Cabinet (Holten Laminar; Thermo Scientific, Denmark). All solutions and equipment were purchased sterile from the manufacturer or sterilised by autoclaving. Materials and solutions that were not purchased in a pre-sterilized state were decontaminated via autoclaving at the University of Bristol in-house facility. Tissue culture plastic flasks, plates and serological pipettes were obtained from Greiner Bio-One and Corning (UK). Glass pipettes and tips were purchased respectively from Fisher and Star-lab (UK). Cells were maintained in incubators at either 33°C or 37°C with 5% CO_2 incubator for proliferation and differentiation respectively.

2.2.4 Passage of cells

If differentiation of the cells was not required, they were split or passaged when a confluency of 80% was reached. Cell culture media was aspirated from the culture flask and cells were washed twice with 3-4ml phosphate buffered saline (PBS) ((mM): 137.5 NaCl; 2.68 KCl; 10 Na_2HPO_4; 1.76 KH_2PO_4; pH 7.4, prepared in dH2O) before 2ml of 0.25% trypsin-EDTA for GEnC or 1% Trypsin-EDTA for podocytes was added. The flask was then incubated at 33°C for 3-5min until cells could be detached by gentle flask tapping (1-3min). Fresh media was then added to the flask at a 1:1 ratio and the cells were centrifuged for 3min at 1500g. The supernatant was discarded, and the pellet resuspended with 4ml of media for a ratio of 1:4. Every 1ml of the cell suspension was put in a new T75 plate with 9ml of media.

2.2.5 Cell freezing and thawing

The re-suspended cells from above were added to an equal volume of freeze solution (80% FBS and 20% dimethyl sulphoxide (DMSO, Sigma, # D8418) in a cryovial. This was frozen slowly using isopropanol filled boxes at -80°C. After 24-48h, cells were transferred to liquid nitrogen for long term storage. To revive cells, the cryovial was held under a warm tap to defrost quickly. As soon as it was thawed, cells were transferred to a flask containing appropriate culture media. The flask was incubated for at least 24h at 33°C to allow cells to attach before changing the media, as DMSO is toxic. Cells in culture were routinely examined using a Nikon TMS phase contrast inverted microscope and culture medium was changed every 2 to 3 days until the cells were sufficiently confluent for passage into new vessels.

2.3 Treatment of GEnC and podocytes

For the different cell treatments, serum-free conditions were applied eliminate any possible confounding factors in the serum. Therefore, treatments were set up using cells that had been subjected to serum starvation. Culture media was removed and replaced with serum-free media (SFM) 3h prior to treatment unless otherwise stated.

2.3.1 High glucose stimulation

When the CiGEnC and CiPod were ready for treatment, high glucose concentration was prepared at 20mM and 14mM respectively, to give a final concentration of 25mM. A media containing normal glucose concentration for the appropriate cell type brought up to 25mM glucose with the addition of L-glucose was used as an osmotic control. Cells were treated in a time-dependent manner for 2, 6 and 24h for short-term exposure and 2, 7 and 14 days for long-term exposure with high glucose unless otherwise stated. After stimulation, the cells were lysed as described in the upcoming section.

2.3.2 Globular adiponectin stimulation

In order to investigate the effects of human recombinant gAd (PeproTech #450-21) on CiGEnC/CiPod, a range of different concentrations were tested to determine the optimum dosage of gAd within CiGEnC. A gAd concentration of 2.5µg/ml was the optimum concentration to be used after the dose-dependent experiment. Control cells were not treated with adiponectin. The phosphorylation of different signalling proteins, including AMPK-α, ACC, Akt and p38 MAPK, within the cells in response to gAd was analysed by Western blot.

2.3.3 TNF-α stimulation

In order to disrupt the endothelial glycocalyx of cultured GEnC, TNF-α was used as a model of the inflammatory aspect of the diabetic milieu, as suggested by Ramnath *at al* [35], at a concentration of 10ng/ml. Similarly, a range of different time points (1, 2, 4 and 24h) were used to determine the optimum time frame needed for the glycocalyx to be disrupted by shedding its main components. Co-treatment of TNF-α and gAd was done by preparing both at final concentrations in the suggested volume of media.

2.4 Protein extraction

2.4.1 Cell lysate preparation

After the designated treatments were completed, cells were extracted at the end of a culture experiment to generate a protein lysate for use in Western blot. Culture media was removed from the cells using a pipette and the cells were rinsed twice with ice-cold PBS. The low temperature minimises the proteolysis, dephosphorylation and denaturation that occur at room temperature. A commercial protein lysis buffer known as cold radio immunoprecipitation assay buffer (RIPA) (ThermoFisher Scientific # 89900) was added at 150µl per 1 well of cells in a 6-well plate or 300µl for cells in T25 flasks. Protease inhibitor cocktail (Sigma Aldrich, #8340) and phosphatase inhibitor cocktail 2 (Sigma Aldrich #5726) was added to the flask (the ratio used was 10µl of protease and phosphatase inhibitor solution per 1ml of RIPA buffer as recommended by the supplier). The cells were then removed from the flask with a cell-scraper (Sarstedt), transferred to 1.5ml micro centrifuge tube (Fisher) and then centrifuged at 13000rpm for 10min at 4°C. The supernatant lysate was transferred into a fresh Eppendorf and then snap-frozen using liquid nitrogen and stored in a -80°C freezer.

2.5 Western blot

2.5.1 Introduction to Western blot

Western blot aims to separate proteins by size using SDS and individual proteins of interest are stained identified using specific antibodies. Equipment used for this technique was acquired from Bio-Rad Laboratories, Hemel Hempstead, UK unless stated otherwise. This technique was used to assess the effect of recombinant gAd on the phosphorylation of different signalling proteins in both GEnC and podocyte cultures. Vertical plate Western blot apparatus using a mini-gel system was used. Glass plates, combs and spacers were sprayed with 70% ethanol and dried thoroughly before assembly, ensuring that potential leaks were eliminated.

2.5.2 Preparation and loading of protein samples

Laemmli sample buffer was added to samples at a 1:4 ratio to the sample volume. Samples were then heated in a heat block at 90°C for 10 minutes to denature proteins before loading. The same volume of each sample was added to the wells of the gel alongside a single well loaded with approximately 3µl of a protein marker ladder (Blue Wide Range Protein Ladder, Cleaver scientific, #47). Electrophoresis was performed at 150V in 1x running buffer (see Appendix for recipe) until the dye front had reached the bottom of the gel.

2.5.3 Wet transfer

Proteins were transferred (transfer buffer, recipe in Appendix) from the polyacrylamide gel to a PolyVinylidine DiFluoride (PVDF, Millipore, UK) membrane using Trans-blot wet

blotting apparatus. The transfer was carried out at 240mA current for 90min. An ice pack was placed in the tank to prevent overheating of the system as well as a magnetic stirrer to maintain an even buffer temperature and ion distribution. Once transfer was completed (indicated by the visibility of the ladder marker on the transfer membrane) the membrane was removed and transferred into a box.

2.5.4 Immunoblotting

The membrane was then incubated in blocking solution (5 % BSA in 1x TBS-T (15.4mM Tris HCl pH 7.6, 137mM NaCl, 0.1% Tween 20)) for 1h at room temperature. Primary antibodies (list in Appendix) were then added after blocking. Primary antibodies were selected to target the protein of interest on the blot and were added to the membrane at a concentration of 1:1000 in BSA (3%) (unless otherwise specified) overnight at 4°C to reduce off-target binding. The membrane was then washed 5 times with TBST (each time for 5min) to remove unbound primary antibody. A secondary antibody (list in Appendix) (targeting the primary antibody) conjugated to horseradish peroxidase (HRP) was then made up at 1:10,000 in BSA (3%) and added to the washed membrane for 1h at room temperature. Excess secondary antibody was then removed with another 5 washes with TBST (each time for 5min).

2.5.5 Chemiluminescence imaging

Chemi-luminescence imaging was then employed. Luminal and Femto peroxidase (Western ECL Substrate, Biorad Clarity) were added in equal volumes (500µl each) to the membrane and the signal analysed using an Amersham imager 600 system. Any subsequent densitometry was performed using ImageJ 1.43m software.

2.6 Messenger RNA (mRNA) gene expression

2.6.1 Total RNA extraction from cultured cells

In order to investigate effects on gene expression at the mRNA level, RNA was extracted from cultured cells under the different conditions. Cell monolayers were lysed directly in 6-well plates or T25 or flasks once cells had been thermoswitched after reaching 80% confluence. Cells were washed once in sterile PBS and cellular RNA extracted using a RNeasy mini kit (Qiagen #74104) according to manufacturer's instructions. The samples were stored at -80^{o}C if not used immediately.

The concentration of RNA was calculated, and quality determined (A260/A280) by using a Nano drop system (Thermo Scientific, Waltham, USA). An A260/280 between 1.8 and 2.0 was considered a good quality of RNA concentration. The system was standardised using nuclease free water and RNA levels were measured at a wavelength of 260nm.

2.6.2 First-strand complementary DNA (cDNA) synthesis by high capacity reverse transcription

Complementary DNA was produced using a high capacity RNA to cDNA kit (Applied Biosystems, #4387406) according to the manufacturer's instructions. One microgram of RNA was converted at a time. A RT buffer mix of 10µl, 1µl of enzyme mix with 1µg of RNA that was diluted to a total of 9µl with nuclease free H_2O was used for the 20µl reaction. Eppendorfs were then put in a PCR thermocycler and underwent heating at 37°C for 1h followed by 95°C for 5min before cooling down to 4°C. The samples were then stored at -20°C or used for real-time PCR directly.

2.6.3 Quantitative polymerase chain reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) is one of the most powerful and sensitive gene analysis techniques available. It is used for a broad range of applications including detection and quantification of mRNA expression. qPCR measures PCR amplification as it occurs. The SYBR® Green dye is used as a fluorescent reporter molecule to monitor the accumulation of PCR product relative to an endogenous housekeeping gene. As the quantity of target amplicon increases, so does the amount of fluorescence emitted from the fluorophore. In this project, qPCR was used to quantify a wide variety of mRNA expressions in CiGEnC and CiPod (Primer table in Appendix). Master mix was made by mixing 5.5µl of SYBR Green (Sigma, #S-4438), 0.8µl primer mix (10µM) and 2.7µl of DEPC water. One microliter of cDNA was added to 9µl master mix for each well of the 96-well qPCR plate (Sarstedt, #72.1981.202). Real-time PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystem). For all samples, a primer set for human β-actin or GAPDH was used as the normalisation standard housekeeping gene.

Melt curve analysis was performed to confirm the specificity of the PCR-product. Briefly, each primer set would have 1 melting point at a specific temperature that would determine the specificity of the primers to the genes of interest. RT-PCR results were analysed using StepOnePlus^{TM} v2.1. software. Fluorescence data from each sample was analysed with the comparative threshold cycle (C_T) method ($\Delta\Delta C_T$ also written $2^{\Delta\Delta CT}$ method). Genes of interest values were normalised to the β-actin value for each sample. Within each experiment, the expression of the genes of interest were calculated relative to the value in untreated controls which was taken as 1.

2.7 Immunofluorescence (IF)

Cells were treated with fluorescently labelled antibodies to detect proteins of interest using immunofluorescent imaging. All incubations were performed at room temperature. Cells were seeded onto glass coverslips (Fisher, UK) in a 6-well plate (Cell star UK, 657160) at 1x 10^5 cells/cm^2. After 5 days of thermoswitching to 37^oC, the cells were washed once with PBS and fixed in 4% paraformaldehyde in PBS (Alfa Aesar UK, J61899) for 15min. Fixative was removed from the cells by three washes with PBS and the cells were then permeabilised (when needed) in a solution of 0.2% Triton X-100 in PBS for 10min followed by another wash in PBS. Cells were then incubated with blocking solution containing 5% normal goat serum (Sigma UK, G9023), 1% BSA (Fischer Scientific UK, BPE 9701-100) and 0.1% Triton X-100 in PBS for 30min. Cells were then washed three times with PBS before incubation with primary antibody of interest diluted 1:100 in blocking solution for 1h. Following that, the cells were washed three times in PBS and then incubated in the dark with appropriate secondary antibodies for 1h. The antibodies were either Alexa Fluor 488 Goat anti-mouse (Life technologies UK, #A11001) or Alexa Fluor 488 Goat anti-rabbit (Life technologies UK, #A11008) at a dilution of 1:500 in blocking solution. Finally, the coverslips were washed three times in PBS and mounted with Vectashield mounting medium (H-1200) with DAPI (4',6-diamidino-2-phenylindole) (Vectashield; Vector Laboratories, UK). Mounted coverslips were examined by fluorescent microscopy using the Leica DMI 6000B microscope. The resulting images are presented with original magnifications indicated. Within each experiment identical microscope and camera settings were used so that images can be directly compared.

2.8 Statistics

Graph and statistics were done using GraphPad Prism and p values of less than 0.05 were considered significant. For comparing differences between two groups, unpaired t-test with Welch's corrections was performed. One-way or two-way analysis of variance (ANOVA) was performed with post-hoc Bonferroni's analysis. Experiments were performed independently at least 3 times (n=3) with each repeat in triplicate for qPCR and Western blot results.

2.9 In vivo studies

2.9.1 UK animal declaration unit

All animals were handled in accordance with the University of Bristol's institutional guidelines and procedures approved by the UK Home Office in accordance with the Animals (Scientific Procedures) Act 1986. All experimental procedures were covered by Dr. Rebecca Foster's project licence PPL 30/3048 and on my own personal licence (39440).

2.9.2 Lean and db/db mice

Db/db and lean mice on the C57BLKS/6 background were obtained from Charles River. They were transported to the University of Bristol experimental housing facility and arrived at 6 weeks of age. (For more details, this part is explained in Chapter 3, section 3.2.2.2)

2.9.3 Wild type mice

Wild type mice that were housed at Level 0 in the Dorothy Hodgkin building (DHB) and not needed by other groups were kindly given to me to be used to determine adiponectin system components by an mRNA and protein expression profile. They were aged between 10 to 12 weeks.

2.9.4 Harvesting of organs

Animals were culled according to Home Office Code of Practice -The Humane Killing of Animals under Schedule 1 to the Animals (Scientific Procedures) Act 1986. After applying schedule 1 culling to the wild type mice which is done in a carbon dioxide (CO_2) chamber, the animal was put in a supine position and was cut open using sterilized surgical instruments to harvest several organs such as liver, adipocytes, muscle and kidneys.

2.9.4.1 Protein Extraction

Tissues were collected, immediately frozen in liquid nitrogen and stored at -80^{o}C until use. Upon thawing, tissue was washed 5 times in PBS in 1.5ml eppendorfs. The final PBS wash was then removed and replaced with 1ml of tissue lysis buffer (Bio Basic Inc. #BSP006) followed by mechanical homogenisation for 5min. The tissue suspension was subsequently incubated in lysis buffer in the cold room at 4^{o}C on a rotator for 1h, before further homogenisation. The samples were then centrifuged at 16,000g for 30min at 4°C. Protein samples were stored at -80^{o}C, until required.

2.9.4.2 RNA extraction

The extraction steps were carried out the same way as cultured cells, using RNeasy mini kit (Qaigen) with the addition of one step that allowed the tissue to be homogenized with the lysis buffer using a mechanical homogenizer to disrupt the membranes and allow extraction of RNA. The samples were stored at -80^{o}C if not used immediately.

2.9.5 Others

Further animal experiments such as glomeruli sieving, and treatments are explained in detail in their respective chapters and sections.

Chapter 3 Adiponectin and Receptors Expression in GEnC

3.1 Introduction

A major complication of diabetes, DN, is characterized by microalbuminuria (>30mg/day) and an impaired GFR (GFR <60 mL/min/1.73 m^2) [9]. During the last two decades, adiponectin has been identified as an insulin-sensitizer, an anti-inflammatory and vasoprotective adipokine [86]. Serum adiponectin levels are correlated with increasing age in healthy individuals and a rise of 1μg/ml of adiponectin has been observed for every 10 years of age [94]. However, low levels of adiponectin and dysregulation of receptor expression has been evident in a number of diseases (obesity, IR, chronic kidney disease (CKD), and types 1 and 2 diabetes) [80, 90, 158]. Importantly, adiponectin has been observed to exert beneficial renal effects by protecting against albuminuria in rodent experiments [90].

As mentioned before, adiponectin's main mode of action is through its receptors, AdipoR1 and AdipoR2. It has become clear that the receptors were also dysregulated in disease states especially in T2D with or without nephropathy [108]. First, the receptors expression is dependent on tissue of origin; that is, it can vary between one organ and another. For this project, I am interested in the level of expression in renal cells, and most specifically GEnC. However, I will also look at CiPod. It has been observed that both receptors were expressed in renal tissues (human and mice) [137]. The importance of the adiponectin system was established in renal tissues of T2D with nephropathy [159-161]. Specifically, the receptors' mRNA expression was found to be reduced in the cortex of

db/db mouse [160, 161]. This potentially results in reduced adiponectin sensitivity and a downregulation of AdipoR1 or AdipoR2 related signalling pathway in the kidneys. Therefore, isolated glomeruli from human and mouse will also be checked for adiponectin receptors in control and diabetic kidneys. On the other hand, an agonist of AdipoRs restored diabetes-induced decrease in level of expression of renal AdipoRs mouse to the levels present in controls [162]. This resulted in an increase of insulin sensitivity and reduction of glucose intolerance as well as improvement in the survival rate of diabetic mice [162].

The aim of this chapter was to identify the expression of adiponectin system components (adiponectin and receptors) in CiGEnC, CiPod and isolated glomeruli in health and disease.

a- To determine the mRNA and protein expression of AdipoRs in CiGEnC and CiPod by qPCR and Western blot.
b- To determine the localization of AdipoR1 in human kidney sections as well as in CiGEnC.
c- To explore the effect of a diabetic milieu (HG and TNF-α) on adiponectin receptors in CiGEnC and CiPod.
d- To work on a db/db model to assess the expression of adiponectin and adiponectin receptors in whole kidney lysates and sieved glomeruli.

3.2 Methods

3.2.1 qPCR validation of primers

Total RNA was extracted from cultured CiGEnC or CiPod as described in the Chapter 2. One µg of RNA was converted to cDNA. Then, serial dilutions of the cDNA were performed

at 1:10, 1:100, 1:1000 and 1:1000. The sequences of primer sets used are detailed in the Appendix. For each set of primers, a melt curve was created to assess the specific detection of a single product shown by a single peak on the melt curve analysis. In addition, the standard curve and the efficiency of each primer was performed and evaluated by the value of R^2 that should be between 0.90 and 1.10 calibration curve. The efficiency of the PCR amplification was calculated by plotting the C_T values of each dilution against the log of the cDNA input. The slope of the standard curve estimates the PCR amplification efficiency of the PCR reaction using the following equation: $(10^{(-1/\text{slope})} - 1)$. Figure 3.1 shows the standard curves of the genes that are to be assessed in this chapter. They all showed a great fit into the line with an R^2 between 0.90 and 1.10. This suggests that all the primers were validated and are specific for the gene of interest.

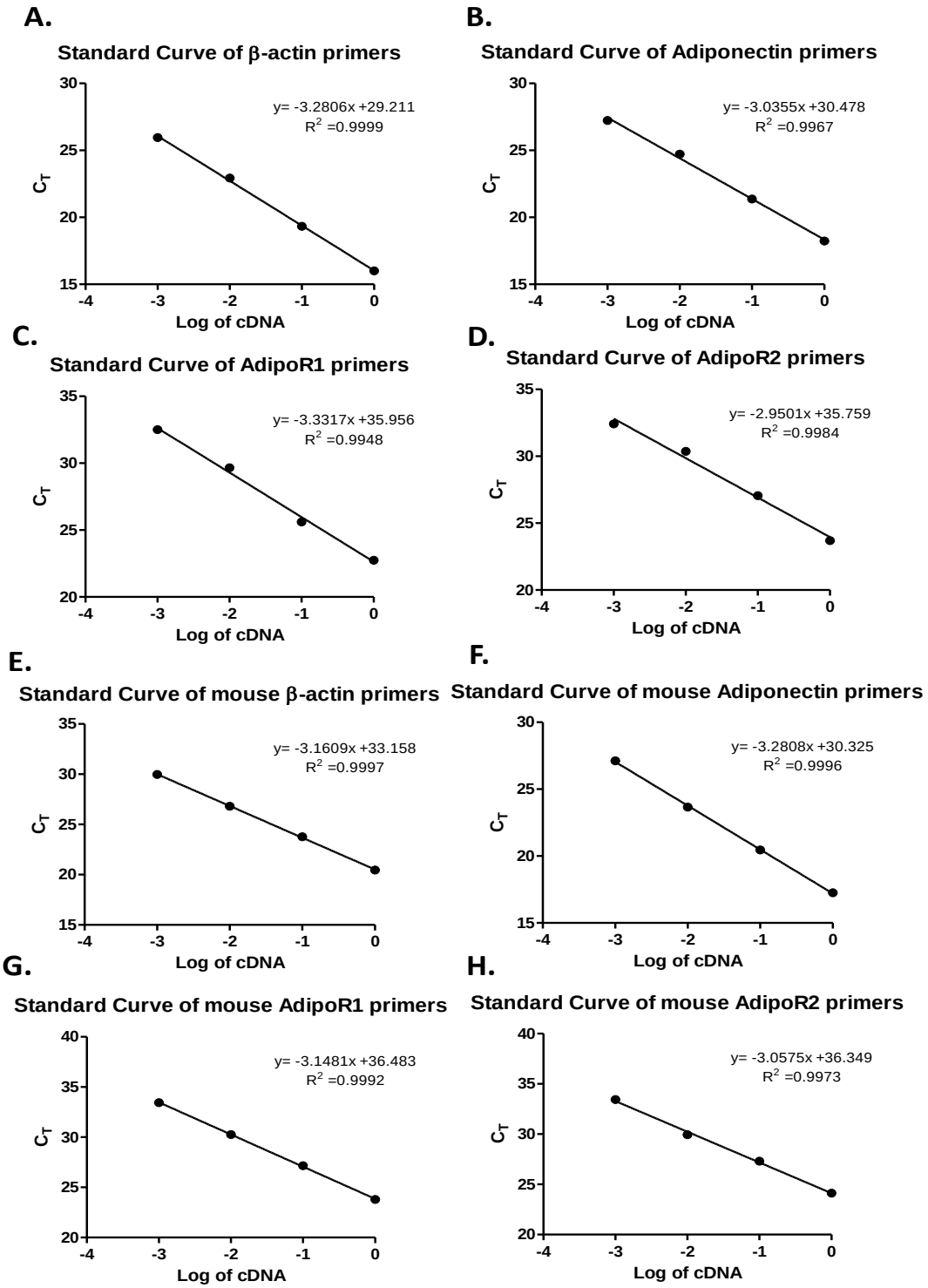

Figure 3.1 Standard curve of PCR amplification efficiency for human and mice adiponectin and its receptors and β-actin control primers

Graphs A to H represents the standard curve of each, human and mouse β-actin, adiponectin, AdipoR1 and AdipoR2. The initial cDNA concentration (1µg) was used and then serial dilutions of 1 in 10 were performed. Standard curves were plotted as C_T values vs. log of cDNA. R^2 is the correlation coefficient that define the fitness of the curves. y is the formula of the line in the form of y=ax+b. The curves were relative to n=1.

3.2.2 Kidney tissue

3.2.2.1 Human source

Human kidney tissue for this study was obtained from kidneys retrieved for transplantation but not subsequently used for clinical reasons. Full ethical approval for the use of these specimens was obtained. Table 4 shows the details of the control transplant kidneys. Sieving of kidney tissue for glomeruli was carried out in a laminar flow hood (MSC/BIO -Envair 89 Rossendale Lancs UK). The kidney was kept on ice during the whole sieving process.

It is rare for diabetic kidney organs to be transferred to our renal unit. However, 2 diabetic kidneys were obtained, but no clinical data were available.

Specimen	Age(yrs)	Reasons unsuitable for transplantation	Comorbidities	Approx. Cold Ischaemic Time (hrs)
HK49	27	No suitable recipients	Brain Tumor, Abnormal chest x-ray	24
HK63	69	Microscopic arterial diffusion, vascular damage	Heavy Drinker	34
HK64	63	Cold Ischaemic time too long		33
HK65	54	Damaged. Deemed non-transferable by surgeon	Motor neuron disease	29

Table 4 Characteristics of human donor kidneys

Age and characteristics of the 'transplant' kidneys unsuitable for transplantation but consented for research use. Kidney cortex was processed for sections for IF of adiponectin receptors and for sieving of glomeruli.

3.2.2.2 Diabetic mice

At present no single mouse model meets the requirements to study all the pathologies within the spectrum of diabetes and models most suited to the aim of each study must be identified. For the purposes of this study, the C57BLKS/J db/db mice mouse model of diabetes was chosen to be the most suitable model in terms of diabetic mice which are prone to nephropathy [163]. This model is characterised by a mutation in the receptor for leptin (adipocyte-derived hormone that regulates satiety and food intake) leading to defective leptin signalling [164]. Lack of leptin signalling in the hypothalamus leads to constant hyperphagia and obesity with resulting high leptin and insulin levels. This mouse strain develops hyperglycaemia by 8 weeks of age [165] and kidney hypertrophy is evident at 16 weeks. As for albuminuria, which is an index used to confirm nephropathy, it can start as early as 8 weeks and persists until 16-24 weeks of age [165, 166]. Matched controls for these animals are the non-diabetic, lean C57BLKS/J-db/+. This model was used to determine the expression levels of adiponectin and adiponectin receptors in the GEnC of the kidney. The db/db and lean mice were purchased from Charles River for Dr. Yan Qiu for her ongoing project concerning diabetic cardiomyopathy. She performed the animal work including ordering them and checking their weight and glucose levels (as seen in Figure 3.2). The db/db mice were confirmed to be diabetic from their respective glucose levels (from 16mM and above). After using the mice for assessment of heart function, the kidneys were made available for this project. One kidney cortex was divided for subsequent protein and RNA extraction (as shown in Chapter 2 sections 2.7.4.1 and 2.7.4.2 respectively). Five db/db and lean cortexes were then used for qPCR and WB.

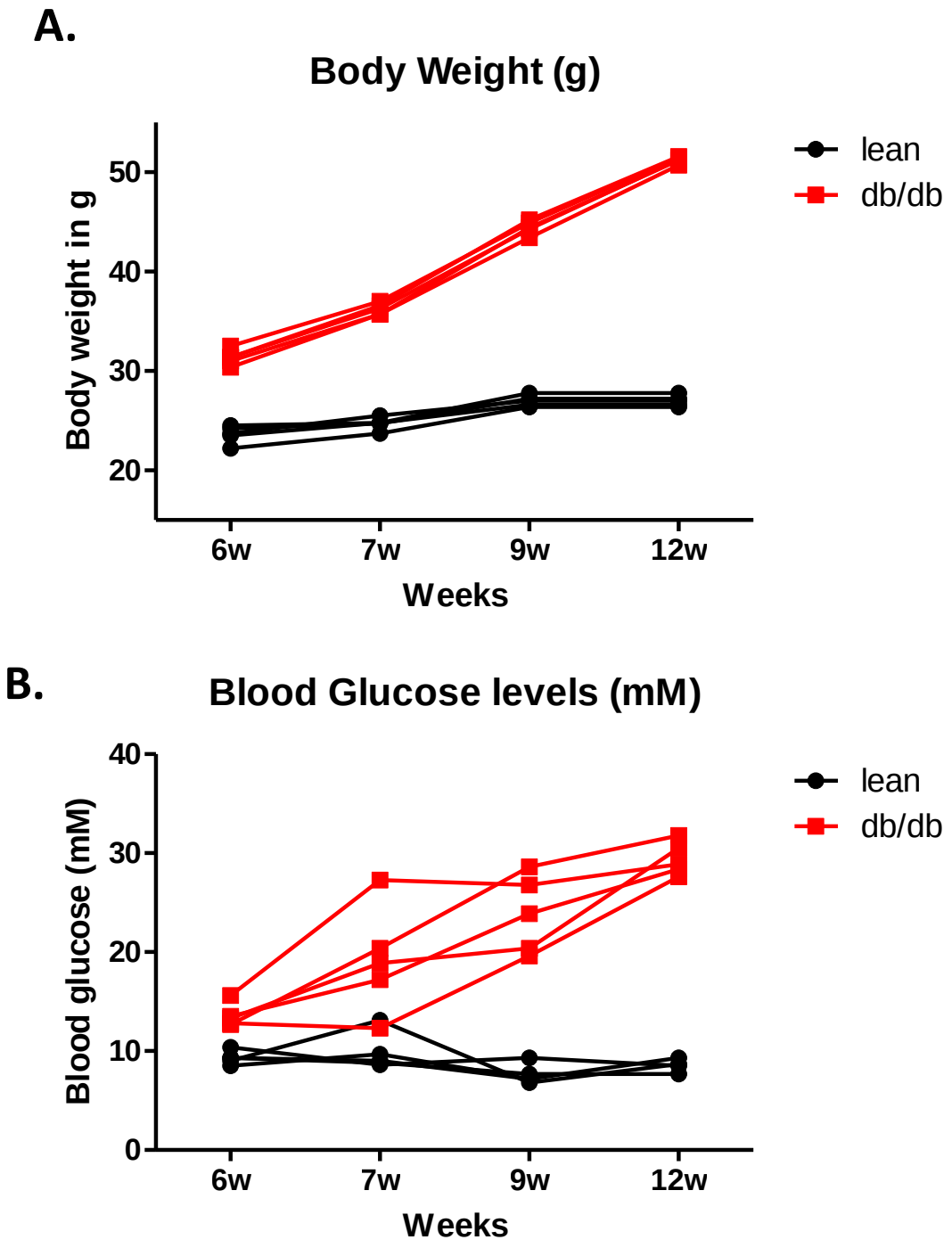

Figure 3.2 Body weight and glucose levels of db/db and lean mice.

A: Graph showing the characteristics of lean and db/db mice in which there is an increase in body weight from 6w to 12w in db/db mice when compared to lean controls. **B**: An increase in blood glucose in db/db mice started at 7w and all mice were hyperglycaemic (diabetic) by 9w.

3.2.2.3 Sieving of glomeruli

The kidney (human or mouse) was collected and placed in sterile, ice-cold PBS before starting the sieving process. The renal cortex from the human kidney was cut into smaller pieces and pushed sequentially with the plunger of a 20ml syringe through wetted sequential metal sieves with pores of 425µm, 180µm and 125µm. Ice-cold PBS was used to wash the tissue through each sieve. Human glomeruli were collected from the 125µm sieve. Whilst, the mouse kidney was cut in half and then in smaller cubes and pushed through the graded mouse sieves with the plunger of 5ml syringe. Mouse kidney cortex was pushed through another sieve (75µm) and glomeruli were collected from the last sieve and transferred to a 50ml falcon tube as shown in Figure 3.3. The suspension was spun at 3000 rpm for 5min at 4^{o}C to form a pellet of glomeruli. Supernatant was discarded and glomeruli were either treated first or re-suspended in protein or RNA lysis buffer.

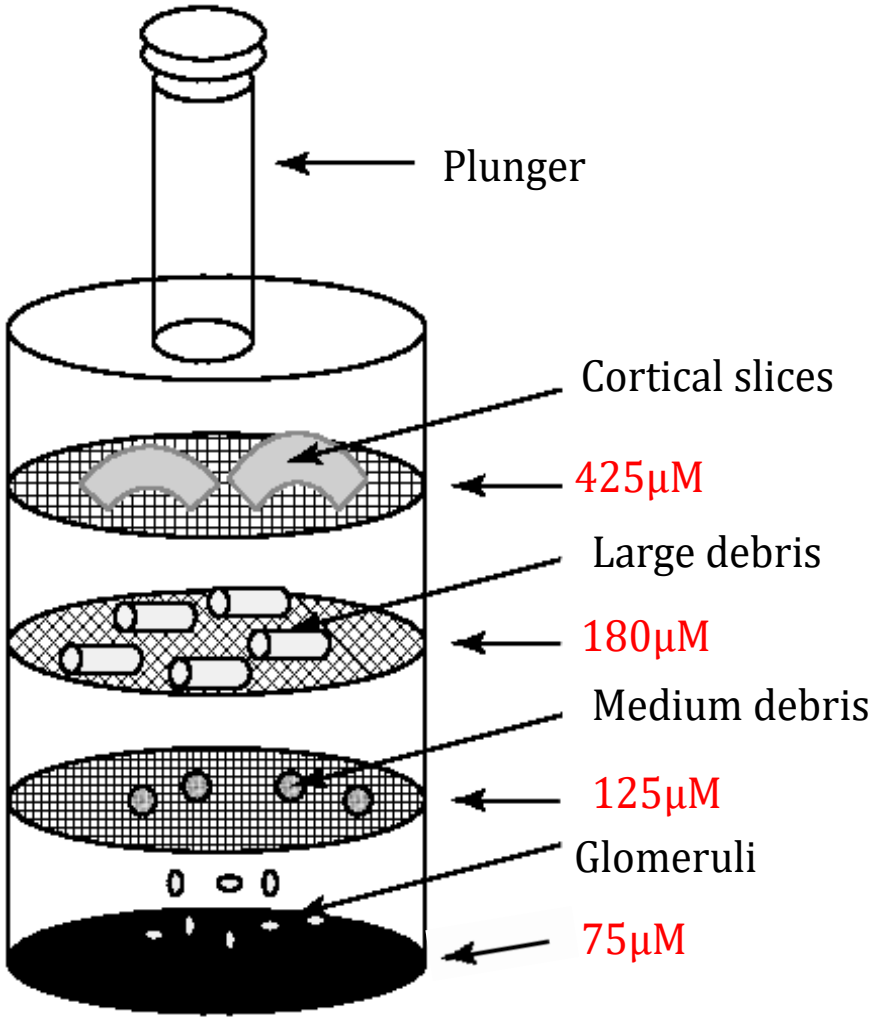

Figure 3.3 A schematic diagram showing the process of sieving mouse glomeruli

A simplified diagram that shows the steps of sieving glomeruli. Renal cortex is cut into smaller pieces and put on the 425µM sieve whilst pushing them through a plunger and washing with PBS. Gradient sieving is used whilst pushing large and medium debris through 180µM and 125µM respectively, until reaching to the last sieve, the 75µM where the glomeruli are collected.

3.2.3 RNA extraction from glomeruli

3.2.3.1 Optimization techniques

Whilst optimizing the RNA extraction from mice glomeruli, it was discovered that very little to no RNA was obtained just by putting them in RNA lysis buffer (RLT) as per the manufacturer's instructions. Therefore, this technique was adapted by adding an extra

step. To aid lysis, a mechanical lysis step was performed. The glomeruli with the lysis buffer in an eppendorf was repeatedly drawn through a 30-gauge needle under high pressure. This additional mechanical stress ensured the glomeruli were broken into smaller pieces allowing the buffer RLT to contact all cells of the glomerulus. This improved dramatically the RNA yields

The low yield of RNA initially meant that samples were not obtained from the first batch of littermate-controlled animals and thus the first successful extraction was carried out in db/db mice which unfortunately did not have matched littermate controls. In this case wild type mice were used as controls. In subsequent experiments mice with appropriately matched littermate controls were available so were used. This is detailed in the results section.

3.2.4 Protein extraction from glomeruli

The glomeruli (human or mice) in PBS were centrifuged at 3000 rpm for 5min to form a pellet. The supernatant media was removed, and PBS was added to wash the glomeruli and then re-centrifuged at same speed and time. The glomeruli were re-suspended in tissue lysis buffer and kept for 30min on a rotator in the cold room (4^{o}C). To ensure that the same problem with the RNA did not occur for the protein, the glomeruli in the lysis buffer were put in a 12-well plate and mechanical stress was added by using by using a 1ml plunger of a syringe. This was used to disrupt the glomeruli and to aid protein extraction. The samples were then centrifuged at 16,000g for 20min at 4°C. Protein samples were either stored at -80°C or Western blot was performed to quantify relative protein expression.

3.2.5 Statistical analysis

Experiments were performed independently at least 3 times (n=3) with each repeat in triplicate for qPCR results. qPCR data were calculated as relative quantification (RQ) to the unstimulated control sample. Data were normalised to housekeeping gene: β-actin and plotted as mean $2^{-\Delta\Delta CT}$ of each triplicate. For comparing data between two different groups, unpaired t-test with Welch's correction was used. Data with more than 2 groups were analysed by one-way ANOVA with post hoc Bonferroni analysis to allow for multiple comparisons.

3.3 Results

3.3.1 Glomerular endothelial cells do not express adiponectin

The first aim of this project was to determine the expression of adiponectin using qPCR and Western blot, in human glomerular cells using human tissue, (from organs not used for transplants) CiGEnC and CiPod and sieved mouse and human glomeruli. Kidney lysates were prepared from tissue taken from the renal cortex and medulla. The fat surrounding the kidney (perinephric fat) was used as a positive control. CiGEnC and CiPod were grown in T25 flasks and then thermoswitched for 5 and 10 days respectively prior to RNA or protein extraction.

Primers for human adiponectin were purchased from Eurofins for qPCR. The results from the qPCR data from different tissues/cells are depicted in Figure 3.4. The comparison of the $2^{-(\Delta\Delta CT)}$ calculation demonstrated no mRNA expression for adiponectin in either the whole kidney cortex, CiGEnC or CiPod (figure 3.4A). Semi-quantitative estimation of

adiponectin protein level expression in cellular lysates of tissues and renal cells was determined using Western blot. An anti-adiponectin antibody (CST #C45B10) was used for detection. Figure 3.4B shows that the Western blot generated a band at 27kDa consistent with the expression of adiponectin only in the adipocyte sample. Although a faint band was seen in the cortex lysate, this might be a false positive due to impurities being introduced from the surrounding fat during the protein extraction.

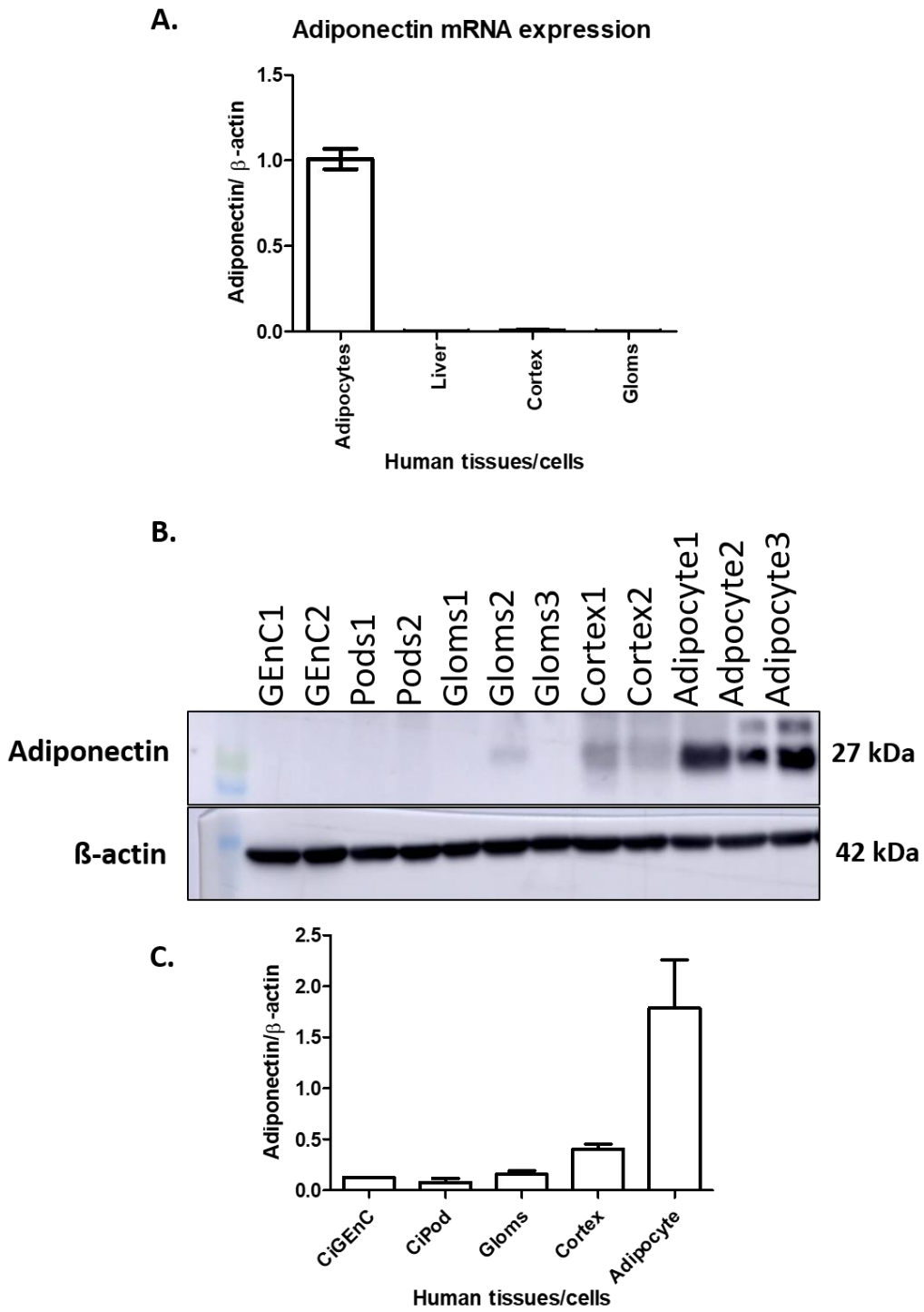

Figure 3.4 Expression levels of adiponectin in kidney tissue/cells

A: qPCR analysis graph representing the mRNA expression of adiponectin between human kidney tissue such as adipocytes or kidney cortex or glomeruli (gloms) and cells (GEnC and Pods). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. n= 3, one-way ANOVA, p>0.05 ns. *post hoc* analysis (Bonferroni). **B** and **C**: Representative western blot and densitometry demonstrating kidney lysates with adipocytes as a positive control normalised to β-actin loading control. Bars represent means ±SEM, n=3, one-way ANOVA, p>0.05 ns when compared to adipocyte column. *post hoc* analysis (Bonferroni).

3.3.2 Glomerular endothelial cells express AdipoR1

AdipoR1 is one of the main receptors for adiponectin. Specific mRNA primers for AdipoR1 were purchased from Eurofins and the respective efficiency curve was evaluated as shown in Figure 3.1C. The expression of AdipoR1 mRNA was demonstrated in CiGEnC and CiPod, kidney cortex tissue and sieved glomeruli (Figure 3.5A). The highest expression for AdipoR1 appeared in the sieved glomeruli.

At the protein level, an antibody of AdipoR1 was purchased from abcam (#126611). Western blot showed the expression of AdipoR1 with a single band at 44kDa (Figure 3.5B) in all samples tested. Again, an adipose tissue sample was used as a positive control.

In addition, IF experiments were performed to complete the profile of expression of AdipoR1 in CiGEnC. Figure 3.6 shows that the staining pattern of AdipoR1 is mainly distributed within the cytoplasm and peri-nuclear area of CiGEnC.

The localization of AdipoR1 was also detected in frozen human kidney cortex sections. Immunofluorescence staining was performed on AdipoR1 (green). It displayed a similar pattern as the cultured cells (Figure 3.7).

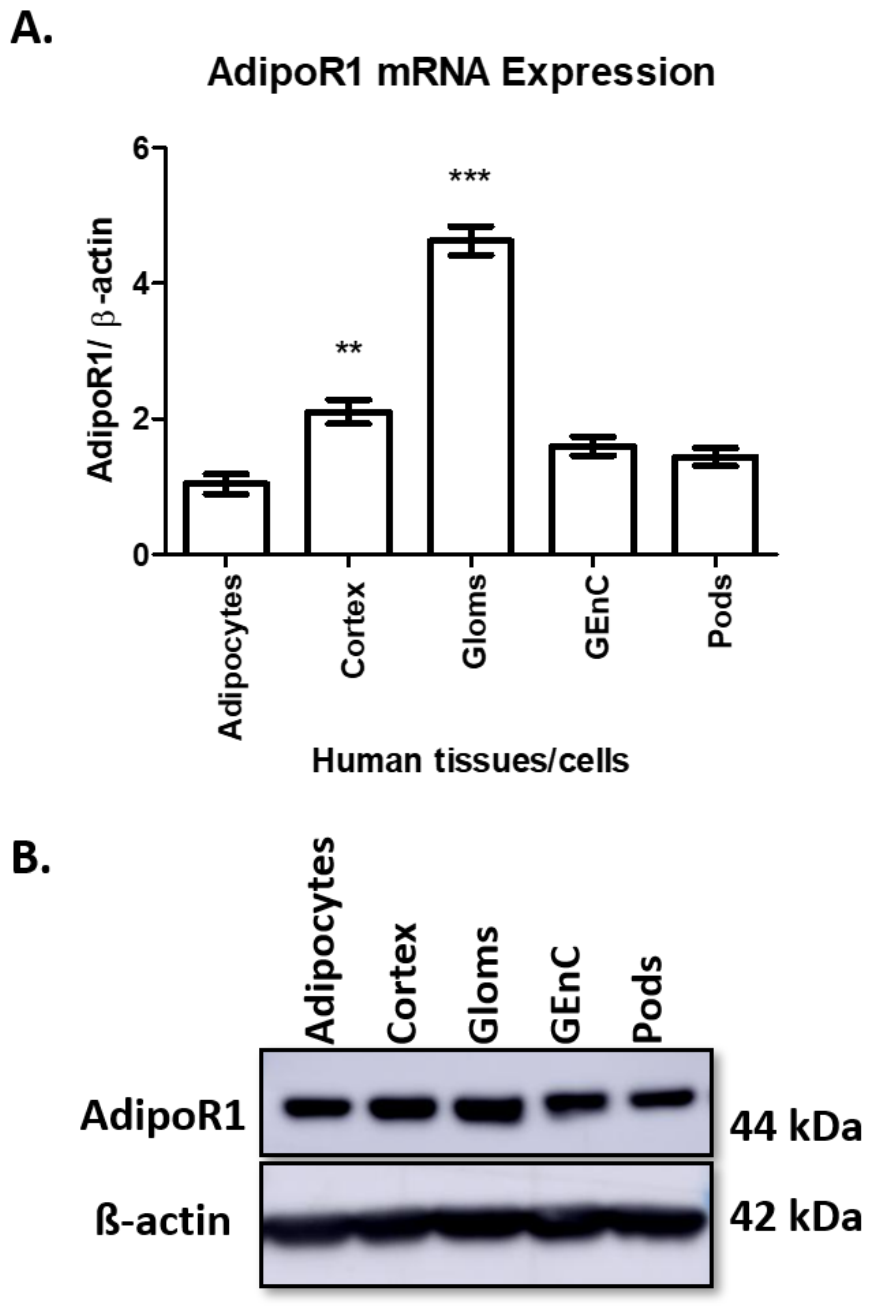

Figure 3.5 Expression of AdipoR1 in kidney tissues/cells

A: qPCR analysis graph representing the mRNA expression of AdipoR1 between human kidney tissue such as adipocytes or kidney cortex or glomeruli (gloms) and cells (GEnC and Pods). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. n= 4, one-way ANOVA, ** p< 0.01, *** p <0.001 compared to adipocytes *post hoc* analysis (Bonferroni). **C**: Representative Western blot demonstrating kidney lysates with adipocytes as a positive control normalised to β-actin loading control.

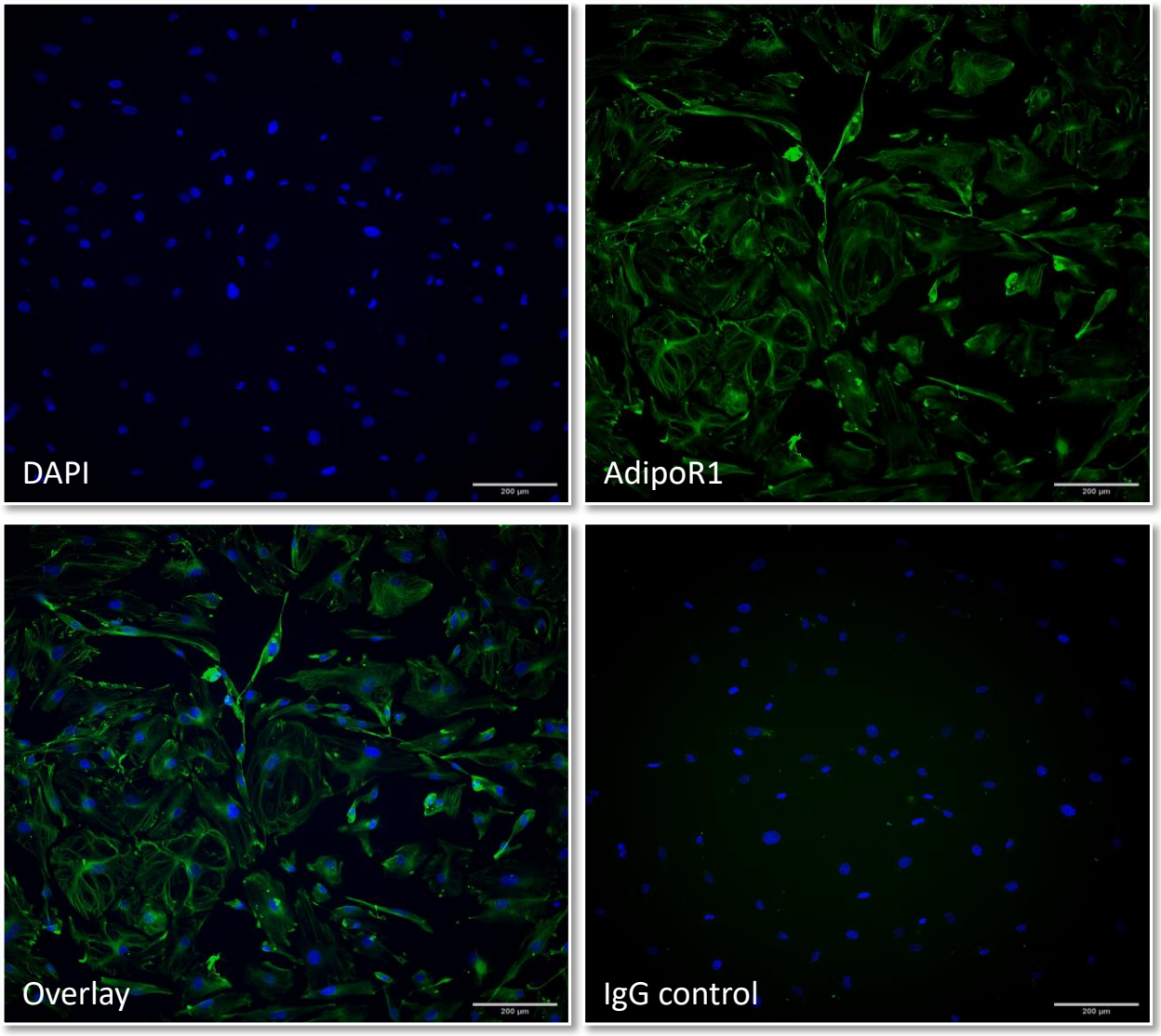

Figure 3.6 Immunofluorescence of AdipoR1 expression in CiGEnC

Primary antibody AdipoR1 (green) with counterstaining for DAPI (nuclei in blue). Peri-nuclear and cytoplasmic staining of AdipoR1 in CiGEnC. A negative control was used for specificity of antibody. Images shown are at x10 magnification.

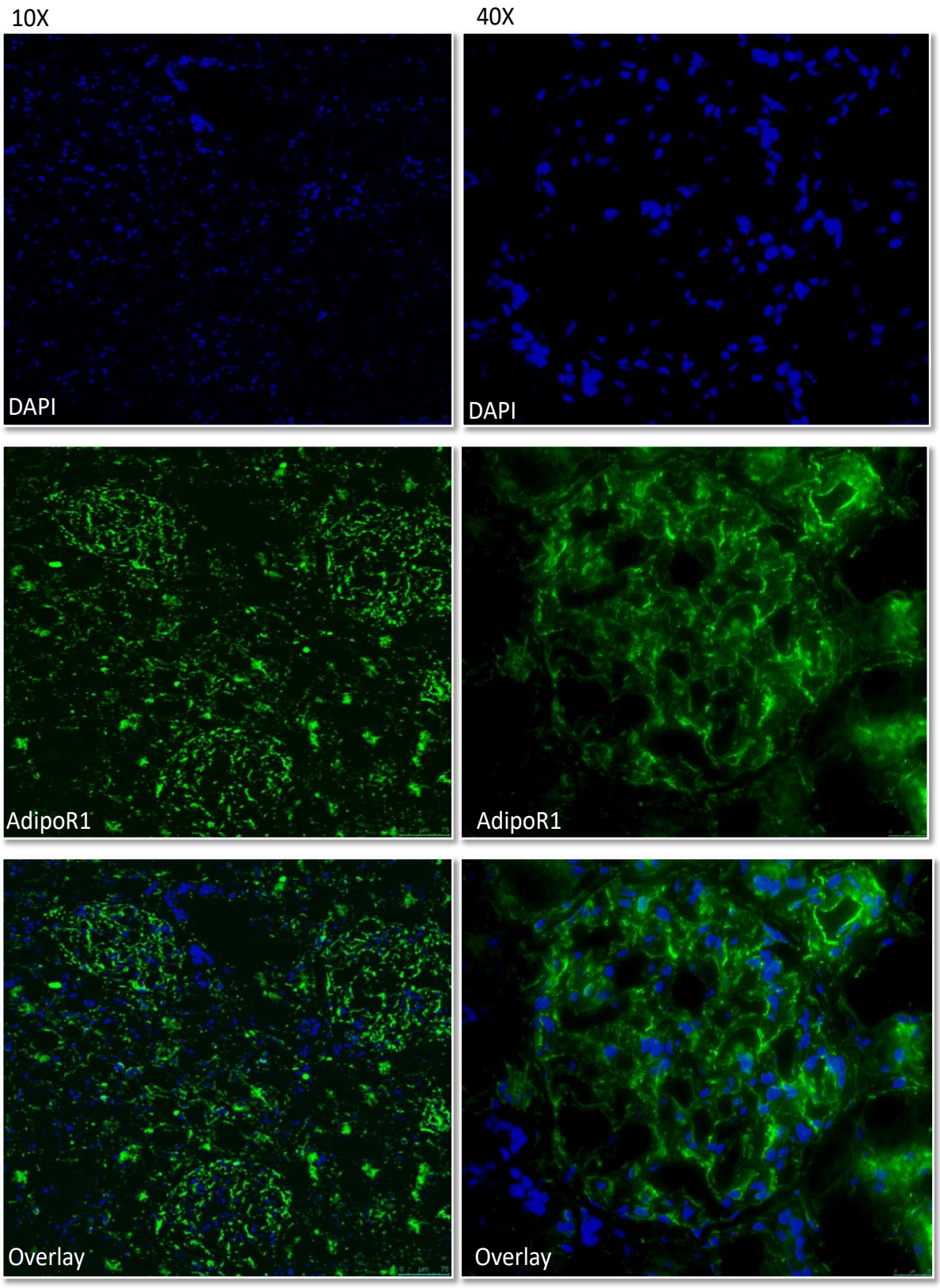

Figure 3.7 IF on human cortex sections for AdipoR1

Primary antibody AdipoR1 (green) with counterstaining for DAPI (nuclei in blue). Peri-nuclear and cytoplasmic staining of AdipoR1 in human kidney sections. Left panel: Images shown are at x10 magnification. Right panel: Images shown are at x40 magnification

3.3.3 Glomerular endothelial cells express AdipoR2

The expression of AdipoR2 mRNA was also determined by qPCR. AdipoR2 was expressed by CiGEnC and there were no significant differences between the cell types tested (Figure 3.8A). Western blot analysis also showed a single band at 43kDa corresponding to the protein expression of AdipoR2 among all the tissues and cell lysates tested (Figure 3.8B). The pattern of AdipoR2 protein expression by IF appeared to be peri nuclear in CiGEnC (Figure 3.9). However, it was difficult to optimize the AdipoR2 antibody in human tissue sections and time constraints meant that I was unable to assess AdipoR2 pattern.

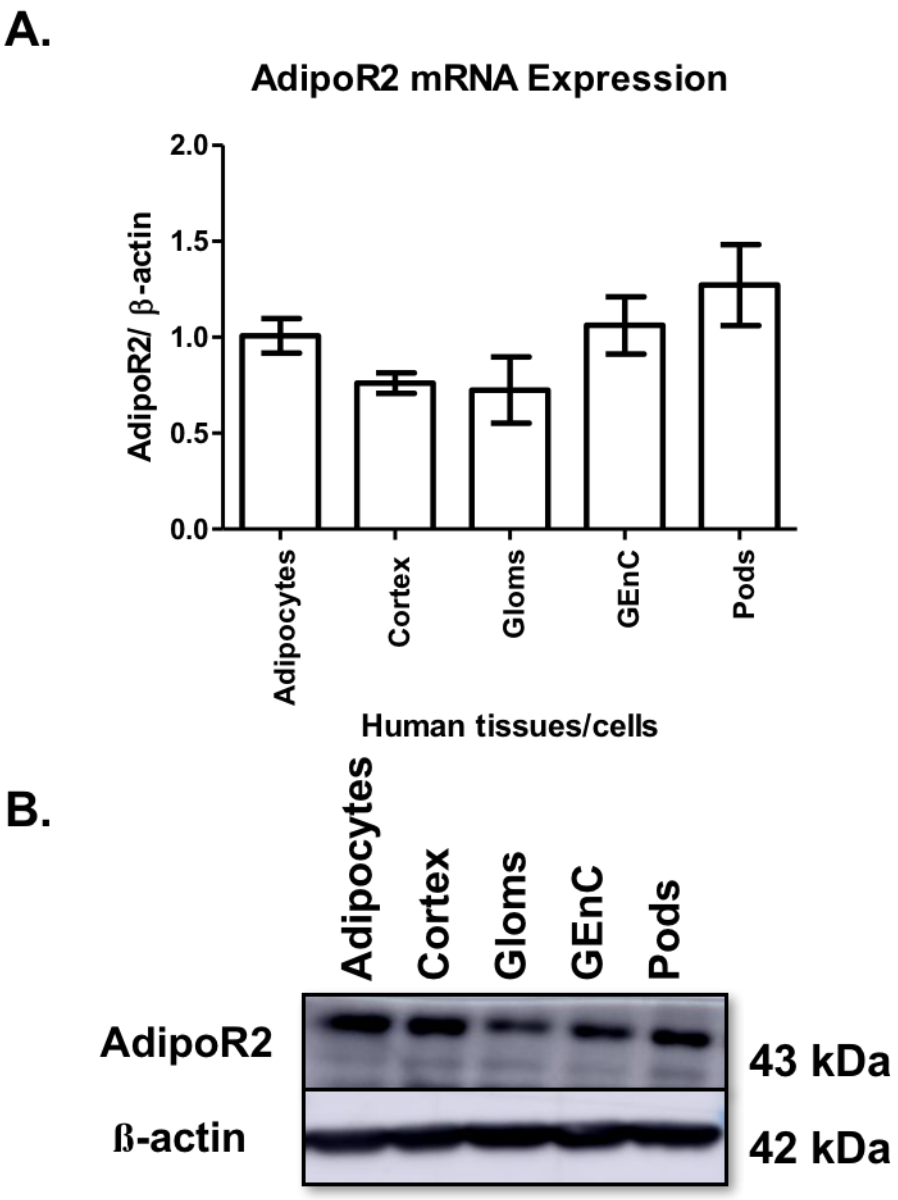

Figure 3.8 Expression of AdipoR2 in kidney tissues/cells

A: qPCR analysis graph representing the mRNA expression of AdipoR2 between human kidney tissue such as adipocytes or kidney cortex or glomeruli (gloms) and cells (GEnC and Pods). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. n= 3, one-way ANOVA, p>0.05 ns *post hoc* analysis (Bonferroni). **B**: Representative western blot for AdipoR2 demonstrating kidney and cell lysates with adipocytes as a positive control normalised to β-actin loading control.

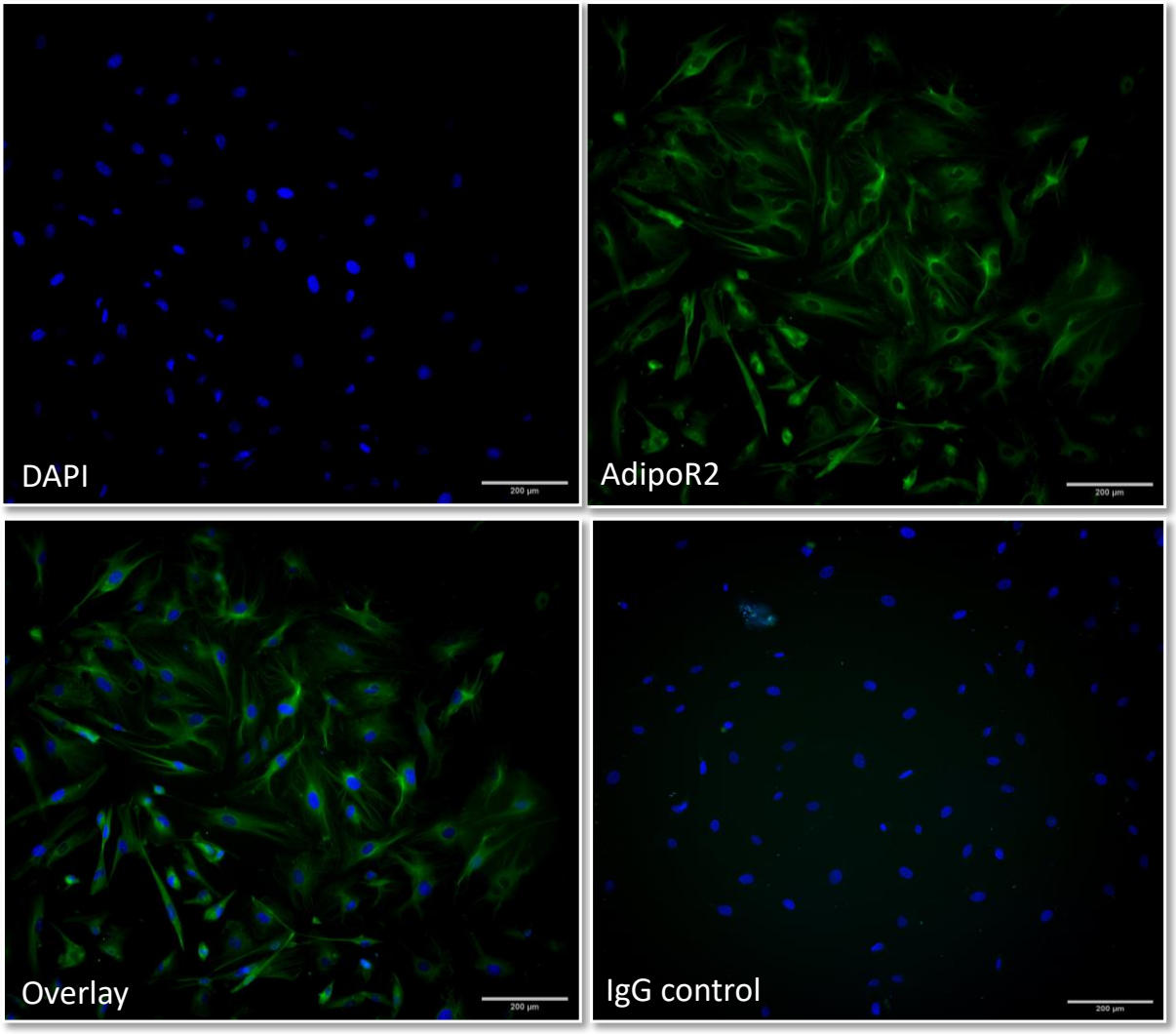

Figure 3.9 Immunofluorescence of AdipoR2 in CiGEnC.

Primary antibody AdipoR2 (green) with counterstaining for DAPI (nuclei in blue). Peri-nuclear staining of AdipoR2 in CiGEnC. An IgG control was used to detect background staining Images shown are at x10 magnification.

3.3.4 AdipoR1 vs AdipoR2 mRNA expression

Comparing between the two receptors in CiGEnC and CiPod, figure 3.10 shows that AdipoR1 is the predominant receptor in CiGEnC. However, in podocytes the receptors do not vary significantly.

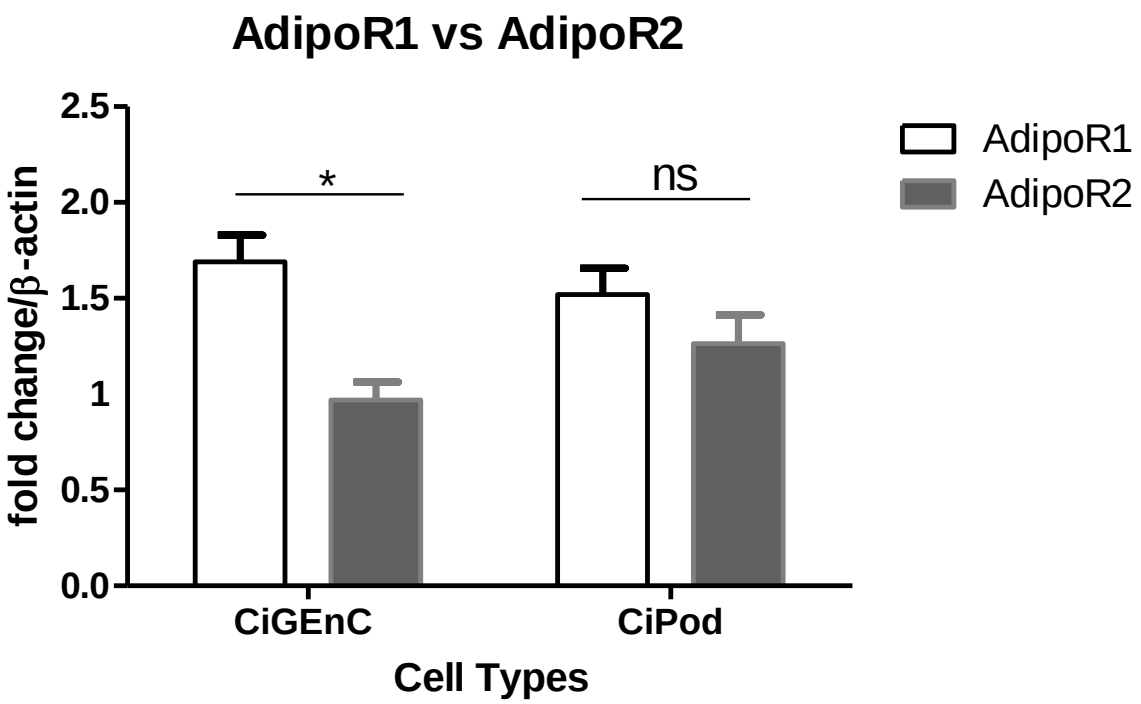

Figure 3.10 AdipoR1 vs AdipoR2 mRNA expression in CiGEnC and CiPod

qPCR data analysis showing the mRNA expression of AdipoR1 and AdipoR2 in CiGEnC and CiPod. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. AdipoR1 is more dominant in CiGEnC than AdipoR2. No differences in AdipoR1 between CiGEnC and CiPod. n=3, unpaired t test was used between the different receptors in each cell type*$p<0.05$ and $p>0.05$ ns.

3.3.5 AdipoR1 and AdipoR2 mRNA expression in human diabetic glomeruli

After the efficient RNA extraction of sieved glomeruli in control and diabetic human tissue samples, mRNA expression was measured. As seen in figure 3.11A, AdipoR1 mRNA was decreased up to 40% and AdipoR2 around 20% in the diabetic samples compared to control. Since there were only 2 samples in the diabetic group, a statistical data output could not be obtained. However, this suggests that there are differences in receptor expression between control and diabetic samples.

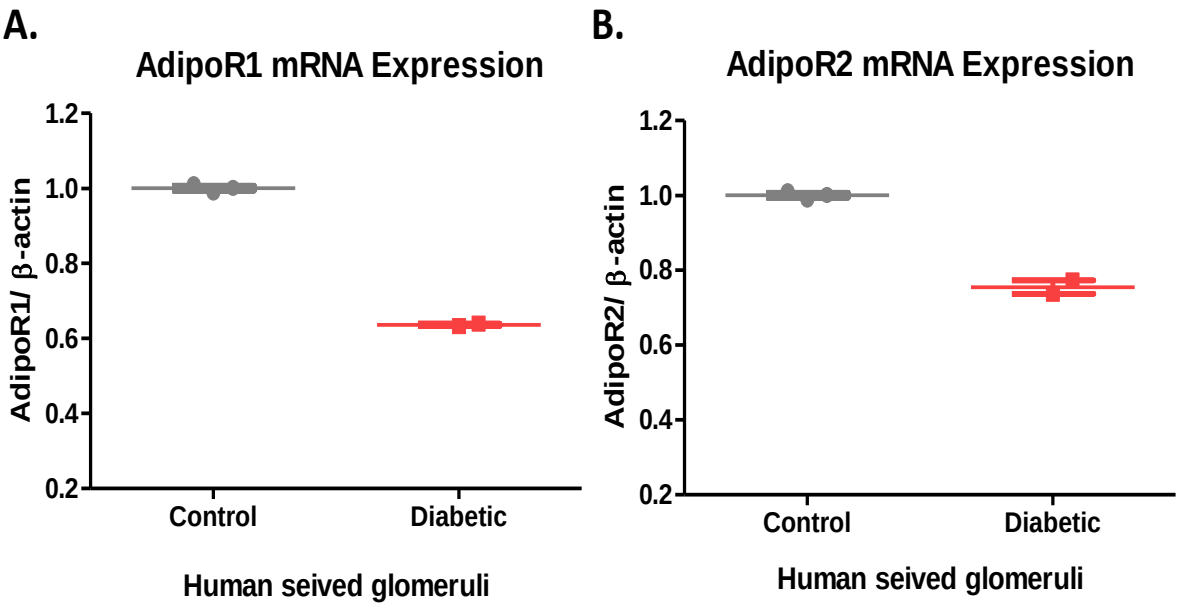

Figure 3.11 AdipoR1 and AdipoR2 mRNA expression are decreased in human diabetic glomeruli

A and **B**: mRNA data analysis between control and diabetic human sieved glomeruli of AdipoR1 and AdipoR2 (n=2). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean and normalized to β-actin loading control.

3.3.6 Effect of high glucose on the expression of AdipoR1 mRNA in CiGEnC and CiPod

After confirming that adiponectin receptors are expressed in CiGEnC as well as in CiPod, the next question considered was whether external stimulus such as high glucose can alter the expression of these receptors in either cell type. A concentration of 25mM of glucose was chosen to stimulate the cells based on the scientific literature data and has been utilised in many published works as a reference dose to mimic a HG environment on endothelial cells as well as other types of cells [167-169].

A short and long exposure of HG was carried out in CiGEnC and CiPod and the level of AdipoR1 assessed. Figure 3.12A and D shows the short exposure of 2h, 6h and 24h of

AdipoR1 on CiGEnC and CiPod. Using qPCR, a significant downregulation by HG was shown after only 6h and this remained consistent for 24h at the mRNA levels for CiGEnC. However, in CiPod, the AdipoR1 mRNA expression did not change over 24h. An osmotic control was used to determine the effects of a concentrated solution at the 24h time point. The high inactive L-glucose concentration is considered an isomer of the active D-Glucose isomer but cannot be degraded within the cell; it only causes a high osmolarity environment. As seen in figure 3.12C, the L-glucose treatment caused an increase in AdipoR1 which was the opposite of the high D-glucose. This suggests that the decrease at 24h was a result of the effect of high glucose and not an osmolarity effect.

A longer exposure of HG was also done to further mimic a diabetic environment. For CiGEnC, the three time points chosen were 2D, 7D and 14D (D for days). The expression of AdipoR1 mRNA was further decreased by almost 60% after 14D exposure to high glucose ($p<0.001$). In CiPod, however, different time points were chosen due to the longer period of thermoswitching (9-12d for CiPod vs. 3-5d for CiGEnC). Due to a worry about the cells dying after a long period of incubation, time points of 2D, 4D and 7D were used for CiPod. There were significant decreases in AdipoR1 after 2D and 4D by almost 50% and a more prominent downregulation after 7D (almost 65%).

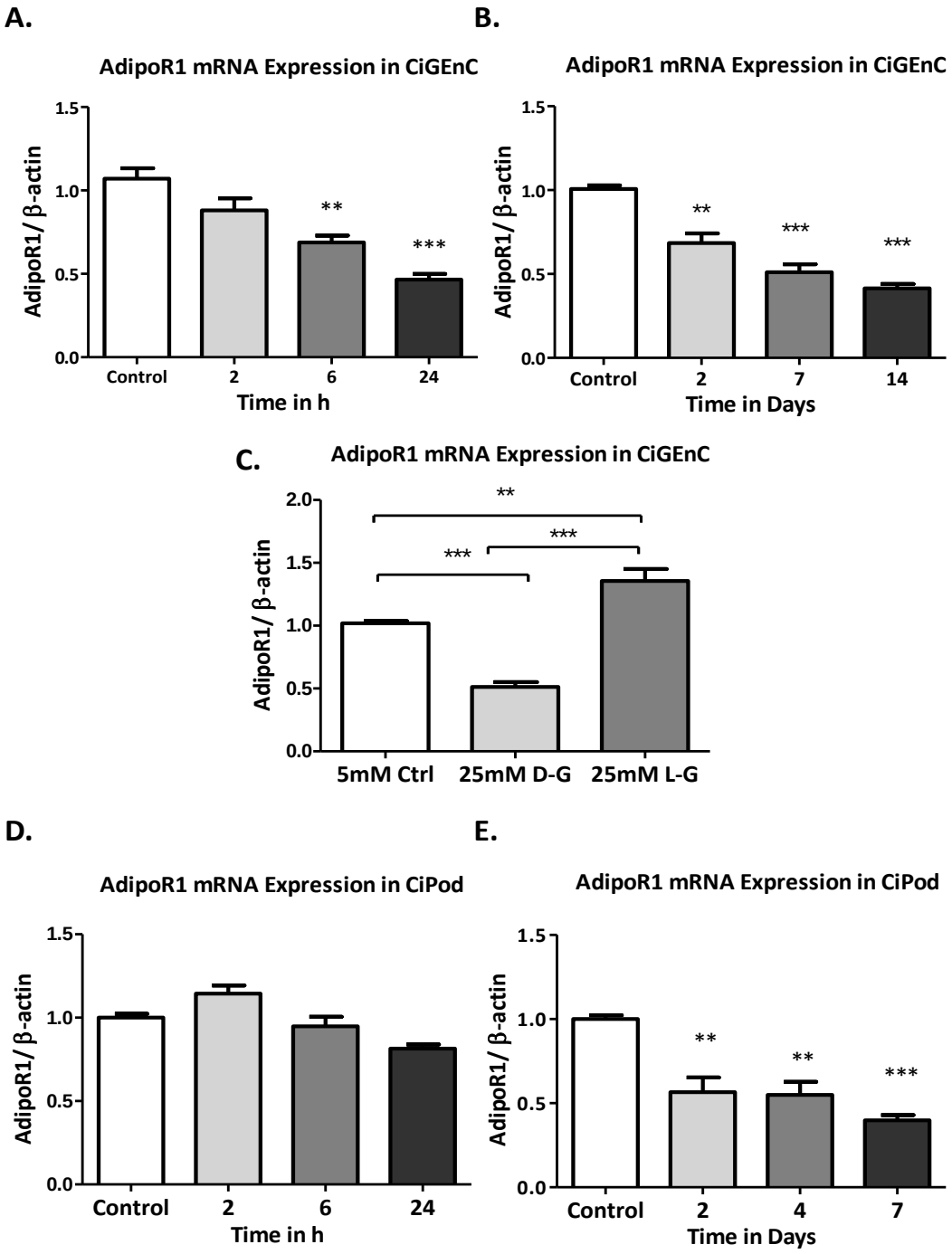

Figure 3.12 Decrease in AdipoR1 mRNA with respect to HG in CiGEnC and CiPod

A and **B**: qPCR analysis of AdipoR1 in CiGEnC treated with high glucose (25mM) over a short time course of 2h, 6h and 24h (hours) **(A)** and long-time course of 2D,7D and 14D (days) **(B)**. **C**: qPCR analysis of AdipoR1 in CiGEnC treated with 25mM D-glucose and L-glucose for 24h. **D** and **E**: qPCR analysis of AdipoR1 in CiPod treated with high glucose (25mM) over a short time course of 2h, 6h and 24h (hours) **(D)** and long-time course of 2D,4D and 7D (days) (**E**). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean (±SEM). β-actin used as the housekeeping gene control. n=3, one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$ *post hoc* analysis (Bonferroni).

3.3.7 Effect of high glucose on the expression of AdipoR2 mRNA in CiGEnC and CiPod.

Similarly, the mRNA expression of AdipoR2 was also evaluated in both cell lines at different time points as indicated in Figure 3.13. However, there was no changes in AdipoR2 mRNA with short-term exposure to HG in either cell types (Figure 3.13A and D). Also, the osmolarity check was done with respect to AdipoR2 (Figure 3.13C). There were also no visible changes. Longer exposure to HG, resulted in a significant decrease in AdipoR2 after both 7D (by 40%) and 14D (by 40%). In CiPod, the decrease in AdipoR2 was more rapid and started as early as 2D by almost 30% and resulted in a 50% loss after 7D.

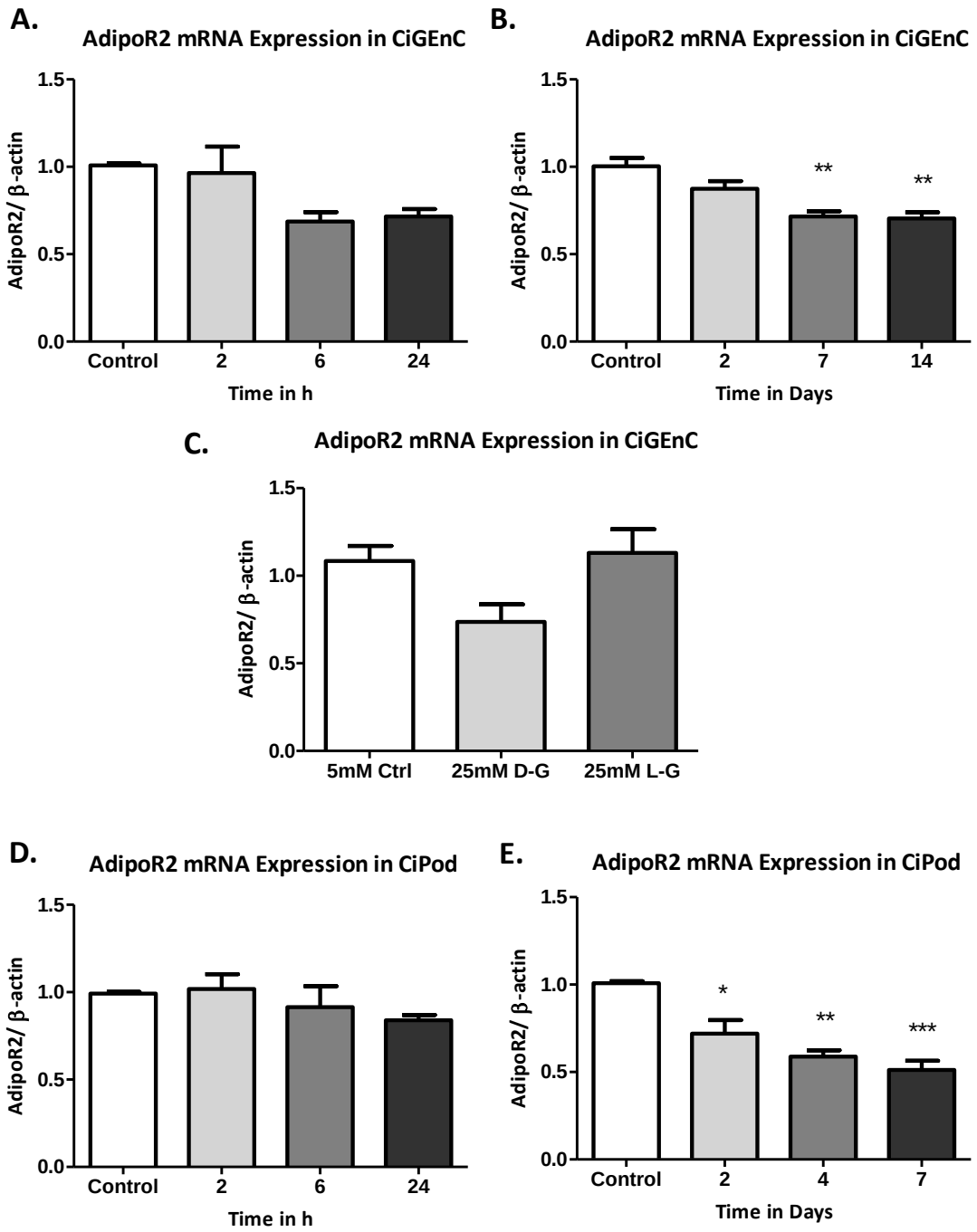

Figure 3.13 Decrease in AdipoR2 mRNA with respect to HG in CiGEnC and CiPod

A and **B**: qPCR analysis of AdipoR2 in CiGEnC treated with high glucose (25mM) over a short time course of 2h, 6h and 24h (hours) **(A)** and long-time course of 2D,7D and 14D (days) **(B)**. **C**: qPCR analysis of AdipoR2 in CiGEnC treated with 25mM D-glucose and L-glucose for 24h. **D** and **E**: qPCR analysis of AdipoR2 in CiPod treated with high glucose (25mM) over a short time course of 2h, 6h and 24h (hours) **(D)** and long-time course of 2D,4D and 7D (**E**). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. β-actin used as the housekeeping gene control. n=3, one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$ *post hoc* analysis (Bonferroni).

3.3.8 Effect of TNF-α on AdipoR1 and AdipoR2 in CiGEnC

Another component of the diabetic milieu was analysed. CiGEnC were treated with TNF-α in a time-dependent manner. Figure 3.14A showed that AdipoR1 was significantly decreased after 2h (by 25%) and 4h (by 35%). However, AdipoR2 mRNA was not changed with TNF-α stimulation for as long as 4h (Figure 3.14B)

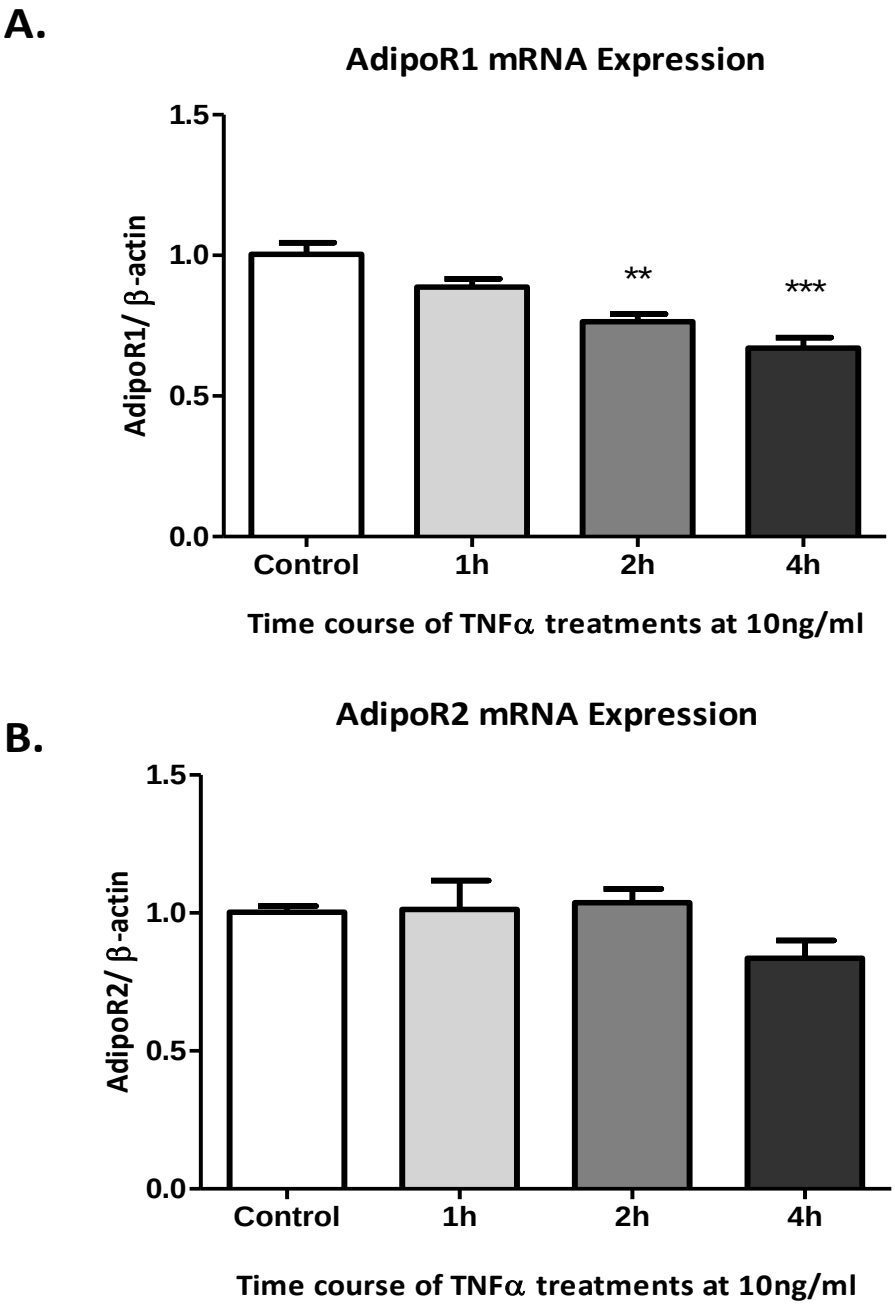

Figure 3.14 Decrease in AdipoR1 but not AdipoR2 mRNA with respect to TNF-α in CiGEnC

A: qPCR analysis of AdipoR1 in CiGEnC treated with TNF-α (10ng/ml) over a short time course of 1h, 2h and 4h (hours**)**. **B**: qPCR analysis of AdipoR2 in CiGEnC treated with TNF-α (10ng/ml) over a short time course of 1h, 2h and 4h (hours). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. β-actin used as the housekeeping gene control. n=3, one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$ *post hoc* analysis (Bonferroni).

3.3.9 Expression of adiponectin and receptors in mice

After analysing that adiponectin and receptors are expressed and changed in CiGEnC in unstimulated and stimulated conditions, we therefore confirmed these findings in a wild type healthy mice model. Wildtype mice were sacrificed, and the adipose tissue, liver, muscle and kidneys were harvested. RNA was extracted as detailed in Chapter 2. Initial findings show that in wildtype mice, adiponectin mRNA is not expressed in either the kidney or in glomeruli (Figure 3.15A). It was shown that AdipoR1 is mainly expressed in muscular tissues and to a lesser extent in the liver (Figure 3.15B). Importantly, kidneys were also shown to express AdipoR1. The liver expresses the most amount of AdipoR2 while muscle and kidneys also demonstrate expression although to a much smaller amount (Figure 3.15C).

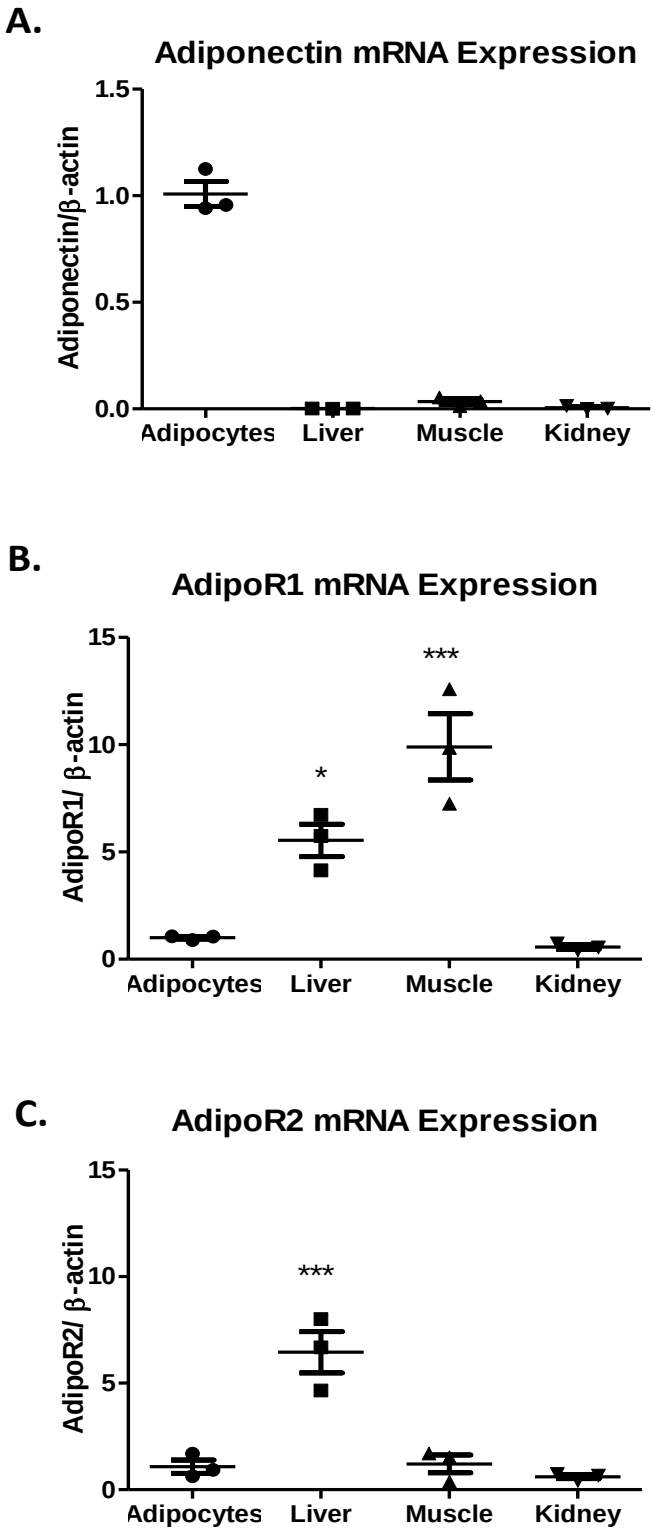

Figure 3.15 Expression of adiponectin and its receptors in different mouse tissues

A, B and **C**: qPCR analysis graph representing the mRNA expression of adiponectin, AdipoR1 and AdipoR2 between mice tissues. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. β-actin used as the loading control. n=3, one-way ANOVA, * $p< 0.05$, ***$p<0.001$ compared to adipocytes *post hoc* analysis (Bonferroni).

3.3.10 Variations in adiponectin receptors in diabetic mice

The next stage was to determine whether the expression of the adiponectin receptors expression changes in a diabetic mice model. As explained in the methods section of this chapter, initially wild type and db/db mice were sacrificed, and adipose tissue and the kidneys were harvested. RNA was extracted using the standard protocol. In adipocytes, adiponectin mRNA levels are downregulated in db/db mice when compared to wt/wt mice (Figure 3.16A). However, the expression of the adiponectin receptors (R1 and R2) did not change between the wild type and diabetic mice adipocytes (Figure 3.16B&C).

The kidneys from the diabetic and non-diabetic-wildtype mice were used to sieve glomeruli. As explained in the methods section, RNA and protein from the *ex vivo* glomeruli were then extracted. At the mRNA level (Figure 3.17A), there was a downregulation of AdipoR1 in the sieved glomeruli (40%). This was confirmed at the protein level by Western blot which also showed a significant decrease in AdipoR1 protein levels in the diabetic mice (Figure 3.17C and E).

Importantly, when a second db/db cortex lysates was compared with their bone fide lean matched littermate controls, a downregulation of AdipoR1 at both the mRNA (by 50%) and protein level (by 40%) was observed (Figure 3.17B, D and F).

AdipoR2 levels were also compared between the diabetics and non-diabetic groups. In the sieved glomeruli, mRNA levels of AdipoR2 was significantly reduced in the diabetic mice (by 30%) (Figure 3.18A). This decrease was even more significant at the protein level (40%) (Figure 3.18C and D). In whole kidney lysates, similar significant decreases in AdipoR2 at both mRNA and protein levels were observed (Figure 3.18B, E and F).

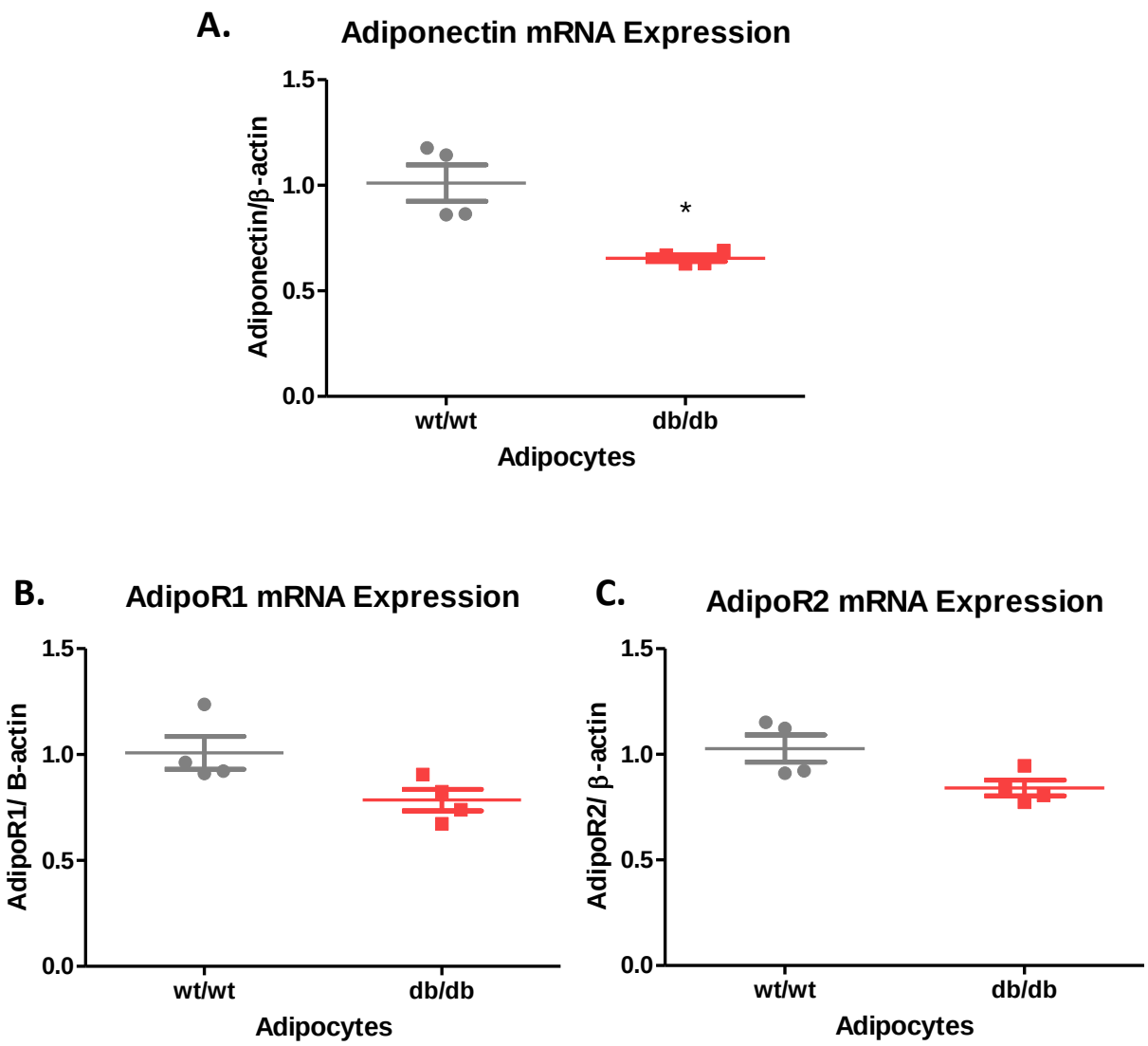

Figure 3.16 Decrease in adiponectin mRNA but not its receptors in the adipocytes of db/db mice

A, **B** and **C**: qPCR analysis graphs representing the mRNA expression of adiponectin, AdipoR1 and AdipoR2 in adipocytes between wildtype and diabetic mice (n=4). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. β-actin used as the loading control. n=4, unpaired t test, *p<0.05

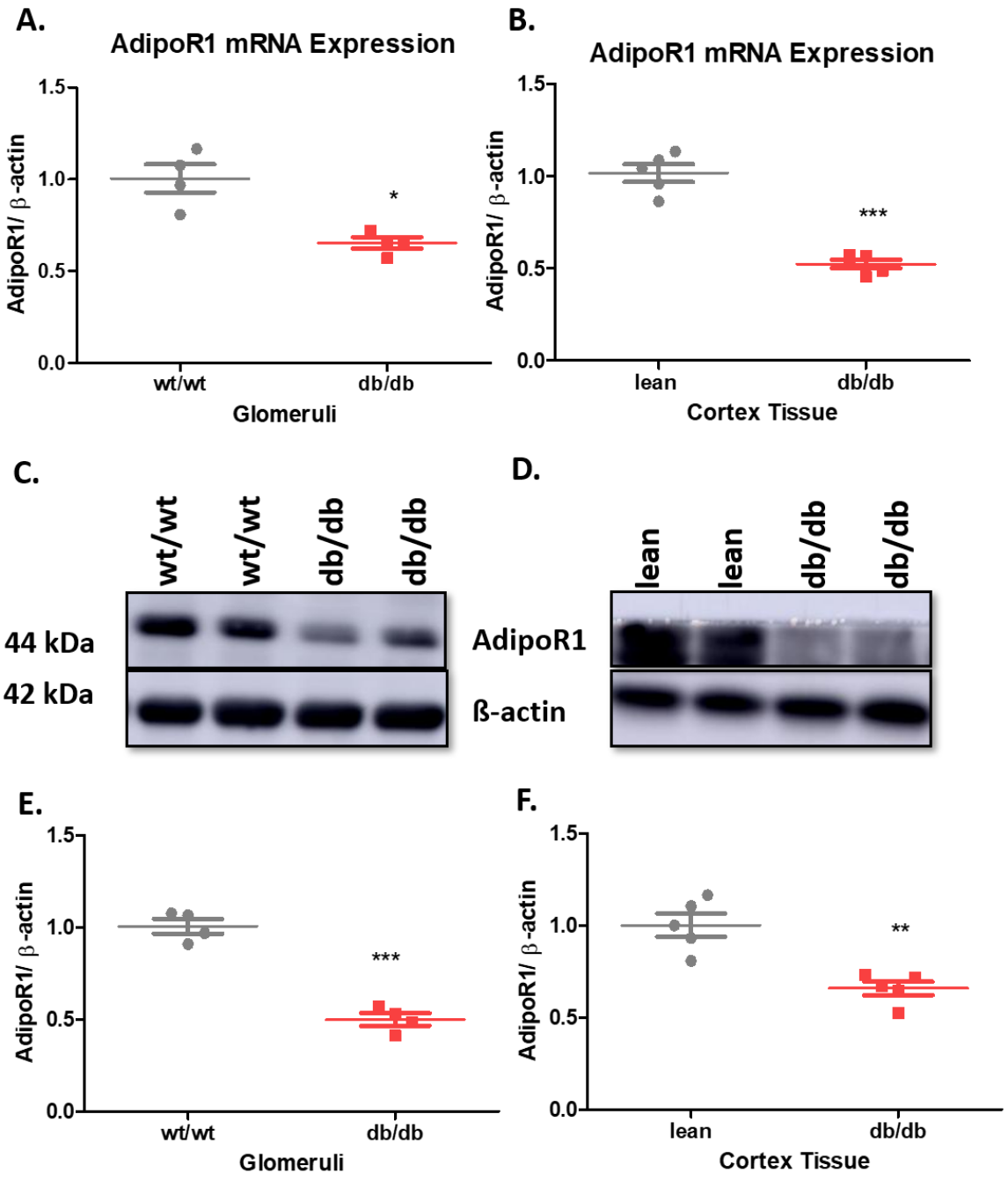

Figure 3.17 Decrease in AdipoR1 mRNA and protein in glomeruli and kidney cortex lysates in db/db mice

A: qPCR analysis graph representing the mRNA expression of AdipoR1 in ex-vivo sieved glomeruli in diabetic mice and **B**: in whole cortex lysates in diabetic mice. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. unpaired t test, *p <0.05 ***p <0.001. **C** and **D**: Representative western blot for 2 repeats and densitometry (for n=4) of AdipoR1 in ex-vivo sieved glomeruli in diabetic mice. (compared to wild type). **E** and **F**: Representative densitometry (for n=5) of AdipoR1 whole cortex lysates in diabetic mice. (compared to lean ones). Normalized to β-actin loading control, points represent means ±SEM, n=4, unpaired t test, ** p < 0.01, ***p <0.001.

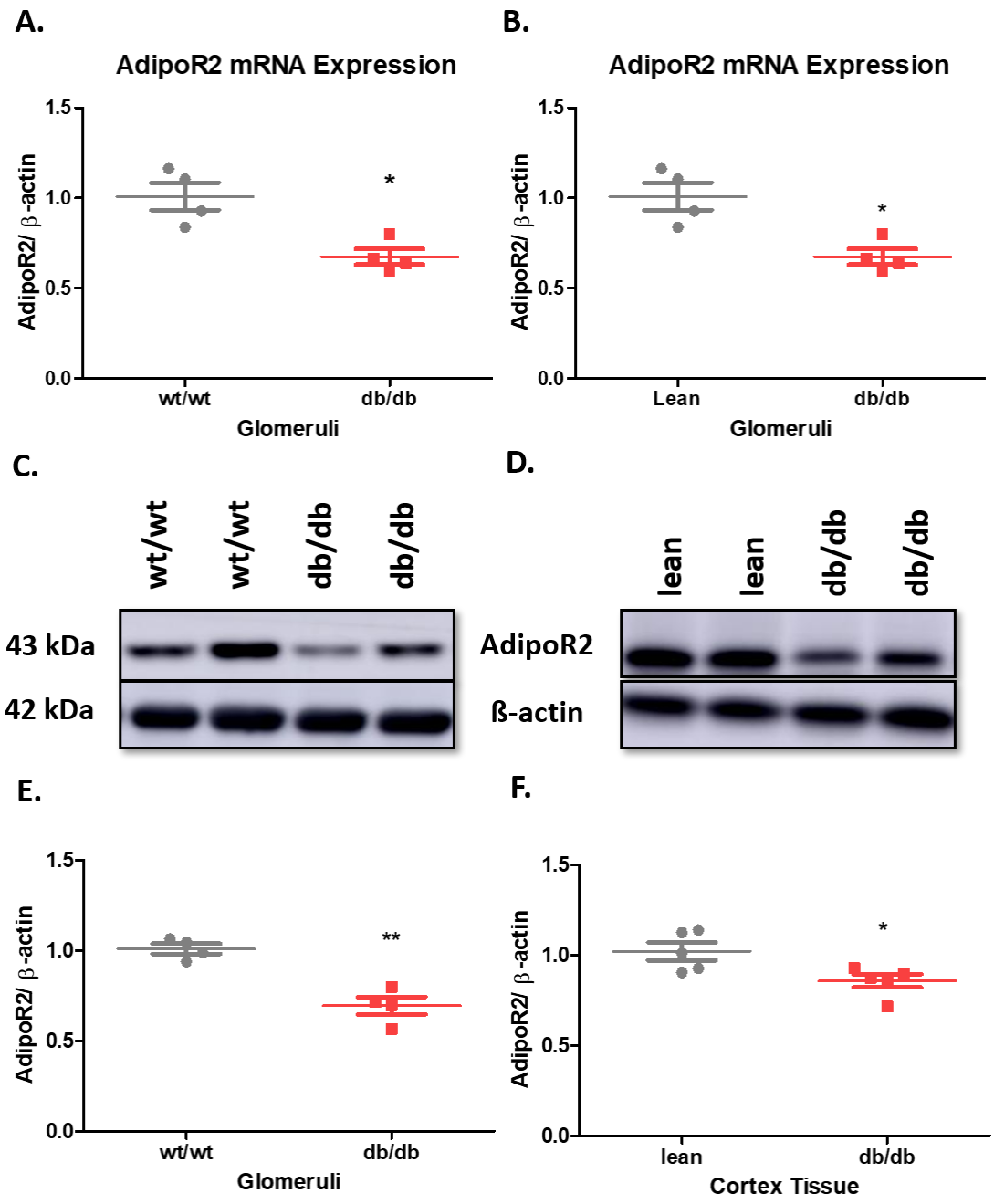

Figure 3.18 Decrease in AdipoR2 mRNA and protein in glomeruli and kidney cortex lysates in db/db mice

A: qPCR analysis graph representing the mRNA expression of AdipoR2 in ex-vivo sieved glomeruli in diabetic mice. (compared to wild type) and **B**: in whole cortex lysates in diabetic mice. (compared to lean ones). n= 4 repeats each in triplicate. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. unpaired t test, *p<0.05. **C** and **D**: Representative western blot for 2 repeats and densitometry (for n=4) of AdipoR2 in ex-vivo sieved glomeruli in diabetic mice. (compared to wild type). **E** and **F**: Representative densitometry (for n=4) of AdipoR2 whole cortex lysates in diabetic mice. (compared to lean ones). Normalized to β-actin loading control, points represent means ±SEM, unpaired t test, *p<0.05 **p<0.01

3.4 Discussion

Adiponectin is an adipose tissue-specific molecule that gained a lot of interest in the last 2 decades. First, it was shown that it makes up to almost 0.01% of plasma proteins within ranges from 5 to 10µg/ml in healthy humans [151]. Importantly, adiponectin plasma levels were shown to be lower in type 2 diabetic patients [170] as well as in obese patients [158]. Furthermore, in specific renal injury (subtotal nephrectomy), hypoadiponectinemia contributed to the exacerbation of renal injury suggesting that replenishing with adiponectin might be therapeutically beneficial in renal disorders [138]. Remarkably, adiponectin levels correlate inversely with weight and body mass index [88]. Although the mechanism of reduced adiponectin levels has not been clear yet in these metabolic disorders, we will try to understand more the relationship of adiponectin and receptors in diabetic kidney-related diseases.

Since the adiponectin gene is expressed solely in both white and brown adipocytes, one of our first findings corroborated this fact. We showed that adiponectin is not expressed nor secreted in any of the healthy unstimulated renal cells (glomeruli and podocytes) at both protein and mRNA levels. This is consistent with other studies that showed that the secretion of adiponectin is exclusive to adipose tissue [88, 158]. It is worth mentioning that adiponectin expression was not assessed in damaged/stimulated CiGEnC. Additionally, Ohashi *et al.* showed abundant staining for adiponectin in the glomeruli of a kidney of wild type mice after subtotal (5/6) nephrectomy but not in the control mice with fully functional kidneys [138].

Since adiponectin has been reported to exert beneficial renal outcomes, such as protecting against the development and progression of albuminuria in mice models [113,

138, 139], questions arose around its two main receptors and their role in these benefits. First, adiponectin receptors were found to be expressed by the four cell types in the kidney; the GEnC, podocytes, proximal tubular cells and mesangial cells [137]. Therefore, the work in this chapter studied the expression levels of the adiponectin receptors in 2 types of cells in the kidney (GEnC and podocytes), as well as how the expression levels are altered in diabetes.

We demonstrated that AdipoR1 and AdipoR2 were expressed at both the mRNA and protein level in unstimulated human CiGEnC as well as CiPod. In GEnC there was a lower level of expression of AdipoR2 compared to AdipoR1 whereas expression levels were comparable in podocytes. This data was consistent with studies by Cammisotto *et al.* [125] and Sharma *et al.* [113], in GEnC and podocytes respectively, that reported lower expression of AdipoR2 in GEnC.

There are a few studies demonstrating adiponectin receptors' expression in adipose tissue or skeletal muscle of T2D patients [171, 172]. These showed that AdipoR1 expression was downregulated in skeletal muscle of diabetic patients compared to controls while AdipoR2 was not affected [172]. In adipose tissue, there was no significant difference in the expression of either AdipoR1 nor AdipoR2 between lean controls and T2D patients [171]. However, there is very little information in the literature concerning the expression of adiponectin receptors in the kidneys of T2D patients. One study showed that in human diabetic kidneys, there is a decreased intraglomerular AdipoR1 and AdipoR2 expression [162]. In our study, mRNA data analysis showed that there was a decreased level of AdipoR1 and AdipoR2 (n=2) in human diabetic kidneys.

A recent study showed that mRNA levels of AdipoR1 and AdipoR2 were decreased in db/db renal cortex and primary human GEnC with HG [20]. In order to understand the

role of renal adiponectin receptors in DN, we investigated the expression levels of AdipoR1 and AdipoR2 *in vitro* in a simulated diabetic milieu environment. CiGEnC and CiPod were cultured in 25mM glucose medium for various time periods. Interestingly, the results in this chapter showed that stimulation with HG resulted in a different profile of AdipoR1 and AdipoR2 in the two cell types. In CiGEnC, AdipoR1 mRNA expression significantly decreased after HG stimulation in 6h and was still decreased at 24h in comparison to unstimulated control cells. Whereas AdipoR2 mRNA expression was not significantly affected by HG even after 24h. However, during a long-time exposure of HG, AdipoR2 expression finally decreased after 7D and lasted till 14D. AdipoR1 mRNA levels also showed a significant decrease after 7D and 14D of 25mM glucose. In comparison, whilst CiPod also demonstrated a down-regulation of AdipoR1 and AdipoR2 mRNA with HG stimulation, it took a longer time frame for the decrease to become apparent (after 2,4 and 7D). These observations are in line such as the one by Park *et al.* which reported a decrease in AdipoR1 and AdipoR2 in commercial human GEnC after 72h with HG induction [159].

The *in vitro* findings were then replicated in an *in vivo* animal model. For the purposes of this study, the db/db mice model of diabetes was chosen to further study the role of adiponectin and its receptors in this model. First, adiponectin mRNA levels were determined in wild type mice in which there is no history of diseases or complications. Our results showed that, in contrast to adipocytes, adiponectin was not present in kidney, liver or muscles. Results also show that in the adipocytes of db/db mice, adiponectin is significantly downregulated when compared to wild type mice. This is consistent with a study that demonstrated that adiponectin plasma levels were reduced in *db/db* mice

[158]. One explanation would be that the secretion of adiponectin from adipocytes may be downregulated in the insulin-resistant state which is depicted in the db/db model.

Furthermore, it is known that AdipoR1 is highly expressed in skeletal muscle while AdipoR2 is highly expressed in the liver under physiological conditions [107, 151]. In this study, it was shown that wild type kidneys express AdipoR1 and AdipoR2 although to a lesser extent to that seen in skeletal muscle and liver. There is evidence that the adiponectin receptor levels are altered in other cell types in the 2 types of diabetes. For example, in type 1 diabetes, it is shown that skeletal muscle AdipoR1 mRNA was increased [173], while it was decreased in type 2 diabetes patients [174]. Similarly, both receptors are decreased significantly in the liver of db/db mice compared to wild type mice [72]. In contrast, cardiac AdipoR1 expression was decreased in both type 1 and type 2 diabetes [161, 175]. However, in this study, we were interested in the receptor expression in db/db cortex tissue and specifically isolated glomeruli. Importantly, AdipoR1 was decreased in the glomeruli and cortex of db/db mice. This is consistent with a study that found that renal AdipoR1 mRNA is decreased in both types of diabetes in rats and mice with nephropathy [161, 176]. This might suggest that two factors are playing a role here, high glucose levels and renal insufficiency, that is significantly decreasing AdipoR1 mRNA and protein expression levels. Moreover, the data about AdipoR2 in renal tissue remains controversial. For example, in type 1 and type 2 diabetes (with DN), AdipoR2 mRNA was not changed at all in renal tissues [161, 176]. However, there was a significant decrease of AdipoR2 in the glomeruli and cortex of db/db mice in our study. Another study also showed a significant reduction of AdipoR2 in db/db mice with DN when compared to their lean control mice [159].

3.5 Conclusion

The reason we are emphasizing on the role of adiponectin receptors is because any reduction or downregulation of adiponectin receptors in DN kidneys may result in reduced adiponectin sensitivity that might affect the protective role of adiponectin in renal physiology. Their location, specifically within the glomerulus, suggests that both adiponectin receptors may change renal physiology and any pathological changes related to diabetes and kidney diseases.

Therefore, in the following chapters, further experiments exploring the influences of AdipoR1 and AdipoR2 will be assessed to better understand how it affects renal function and the adiponectin signalling pathway. Adiponectin treatments in injured CiGEnC will be done to understand more the potent effects of adiponectin under stressful conditions. The role of the receptors will be assessed individually by specific adiponectin receptor knockdowns in cultured CiGEnC to decipher changes in signalling pathways.

Chapter 4 Activation of Signalling Pathways with Adiponectin

4.1 Introduction

Due to its role as an adipose tissue hormone that can exert important biological activities, the effects of adiponectin have been the focus of much research in different target tissues such as skeletal muscle and liver over the past 20 years [68]. The way adiponectin exerts its effects in different targeted tissues, as well as the major signalling pathways involved, were discussed in detail in the introduction to this thesis. Specifically, adiponectin has anti-apoptotic effects in cardiac myocytes [110] and pancreatic β-cells [111], and alleviates oxidative stress in endothelial cells [112] and podocytes [113]. However, it has not been studied specifically in GEnC. Therefore, one of the aims of this project is to decipher the role of adiponectin in GEnC. As discussed in Chapter 1, several signalling pathways has been activated by adiponectin. Importantly, the AMPK pathway and its downstream effect, ACC. There is activation and phosphorylation of AMPKα in skeletal muscle liver, endothelial cells and adipocytes leading to the regulation of glucose and fatty acid metabolism [120]. Specifically, adiponectin has been involved in phosphorylating AMPKα in renal rat glomeruli and its effect was mediated specifically by AdipoR1 [103]. Furthermore, other pathways have been activated in myocytes by gAd such as p38 MAPK pathway [102] and Akt pathway [133]. However, it has not been assessed specifically in GEnC. In the previous chapter, I established that adiponectin receptors are expressed in CiGEnC as well as in human and mouse glomeruli. Therefore, I wanted to determine how adiponectin implements its effects in GEnC (cultured or isolated glomeruli) via its receptors, and which signalling pathways are activated.

Specifically, it is of great importance to determine which receptor is responsible for the activation. A few studies have showed the role of the receptors in other tissues. A model of whole body AdipoR1-knockout in mice showed an impaired glucose tolerance and a decreased phosphorylation of AMPK in the liver, even in the presence of adiponectin, whilst AdipoR2 knockout mouse exhibited insulin resistance and decreased PPARα expression [108]. More importantly, a double receptor knockout mouse resulted in abolition of adiponectin binding and actions, leading to both marked glucose intolerance and insulin resistance [108]. Although these actions were not GEnC-specific, they do point to the importance of both receptors in diabetes. Studies that investigated the distribution of adiponectin receptors and their function in the kidney, suggested that the activation of the receptors could prevent and ameliorate DN [160]. Therefore, an AdipoR1 and AdipoR2 knockdown in CiGEnC, *in vitro,* could be used to study how adiponectin is exerting its' biological effects. Then, I wanted to show whether adiponectin treatment on CiGEnC could reverse changes in signalling cascades under stressful conditions. In fact, it has been shown that HG conditions reduced AMPK activity and it was restored by gAd in mouse podocytes [113]. However, the effect of adiponectin on GEnC in diabetic conditions is not well known.

The aim of this chapter is to understand how adiponectin exerts its effects on cultured GEnC and isolated glomeruli to activate several signalling pathways through its receptors, in health and disease conditions.

a- To show the activation of signalling pathways in response to exogenous gAd in CiGEnC and in *ex vivo* human and mice glomeruli.

b- To investigate the role of AdipoR1 and AdipoR2 in activating intracellular signalling pathways by knocking down either AdipoR1 or AdipoR2.

c- To investigate how adiponectin signalling pathways are modified by changes induced by high-glucose conditions (25mM) and TNF-α (10ng/ml) in CiGEnC.

d- To compare the different signalling effects of gAd in glomeruli from lean and db/db mice.

4.2 Methods

4.2.1 Knockdown of AdipoR1 and AdipoR2 in CiGEnC

Short hairpin RNA (shRNA) sequences are usually encoded in a DNA vector that can be introduced into cells via plasmid transfection or viral transduction. Optimal gene knockdown is a requirement for understanding how the gene is involved in a certain pathway and how vital its function is within the cell. The shRNA constructs of each AdipoR1 and AdipoR2 were purchased from Dharmacon Horizon Inspired cell solutions. Each kit provided 3 different shRNA sequences that targeted the named gene plus a scrambled control shRNA. They were stored immediately at -80^{o}C and avoided any freeze/thaw cycle prior to usage.

4.2.1.1 Detection of puromycin concentration

To generate a fully transduced population of cells, it is crucial to determine the minimum amount of antibiotic required to kill 100% non-transduced cells over a certain period. It is well known that non-viral cells are puromycin resistant. This is done by performing a kill curve to determine the optimal puromycin concentration needed to eliminate non-transduced cells. A dose-response experiment (kill curve) was done where the cells were subjected to increasing amounts of antibiotic to determine the minimum antibiotic concentration needed to kill all the cells over the course of 2 to 5 days. For puromycin, the optimal concentration is the lowest concentration that kills 100% of non-transduced cells and shows maximal survival of transduced cells in 48-72h. Therefore, a dose response experiment of puromycin (0.1, 0.5, 1, 2 and 5µg/ml) was carried out on CiGEnC. The below graph shows the death curve of increasing puromycin concentration on

CiGEnC. The lowest concentration that killed 100% of the cells over a 3-day period was calculated to be 0.8µg/ml.

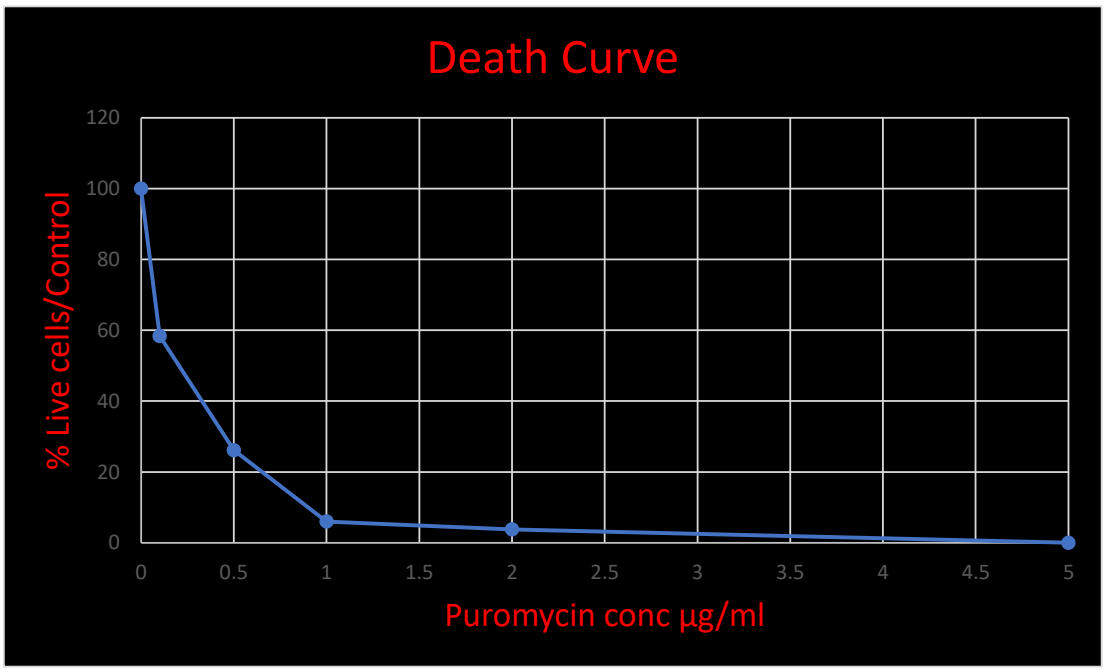

Figure 4.1 Puromycin death curve

Increasing concentration of puromycin (0.1, 0.5, 1, 2 and 5µg/ml) was given to the cells every 24h (changed 3 times during 72h) and then cells were counted to determine the live % of cells after 3 days.

4.2.1.2 Titration of virus by serial dilution

The titration of each shRNA was performed as indicated in the table below. Briefly, CiGEnC were grown in a 6-well plate over a period of 2 days at 33°C (40% confluent). The media was then changed to SFM with polybrene at 575µl in the first well, 500µl in wells 2 to 5, and 400µl in well 6. The whole shRNA vial (25µl) was put in the first well to bring up the total to 600µl. By means of serial dilutions, 100µl was titrated each time into the next well (for a total of 500µl of media) as shown in figure 4.2. This step was crucial to ensure that the concentrated virus will not destroy all the cells at once. After 4h, 1ml of EBM-2MV media was added in each well and left for 48h at 33°C. The cells were then observed under

the microscope to check if they were Green Fluorescent Protein (GFP)-tagged, hence ensuring that the virus is integrated within the cells.

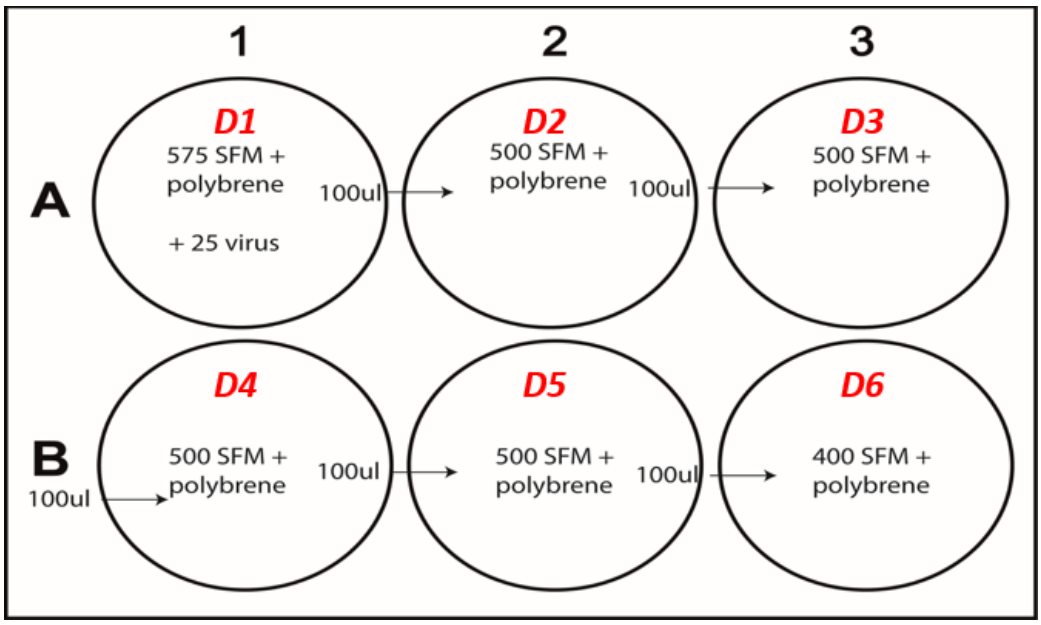

Figure 4.2 Titration of virus into wells

A schematic diagram that shows the serial dilution of the virus into the wells (D1-D6)

4.2.1.3 Selection of cells by puromycin

To ensure that the virus has been integrated into the CiGEnC to generate a stable knockdown cell line, it is important to get rid of the non-transduced CiGEnC. Therefore, after the 48h virus period from the step above, media was changed in each well to a media with puromycin (0.8µg/ml) for 3 consecutive days.

4.2.1.4 Trypsinization of virus cells and storage

Before splitting the cells in the wells and plating them into new flasks, the cells were checked again under the microscope. This confirmed that the first, with the most concentrated virus, well had no viable cells. The 6th well (D6) was discarded because it

had the least concentration of the virus (according to the titration method as seen in figure 4.2. The other 4 wells were washed with PBS before adding 0.25% trypsin-EDTA. The same method was conducted regarding splitting and storing as mentioned in sections 2.2.4 and 2.2.5 in chapter 2.

4.2.1.5 Validation of knockdown

Further RNA and protein were extracted from each shRNA variant to determine the percentage of knockdown of gene expression. RNA and protein analyses were done by qPCR and Western blot as mentioned in chapter 2 sections 2.4 and 2.5. The results are explained in the next section.

4.2.2 Treatment with gAd, HG and TNF-α

CiGEnC and CiPod (when applicable) were treated with gAd (2.5µg/ml) at different time points in SFM (unless otherwise stated) before further protein extraction. Cells were also treated with TNF-α and HG (with or without gAd) as previously described (10ng/ml and 25mM respectively) [35, 177].

4.2.3 Ex-vivo glomeruli treatments

Glomeruli were isolated as previously described in Chapter 3, section 3.2.2.3 and treatments were given as follows: human and mouse glomeruli were treated with gAd (in SFM), for the time points suggested, in a water bath at 37^{o}C and subsequently RNA and protein were extracted as detailed in Chapter 3, sections 3.2.3 and 3.2.4.

4.3 Results

4.3.1 Adiponectin stimulates p-AMPK-α in a dose-dependent and time-dependent manner in CiGEnC

In order to understand how gAd exerts its effects on CiGEnC, initially both a dose response and time course of adiponectin treatment was carried out (Figure 4.3). According to a review of the literature, the physiological level of gAd is around 2.5μg/ml [113]. Therefore, a dose response between 0 and 25μg/ml and a time response between 0 and 24h as carried out. As described in Chapter 1, the AMPK pathway has a major role in adiponectin signalling. Treatment of CiGEnC with gAd caused a sustained phosphorylation of threonine 172 in the α subunit of AMPK (AMPKα1) in a dose-dependent manner as seen by densitometry analysis of the western blots with an expected size of 62kDa. The effect peaked with a 2.5 and 2.7-fold rise after 30min at 2.5 and 10μg/ml respectively (Figure 4.3A and B). Although both concentrations yielded a similar increase in the phosphorylation of AMPK, for future experiments, unless otherwise stated, a concentration of 2.5μg/ml was used since it represents a more physiological concentration. A time course experiment was then carried out to determine whether gAd stimulates the phosphorylation of AMPK in a time-dependent manner. Results from these experiments showed that phosphorylation was sustained even after 1, 2 and 4h but then returned to almost basal levels after 24h (Figure 4.3C and D)

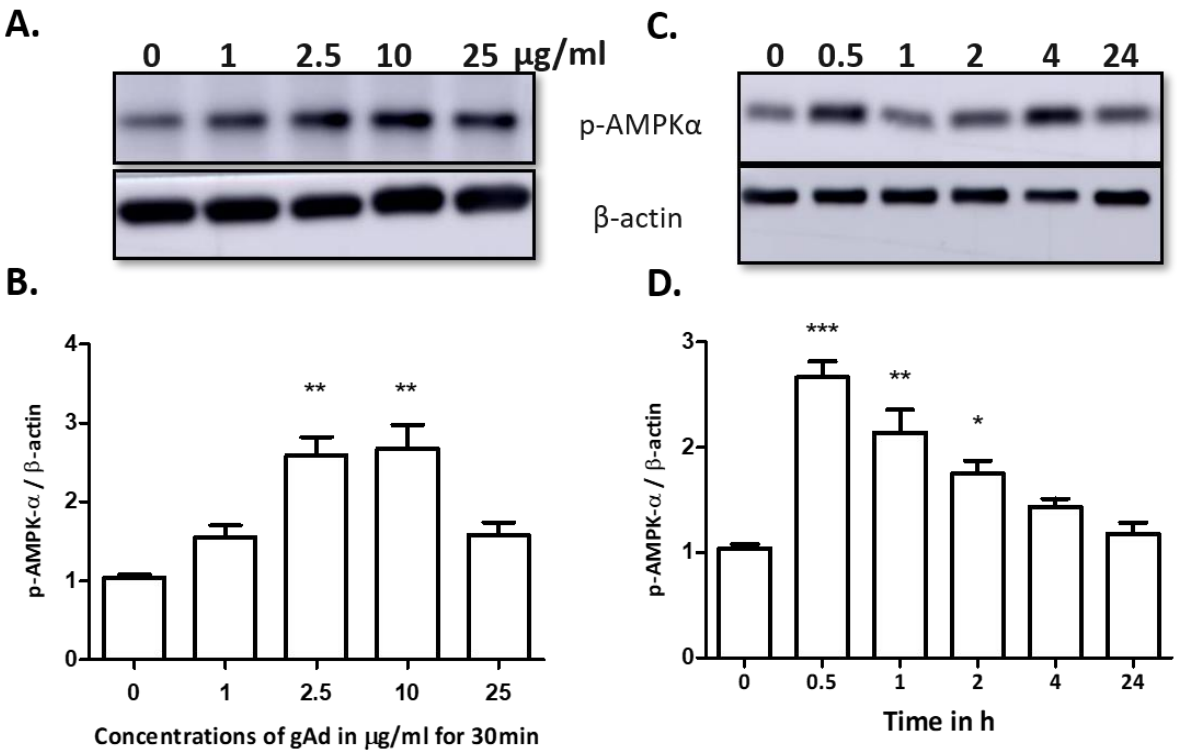

Figure 4.3 A dose and time-dependent effect of gAd in CiGEnC on AMPK pathway

A: Representative western blot demonstrating the concentration dependent effect of gAd (1, 2.5, 10, and 25µg/ml) and its densitometry (**B**) that confirmed the phosphorylation of AMPK in response to gAd in a dose-dependent manner. **C**: Representative western blot demonstrating the time dependent effect of gAd at 2.5µg/ml (0.5, 1, 2, 4, 24h) in CiGEnC on AMPK-α phosphorylation and its densitometry (**D):** Densitometry was performed on 3 representative blots from 3 independent repeats (n=3) showing levels of protein of interest normalised to β-actin loading control. Bars represent means ±SEM, n= 3, one-way ANOVA, ** $p < 0.01$ *** $p < 0.001$. *post hoc* analysis (Bonferroni).

4.3.2 Adiponectin stimulates the AMPK and ACC pathway in CiPod.

The AMPK pathway and its downstream target ACC were also investigated in human CiPod. The CiPod were treated at different time points at a concentration of 2.5µg/ml (Figure 4.4). As predicted, the phosphorylation of AMPK-α was also significantly increased

about 3-fold after 30min. However, the phosphorylation of ACC was only significant after 1h (2-fold) but not 30min.

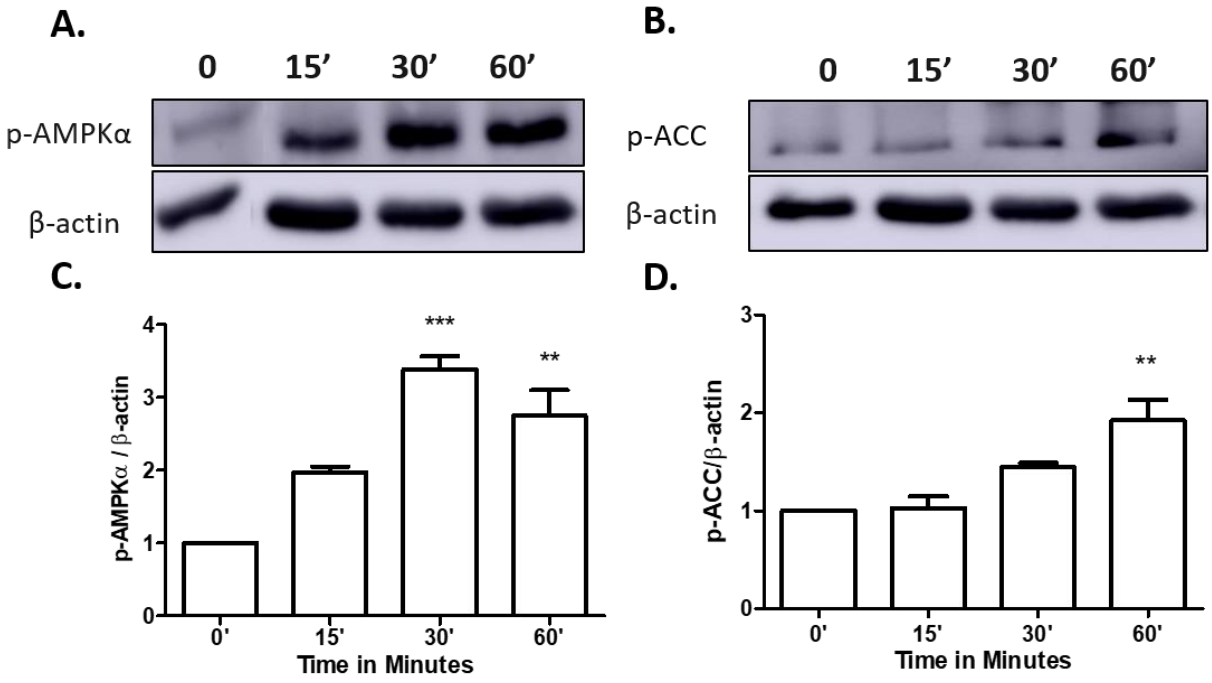

Figure 4.4 Adiponectin activates and phosphorylates AMPKα and ACC in CiPod.

A and B: Representative western blot demonstrating gAd (2.5ug/ml) effects in a timely manner (0, 15, 30 and 60 minutes) in CiPod on AMPK and ACC phosphorylation respectively. **C and D:** Densitometry confirmed the phosphorylation of AMPK and ACC in response to gAd at a maximum after 30min for AMPK and 60min for ACC. Densitometry was performed on 3 representative blots from 3 independent repeats (n=3) showing levels of phosphorylated protein of interest normalised to β-actin loading control, bars represent means ±SEM, one-way ANOVA, ** $p < 0.01$, *** $p < 0.001$, *post hoc* analysis (Bonferroni).

4.3.3 Adiponectin activates several signalling pathways in CiGEnC

The effect of gAd administration can help us understand how adiponectin signalling is mediated in CiGEnC through different signalling pathways. As discussed above, the major signalling pathway activated by gAd is the AMPK pathway and one of its downstream

effects, ACC. Therefore, a different time course of exogenous gAd at 2.5µg/ml was done in CiGEnC to determine not only phosphorylation levels but also their total protein expression. There was a significant increase (3.5-fold,) in p-AMPK-α after 30min (Figure 4.5A and B). Similarly, there was also an increase in the phosphorylation of ACC with a notable 2.8-fold rise after 30min (Figure 4.5C and D).

Furthermore, to study the underlying mechanism of gAd in glucose metabolism, we investigated the role of the Akt signalling pathway. One of the major phosphorylation sites on Akt is the serine at 473 generating p-Akt, which leads to activation of this enzyme and resultant downstream effects such as increased glucose uptake into the cell. As expected, the phosphorylation of Akt at ser473 was significantly increased by 3-fold after 30min treatment with gAd (Figure 4.5E and F).

It is also well known that the p38 MAPK pathway is activated in muscle cells after adiponectin binding to its receptors [47]. Therefore, the phosphorylation of p38 in CiGEnC upon treatment with gAd was also studied. Adiponectin treatment led to a significant increase in the phosphorylation of p38 MAPK which peaked after 30min (Figure 4.5G and H).

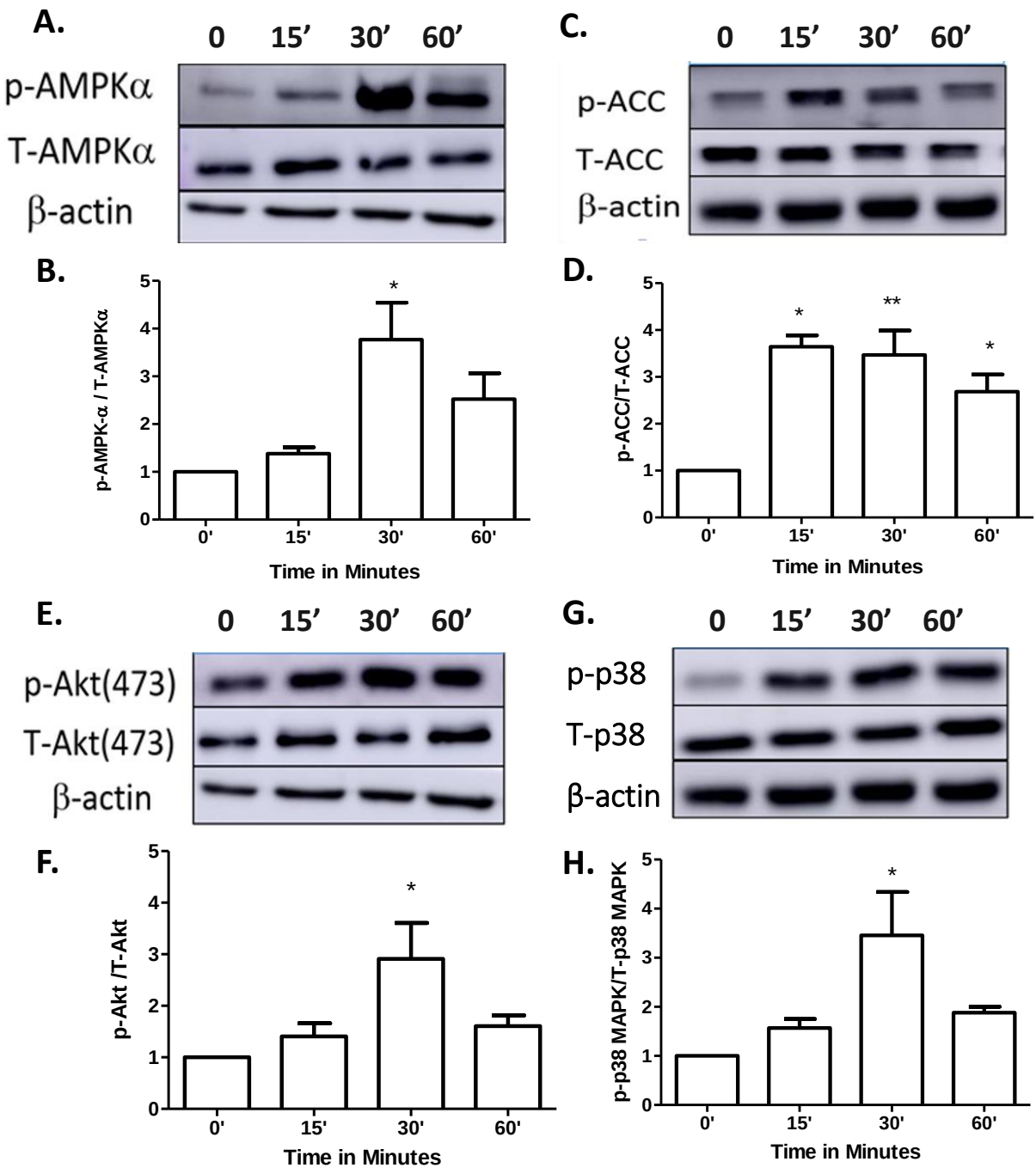

Figure 4.5 Adiponectin stimulates AMPK, ACC, Akt and p38MAPK phosphorylation in CiGEnC

A, C, E,G: Representative western blot demonstrating time course of gAd effects (0, 15, 30 and 60min) in CiGEnC on phospho AMPK, ACC, Akt and P38 MAPK and their respective total levels. **B, D,F,H:** Densitometry confirmed the phosphorylation of AMPK, ACC, Akt and P38 MAPK in response to gAd which peaked after 30 minutes. Densitometry was performed on 3 representative blots from 3 independent repeats (n=3) showing levels of protein phosphorylation of interest normalised to their respective totals then β-actin loading control, bars represent means ±SEM, one-way ANOVA, *p <0.05, **p < 0.01. *post hoc* analysis (Bonferroni).

4.3.4 Are the effects of gAd mediated by AdipoR1 and/or AdipoR2 in CiGEnC?

It was shown in Chapter 3 that both adiponectin receptors are expressed in CiGEnC therefore, I determined whether the signalling pathways activated in CiGEnC by gAd are dependent on AdipoR1 and/or AdipoR2, or there are other factors contributing to the increased phosphorylation of certain proteins.

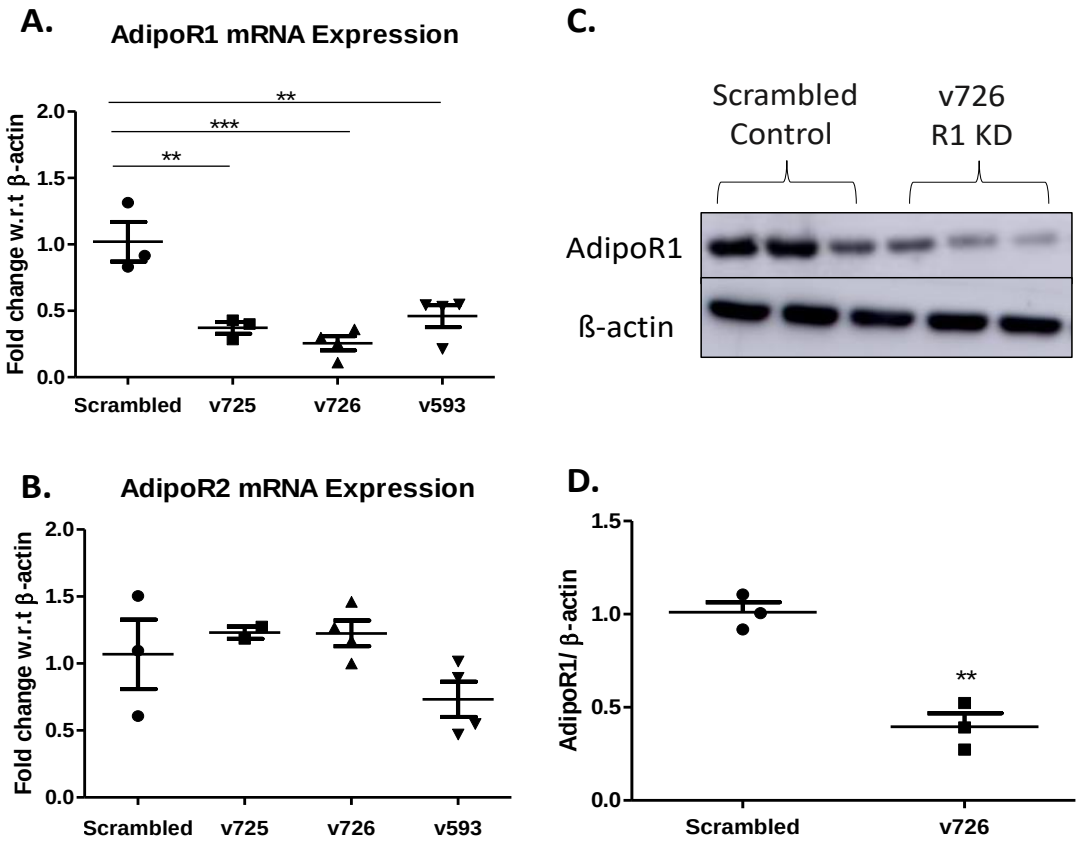

Figure 4.6 Confirmation of AdipoR1 knockdown by shRNA in CiGEnC at mRNA and protein levels.

A: qPCR data analysis highlighting the decreased expression of AdipoR1 mRNA in CiGEnC using 3 different shRNA constructs. One-way ANOVA,**p<0.01, *** p <0.001 *post hoc* analysis (Bonferroni) **B:** qPCR data analysis showing AdipoR2 mRNA expression was not affected by the 3 shRNA of AdipoR1 (ANOVA, ns p>0.05) **C:** Representative western blot demonstrating the knockdown extent of AdipoR1 in shRNA v726 **D:** Densitometry confirmed the knockdown of AdipoR1 protein expression in CiGEnC. Data normalised to β-actin loading control, bars represent means ±SEM, n= 3, unpaired t test, ** p <0.01.

As described in the methods, shRNA sequences can be used for knockdown studies. Three different commercially available shRNA sequences were tested in CiGEnC. Knockdown was confirmed using qPCR for the detection of mRNA expression. As seen in figure 4.6A, there was a significant decrease (70-80%) in AdipoR1 mRNA using all the shRNA variants most notably with v726. It is also worth pointing out that AdipoR2 mRNA levels were not significantly affected by any of the variants of the AdipoR1 KD shRNA used (Figure 4.6B). At the protein level, there was also a significant knockdown (60%) using the AdipoR1 v726 variant (Figure 4.6C and D). Therefore, for future AdipoR1 knockdown studies, shRNA v726 cells were used.

As for AdipoR2, there was also a significant mRNA knockdown (60-80%) of AdipoR2 using all the different shRNA variants tested and specifically v939 (Figure 4.7A). Although significant knockdown was observed at the mRNA level for all shRNA, knockdown at protein expression was only observed in shRNA v939(Figure 4.7C and D). Importantly, there was no change in the AdipoR1 mRNA (ns $p>0.05$) in the AdipoR2 knockdown cells suggesting that the knockdown was specific for AdipoR2 and not AdipoR1 (Figure 4.7B).

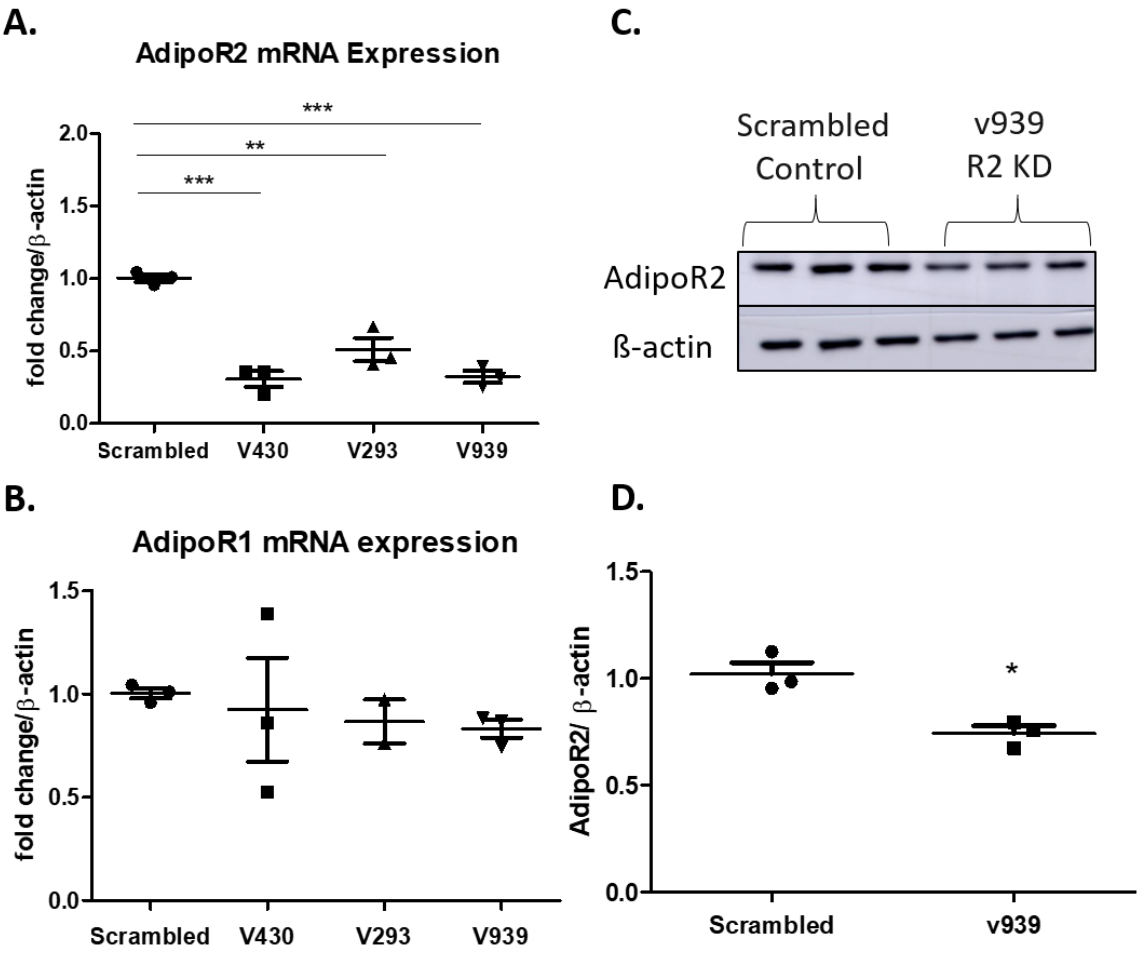

Figure 4.7 Confirmation of AdipoR2 knockdown by shRNA in CiGEnC at mRNA and protein level.

A: qPCR data analysis highlighting the decreased expression of AdipoR2 mRNA using 3 different shRNA knockdown CiGEnC of AdipoR2. One-way ANOVA,**p<0.01, *** p <0.001 *post hoc* analysis (Bonferroni) **B:** qPCR data analysis showing AdipoR1 mRNA expression not affected by the 3 shRNA knockdown of AdipoR2 (ANOVA, ns p>0.05) **C:** Representative western blot demonstrating the knockdown extent of AdipoR2 in shRNA v939 **D:** Densitometry showed significant knockdown of AdipoR2 protein expression in v939 shRNA . Data normalised to β-actin loading control, scatter dot represent means ±SEM, n= 3, unpaired t test, *p <0.05

4.3.5 Adiponectin effects are dependent on AdipoR1

Following the knockdown of AdipoR1 and AdipoR2 in CiGEnC, the knockdown cells were treated with gAd at 2.5µg/ml for 30min and the responses compared to scrambled control cells. As shown in figure 4.8A, there was an activation and phosphorylation of AMPK (2.4-fold) and ACC (2.1-fold) in the scrambled controls as predicted. However, there was a

significant decrease in phosphorylation of AMPKα and ACC in AdipoR1 v726 knockdown CiGEnC when treated with gAd for 30min (compared to 30min scrambled controls). Meanwhile, in the AdipoR2 knockdown CiGEnC, there was no change in phosphorylation of AMPKα and ACC suggesting that AMPK pathway is independent of AdipoR2 but might be still operating via AdipoR1 in CiGEnC.

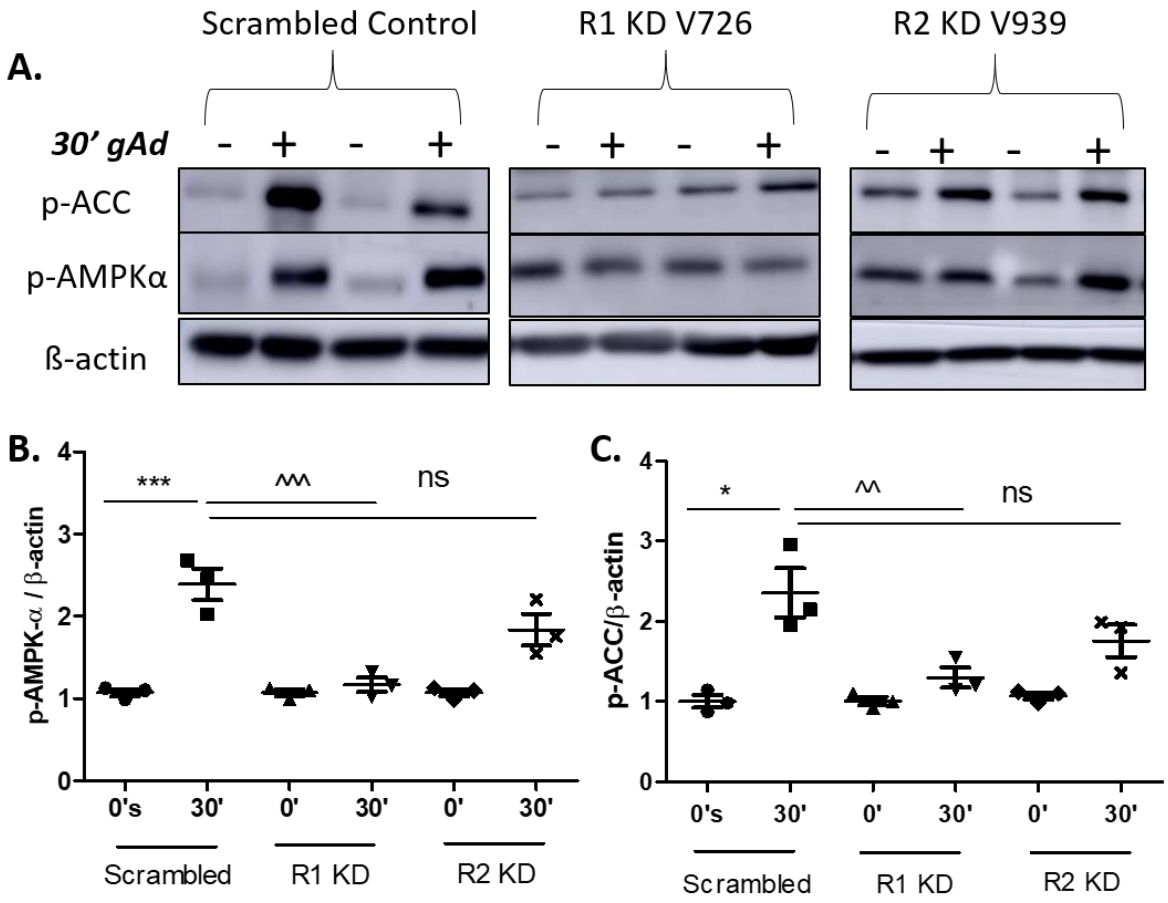

Figure 4.8 Adiponectin signalling through AMPK and ACC is impaired in AdipoR1 and not AdipoR2 knockdown cells.

A: Representative western blot demonstrating gAd effects in a scrambled control, AdipoR1 and AdipoR2 knockdown on AMPK and ACC phosphorylation. **B and C:** Densitometry showing the extent of phosphorylation of AMPK and ACC in the 3 cell lines after 30min. Data normalised to β-actin loading control, dots represent means ±SEM, n= 3, one-way ANOVA, *p <0.05, ***p <0.001 when compared to 0min scrambled control, and ^^p <0.01, ^^^p<0.001 when compared to 30min scrambled. *post hoc* analysis (Bonferroni).

4.3.6 Effect of high glucose on adiponectin signalling pathways.

In order to induce diabetic conditions *in vitro*, CiGEnC were cultured in either 5.5mM or 25mM of glucose for 48h. Figure 4.9 shows the effects of high glucose and gAd in CiGEnC on signalling proteins. In unstimulated cells, HG significantly decreased the phosphorylation of AMPKα (Figure 4.9B) and ACC (Figure 4.9C) but not p38 MAPK (Figure 4.9E) and led to elevated levels of p-Akt. (Figure 4.9D). As shown before, there was a significant increase in all phospho-proteins after 30min of gAd in cells cultured in normal glucose. However, after the culture of CiGEnC for 48h in HG and the last 30min with gAd, p-AMPK, p-ACC and p-p38 were still activated by gAd but to a lesser extent. As for the phosphorylation of Akt, the treatment of gAd for 30min after HG for 48h did not add any further to the observed increase in the p-Akt levels.

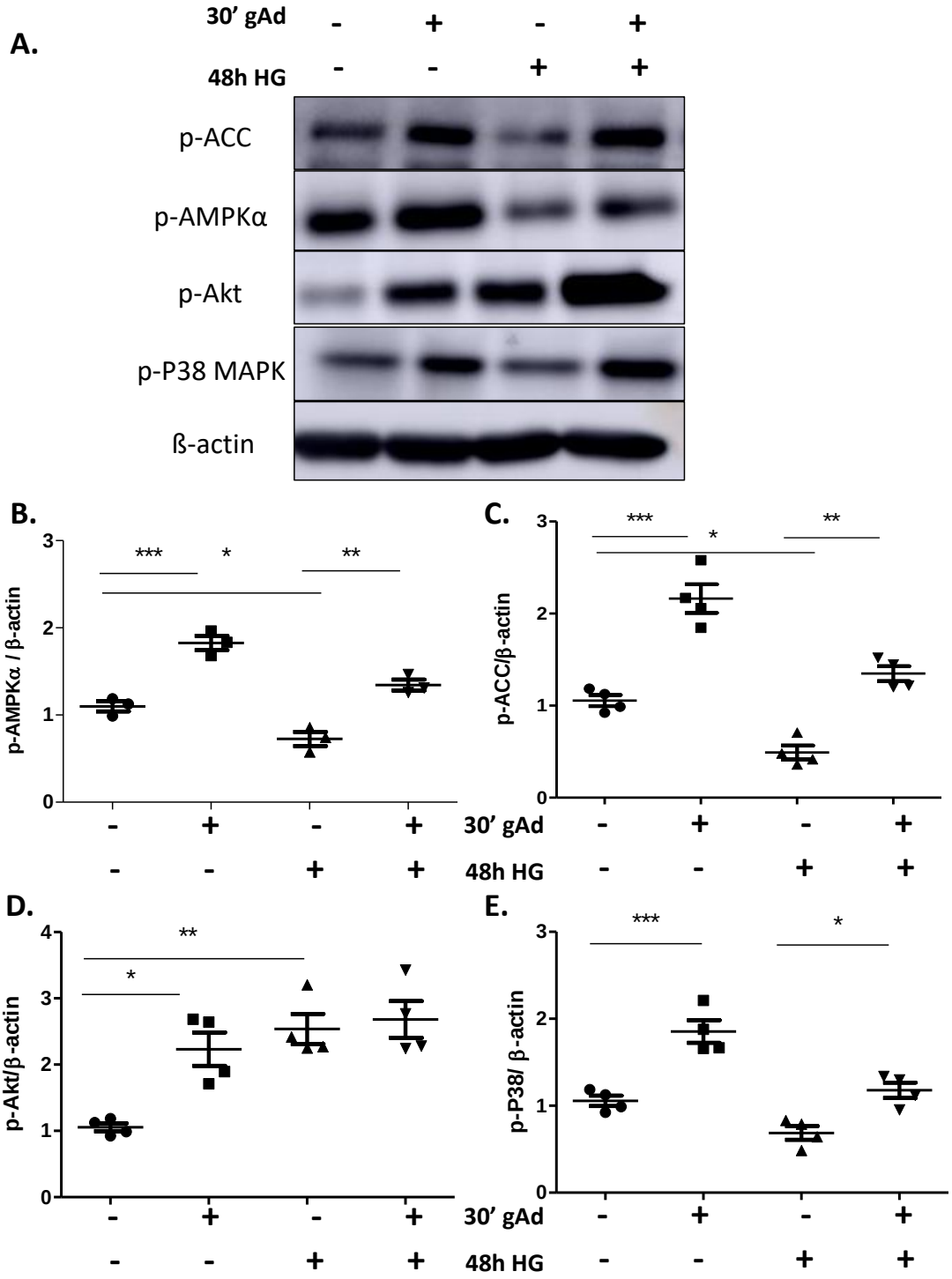

Figure 4.9 Adiponectin reversed decreases of phosphorylation of AMPK, ACC and p38 in high glucose induced CiGEnC

A: Representative western blot and densitometry of p-AMPK, p-ACC, p-Akt and p-P38 proteins (**B, C, D and E)** demonstrating gAd (2.5 µg/ml) effects in normal (5mM) and HG (25mM) for 48h after 30min. Data normalised to their respective totals then β-actin loading control, dots represent means ±SEM, n>3, one-way ANOVA, * p <0.05, ** p < 0.01 ***p<0.001. *post hoc* analysis (Bonferroni).

4.3.7 Effect of TNF-α on adiponectin gAd on signalling pathways.

Another way of inducing diabetic conditions is the treatment of CiGEnC with TNF-α, which is a component of the inflammatory milieu. Therefore, the effects of TNF-α with or without gAd were also evaluated. The dose of TNF-α was chosen according to a study previously done within the group [35]. Briefly, CiGEnC were either treated with TNF-α at 10ng/ml and/or gAd at 2.5µg/ml for 2h. Figure 4.10 represents the Western blots and the respective densitometry. As shown in Figure 4.10, TNF-α induction for 2h led to a significant decrease in both p-AMPKα and p-ACC. Adiponectin treatment reversed this decrease.

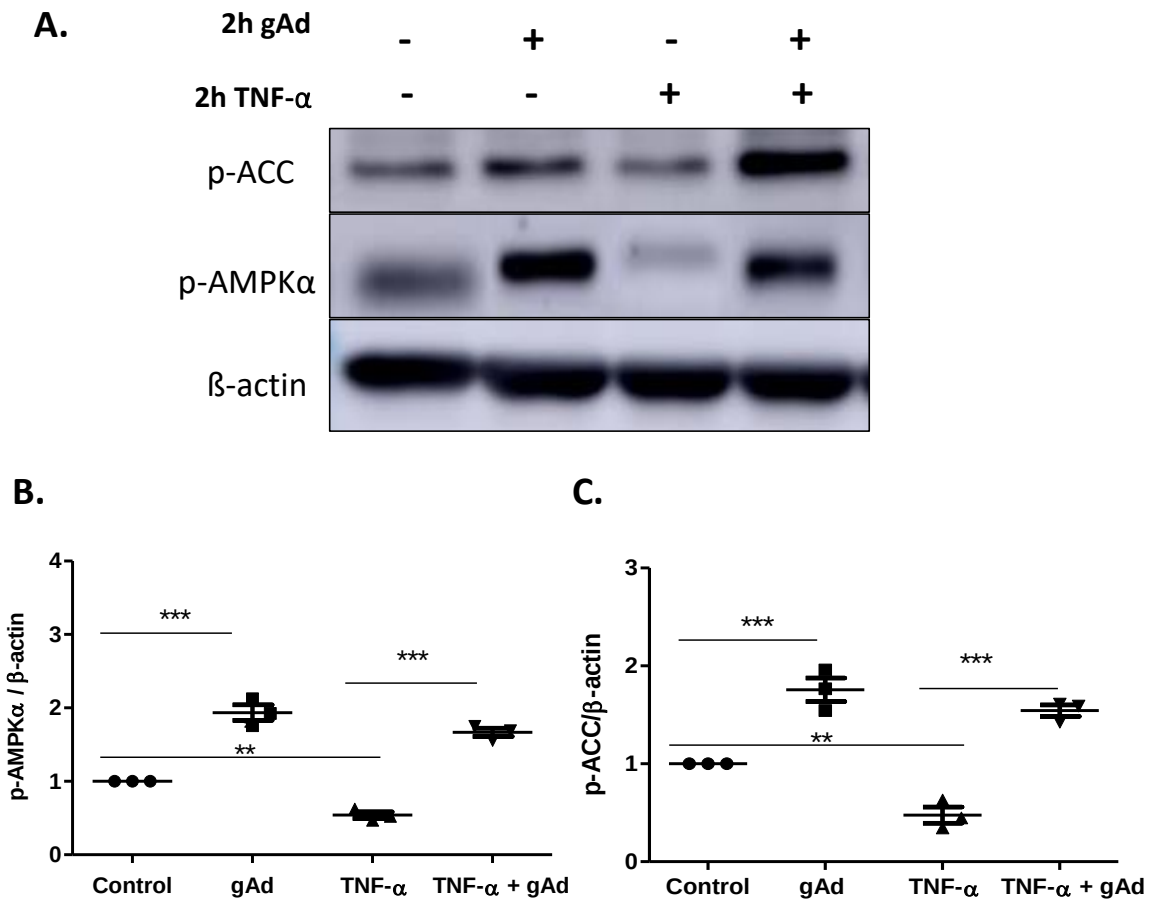

Figure 4.10 Adiponectin reversed decreases of phosphorylation of AMPK and ACC in TNF-α induced CiGEnC

A: Representative western blot and densitometry (**B and C)** demonstrating gAd (2.5µg/ml) effects CiGEnC co-treated with or without TNF-α (10ng/ml) for 2h. Data normalised to their respective totals then β-actin loading control, bars represent means ±SEM, n= 3, one-way ANOVA, **p<0.01 ***p<0.001. *post hoc* analysis (Bonferroni).

4.3.8 AMPK-α subunit is expressed in human and mice cortex

Different subunits of AMPK are expressed differently in tissues. Therefore, I wanted to confirm the alpha subunit of AMPK is expressed in human and mice cortex. Figure 4.10A shows the phosphorylation of AMPK at alpha subunit in both human and mice kidney cortex lysates. Furthermore, we showed that the phosphorylation of AMPK-α is disrupted

and decreased in db/db kidney lysates of mice when compared to lean controls (Figure 4.11B).

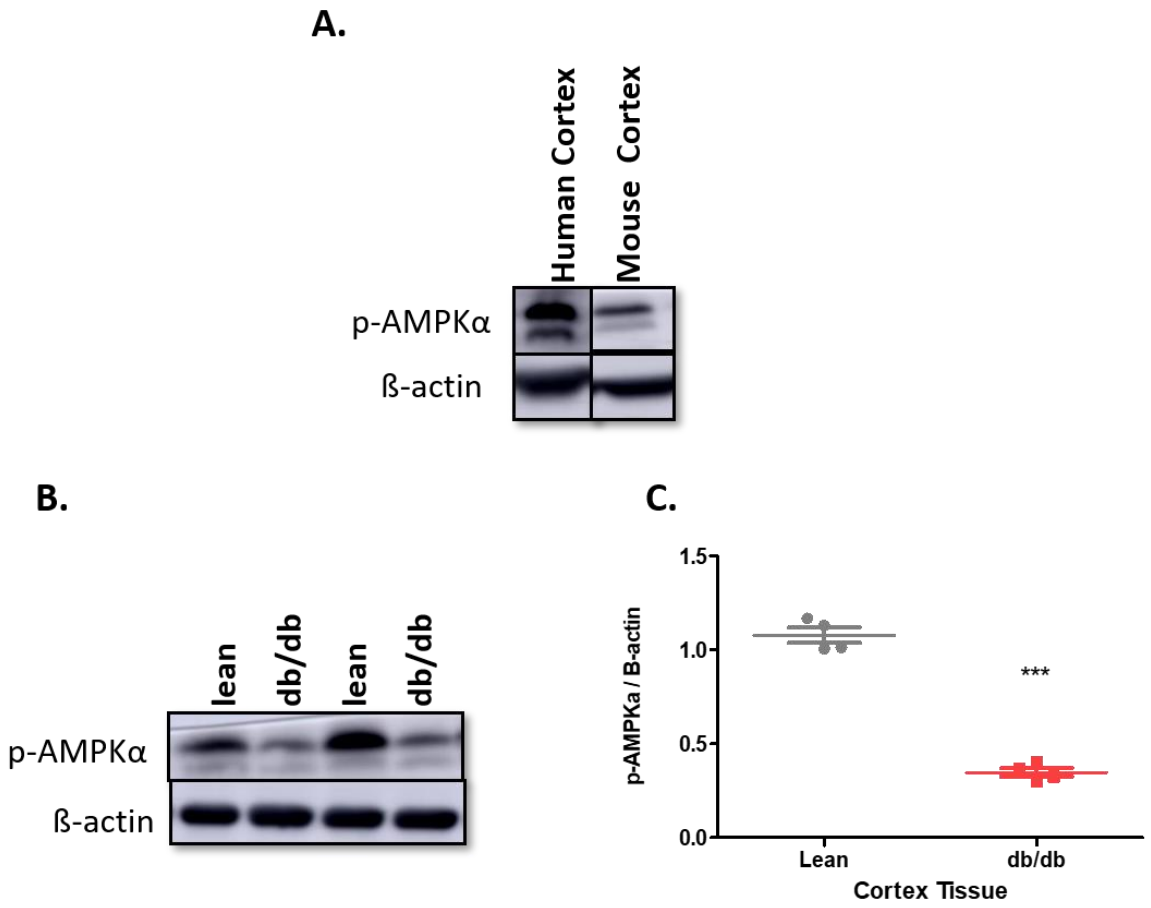

Figure 4.11 Expression of p-AMPK-α in human and mice cortex lysates

A: Representative western blot demonstrating p-AMPK-α subunit in human and wild type mouse cortex. **B:** Representative western blot showing the expression of p-AMPK-α in lean vs db/db mice cortex. **C**: Densitometry confirmed the decrease in phosphorylation of AMPK-α in db/db cortex. Densitometry was performed on 4 representative blots from independent repeats (n=4) showing levels of protein of interest normalised to β-actin loading control, dots represent means ±SEM, unpaired t test, * ***p<0.001

4.3.9 Adiponectin stimulates p-AMPK-α in human and mice glomeruli

Since gAd stimulated signalling pathways in *in vitro* cultured cells, the next step was to confirm the effects in *ex vivo* sieved glomeruli. Briefly, *ex vivo* glomeruli were sieved from healthy

human and lean mice kidney tissue as described in Chapter 3 section 3.2.2.3. Following that, they were incubated with gAd at 2.5µg/ml for the specified time points (0, 0.5, 1 and 2h). Western blot analysis revealed that the activation of AMPKα was significantly increased after 30min in human glomeruli (2-fold) (Figure 4.12A and C). In isolated mice glomeruli, the phosphorylation of AMPKα was seen after 30min and peaked at 1h (2-fold) (Figure 4.12B and D)

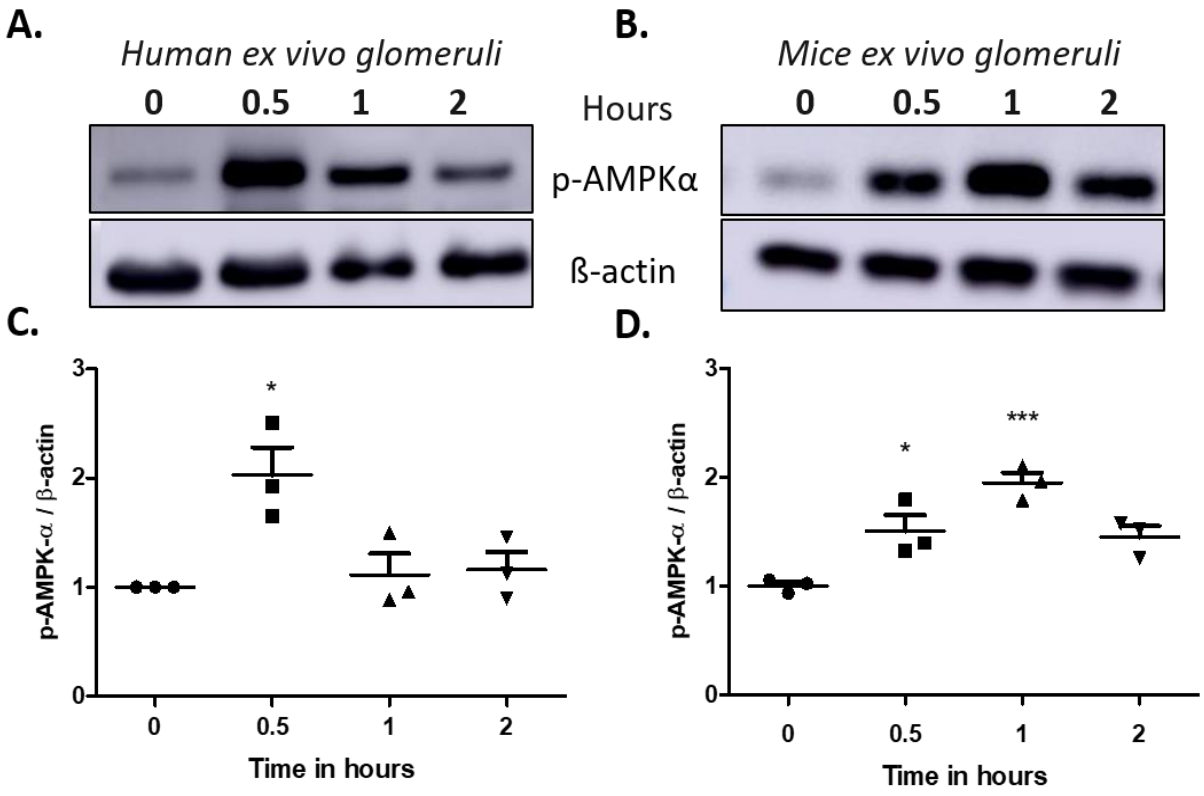

Figure 4.12 Adiponectin stimulates phosphorylation of AMPK-α in human and mice ex vivo glomeruli

A and B: Representative western blot demonstrating gAd effects at different time points (0, 0.5,1 and 2h) in human (**A**) and mice (**B**) ex vivo glomeruli on AMPK phosphorylation. **C and D:** Densitometry confirmed the phosphorylation of AMPK in response to gAd at a maximum after 0.5h for human and at both 0.5h and 1h for mice for AMPK. Densitometry

was performed on 3 representative blots from independent repeats (n=3) showing levels of protein of interest normalised to β-actin loading control, dots represent means ±SEM, one-way ANOVA, * p <0.05, ** p < 0.01, *post hoc* analysis (Bonferroni).

4.3.10 Functional response of gAd on diabetic *ex vivo* glomeruli

Following AMPKα phosphorylation by gAd in ex vivo control mouse glomeruli, I sought to determine whether this is also seen in diabetic glomeruli. As described in the methods, db/db mice were culled, and kidneys were harvested. Following that, the glomeruli were sieved and then treated with gAd for 1h at 37^{o}C in a water bath. The extraction of protein was also done according to the protocol described. Figure 4.13 shows the western blots and their respective densitometry.

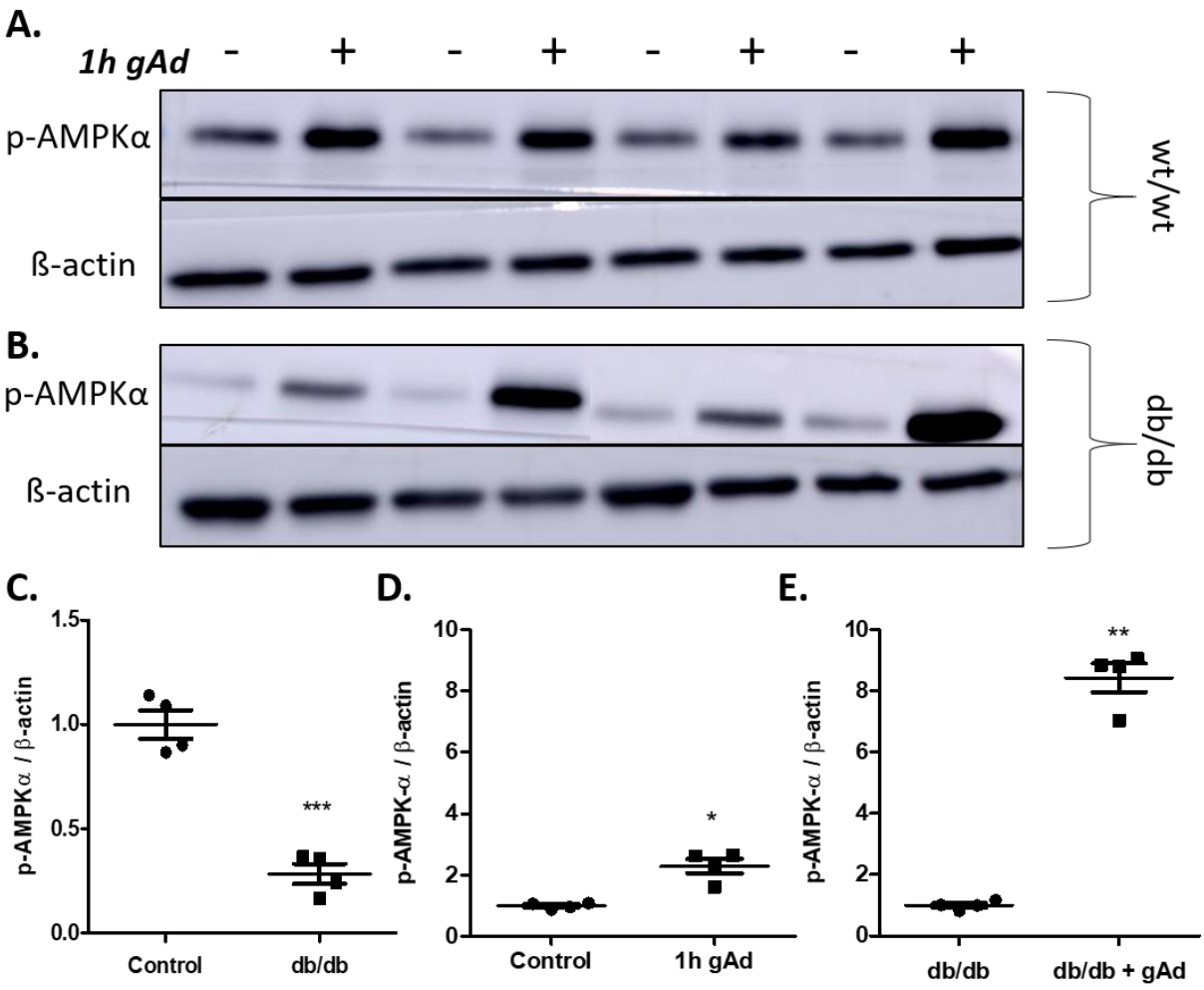

Figure 4.13 Adiponectin stimulates the phosphorylation of AMPK-α in ex vivo db/db glomeruli

Representative western blot demonstrating gAd at 2.5ug/ml effects after 1 hr in wt/wt (n=4) (**A**) and db/db (n=4) (**B**) ex vivo glomeruli on AMPK phosphorylation. **C and D:** Densitometry confirmed the phosphorylation of AMPK in response to gAd at a maximum after 1h for wild type (ANOVA, * p<0.05) and db/db (ANOVA, **p<0.01). Data normalised to β-actin loading control, dots represent means ±SEM, n= 4, one-way ANOVA, * p <0.05, ** p < 0.01, *post hoc* analysis (Bonferroni).

Densitometry shows that the phosphorylation of AMPKα is decreased in diabetic when compared to control glomeruli (Figure 4.13C). Furthermore, as shown also previously in Figure 4.12, gAd treatment for 1h increased p-AMPKα almost 2-fold in wild type glomeruli (Figure 4.13D). Interestingly, this increase of p-AMPKα was magnified up to 8-fold in diabetic glomeruli gAd treatment for 1h (Figure 4.13E).

4.4 Discussion

The aims of this chapter were to determine the exogenous effect of gAd on the phosphorylation of proteins reported to be involved in adiponectin downstream signalling pathways in other cells or tissues. One of the major pathways is the AMPK pathway, a major metabolic switch that senses an increase in the ratio of AMP:ATP. This pathway is known to be activated by gAd in various tissues as liver, skeletal muscle and also cardiomyocytes [103, 173, 178]. This study is the first to have investigated the different signalling pathways induced by gAd in CiGEnC. The data shows promising results, in which there is an activation of more than one of the major pathways in GEnC in response to gAd. Exogenous gAd treatment on CiPod is also shown to activate the AMPK pathway suggesting that its effects on renal cells are not GEnC specific. Increased phosphorylation of AMPK-α in response to gAd has also been reported previously in conditionally immortalized mouse podocytes [113]. Nevertheless, for the purpose of this thesis, subsequent work focused on its roles in GEnC only.

One the downstream molecules of the AMPK pathway, ACC, was also investigated. ACC is the rate limiting enzyme in fatty acid synthesis that is inhibited by phosphorylation and activated by dephosphorylation[179]. Treatment of gAd in CiGEnC resulted in an increased phosphorylation of ACC, hence inhibiting fatty acid synthesis, thereby increasing energy expenditure. In contrast, high concentration of insulin in the fed state results in low levels of p-ACC [180]. This regulation promotes fatty acid oxidation in the fasted state when there are low levels of AMP and inhibits it in the fed state when there is enough energy in the form of ATP [179].

In addition to the AMPK pathway, adiponectin can also mediate a signalling cascade through the p38 MAPK pathway. It is considered as a major kinase in the MAPK family and plays essential roles in regulating inflammation and immune responses [181]. It is also a notable phospho-signalling cascade which is regulated by insulin [182]. Therefore, the effects of the phospho-protein p38 upon gAd stimulation were studied, even though the molecular mechanism underlying adiponectin-stimulated p38 MAPK activation remains largely unknown. The p38 MAPK was phosphorylated in CiGEnC when stimulated with gAd and the rise was noticeable after only 30min.

While insulin is an anabolic hormone promoting synthesis of fatty acids, glycogen and use of ATP, adiponectin through its major activator AMPK, results in catabolic processes such as fatty acid oxidation to increase ATP formation [102]. However, in tissues requiring glucose regulation, AMPK and insulin work in increasing glucose uptake through increased GLUT4 translocation to the plasma membrane[183]. Studying other signalling pathways that may define gAd as an insulin sensitizer might be the answer to the hypothesis proposed. As mentioned before, insulin receptor activation leads to the phosphorylation of Akt at Ser 473 through the insulin-Akt pathway [24]. Akt activation is responsible for the downstream effect such as increased glucose uptake into the cell. Taking that into account, the effects of gAd on the activation of Akt were studied. There was a robust phosphorylation of Akt that peaked with a 3-fold rise at 30min. Insulin as well as gAd can activate Akt, thereby we can speculate that they both work synergistically to increase glucose uptake specifically in CiGEnC.

Initial reports show that silencing AdipoR1 and/or AdipoR2 abolished any downstream signalling in human glomerular cells [159], rat myocytes [184] and hepatocytes [185]. In human GEnC, AMPK activation was decreased when AdipoR1 was silenced by small

interfering RNA (siRNA). However, it was restored after treatment with an adiponectin receptor agonist (AdipoRon) [159]. Still, it is unknown if AdipoR1 or AdipoR2 or other factors are the driver of gAd's effect on GEnC. Therefore, to further elucidate the molecular events involved in CiGEnC by adiponectin through its receptors I knocked down AdipoR1 or AdipoR2 by lentiviral transduction. The results showed that knocking down AdipoR1 but not AdipoR2 attenuated the gAd-induced AMPK and ACC activation. Similarly, these pathways were also reduced in rat myocytes when AdipoR1 was knocked-out [184]. Furthermore, overexpression of AdipoR1 in C2C12 myocytes was associated with increased phosphorylation of AMPK, ACC and p38 MAPK upon stimulation with gAd [102]. In contrast to the observed role of AdipoR1 in AMPK activation, other groups demonstrated the involvement of AdipoR2 in the activation of AMPK signalling [186]. However, we could not observe the same effects in CiGEnC by AdipoR2.Therefore, from the results that I have showed, I can suggest that adiponectin actions in CiGEnC are via AdipoR1.

After showing that gAd can activate several signalling pathways in unstimulated CiGEnC majorly through AdipoR1, the next step was to determine the role of adiponectin in a diseased model to be able to identify adiponectin as a protective marker against diseases. Supporting evidence suggests that the AMPK pathway mediates initiation of kidney disease induced by chronic high glucose and is considered one of the key molecules to be dysregulated during DN [126]. Prior to any major experiments that will be done in an *in vivo* diabetic animal model, it was essential to establish what happens on the intracellular level by the different components of diabetes. Therefore, the effects of HG and TNF-α were tested separately. Although HG downregulates AMPK activity in renal cells, the underlying mechanism is largely unknown. For example, HG environments decreased the

phosphorylation of AMPKα in rat glomerular epithelial cells but metformin and AICAR reversed this p-AMPKα reduction [126]. In this study, I showed a significant decrease in p-AMPKα and p-ACC under HG conditions in CiGEnC. However, gAd treatment restored the phosphorylation of AMPK-α and ACC under HG conditions. Decreased AMPK and ACC activity is associated with increased synthesis of FA and accumulation of TG in chronic kidney disease [187]. Thus, by increasing the phosphorylation of AMPK and ACC, we could thereby assume that adiponectin might play a beneficial role in protecting against kidney disease. Furthermore, other pathways are also dysregulated in DN. Specifically, high levels of p38 MAPK have been reported in diabetic complications such as DN [182]. However, the results show that the induction of HG for 48h did not change the levels of p-p38. In contrast, a few studies have demonstrated that p38 MAPK is stimulated by HG in different cell types such as mesangial cells [188] and HUVEC [189]. The one noticeable difference was the incubation period. For instance, in HUVEC, they were incubated for 72h and increase in levels of phosphorylated p38 MAPK protein was seen [189]. Another study showed that this increase was shown after 7 days of HG in human mesangial cells but not for a shorter time [188]. However, we could not see similar increase in p-p38 in CiGEnC in the presence of HG, and this might be partially due to the differences in the timing of activation. Moreover, the addition of adiponectin did have a marginal increased effect on phosphorylated levels of p38 in the presence of HG with respect to control.

The PI3K/ Akt signaling pathway plays a main role in controlling cell proliferation, survival and motility. Our study shows that HG increased the activation of p-Akt. These results are similar to previous studies that showed that HG affects the Akt pathway in other renal cells such as mesangial cells [190] and proximal tubular cells [168]. Adding gAd to HG does not further increase p-Akt levels. The reasons are still unknown and further investigations

are needed to understand adiponectin effect in NG and HG on Akt levels. However, it is well documented that adiponectin works as an insulin sensitizer, although maybe not directly through its major pathway, the PI3K/Akt pathway. Ongoing experiments will focus more on the AMPK pathway as a major pathway for adiponectin signalling but not p38 MAPK or Akt pathways.

Other than HG, the model of TNF-α simulation is widely used to mimic the effects of inflammation induced stress in diabetes and other diseases [191]. Endothelial cells are known to be extremely sensitive to the effects of pro-inflammatory cytokines such as TNF-α [192]. Therefore, treatments for 2h with TNF-α were used as a model to stress CiGEnC as suggested by Ramnath *et al*. [35]. To understand the mechanism of TNF-α with gAd, we further explored the AMPK and ACC pathway in CiGEnC. Our results indicated that TNF-α treatment reduced AMPK and ACC phosphorylation levels, however, gAd attenuated the inhibitory effects of TNF-α on their phosphorylation levels. A decrease or inhibition of the phosphorylation of AMPK in the presence of TNF-α has been also shown in different cell lines including human fibroblasts, [193] adipocytes [194], C2C12 myoblasts [195] and HUVEC [196]. But no further explorations were done in these studies. Therefore, this study is the first to demonstrate that gAd attenuates the TNF-α-induced effect on AMPK signalling pathway in CiGEnC. Demonstrating that adiponectin can protect GEnC from external stimuli at the molecular level might help finding new methods in the treatment of diabetic nephropathy.

At this stage in the project, to further demonstrate or understand gAd mode of action, I performed *ex-vivo* experiments using isolated healthy human and mouse glomeruli. These were then treated with gAd as described. It showed that gAd exerts its effects on p-AMPK-α after 30min in human and mice glomeruli. However, it increased further in

mice glomeruli after 1h. It is noteworthy to mention that gAd also activated p-AMPKα in isolated rat glomeruli after 1h of incubation and this action was directly mediated through AdipoR1 [103].

Similarly, diabetic ex vivo glomeruli were treated with gAd to assess the we AMPK pathway. The db/db mice are hyperglycaemic and insulin-resistant, hence glucose levels are already abundant. The results showed a downregulation of phosphorylation of AMPKα in the db/db glomeruli when compared to controls. However, when replenished with gAd, there was a robust increase in p-AMPK-α. Activation of AMPK in DN models has proven beneficial by different drugs such as the anti-diabetic drug, metformin, which activated AMPK [197]. Hence, the phosphorylation of renal AMPK by gAd could be an encouraging and reliable approach to treat and manage DN. A few reports show that renal AMPK phosphorylation is reduced in db/db mice [126, 165]. The signals that alter the activity of AMPK in a diabetic kidney are still unknown, but this might include changes in the AMP:ATP ratios.

4.5 Conclusion

In conclusion, gAd can exert its effects positively on CiGEnC via the AMPK pathway through one of its major receptors, AdipoR1. Moreover, this pathway was inhibited in diabetic conditions, such as HG or TNF-α-induced conditions. Additionally, gAd reversed this reduction in AMPK and ACC phosphorylation.

Having established how gAd exerts its effects on CiGEnC, the last aim of this project is to understand gAd extended effects on the endothelial glycocalyx while in a disease state. This will be discussed in the last results chapter.

Chapter 5 Adiponectin can modify CiGEnC glycocalyx Components

5.1 Introduction

The previous chapter focused on the signalling pathways that are activated by adiponectin through its receptors in GEnC and in *ex vivo* isolated glomeruli. The results from chapters 3 and 4 demonstrated that TNF-α and HG treatments (components of the diabetic milieu) reduced adiponectin receptor availability and dysregulated the AMPK and ACC pathway. These changes were attenuated by gAd treatment. This led to the question as to whether adiponectin could implement its effects on albuminuria in part by modulating the glomerular endothelial glycocalyx through regulation of components known to be altered by HG [177] and TNF-α [198], in diabetes. Under normal conditions the GFB, which consists of GEnC and its glycocalyx, GBM and podocytes, prevents the passage of albumin into the urine [199]. However, it is known that diabetes causes disruption to all components of the filtration barrier, but GEnC and glycocalyx dysfunction are particularly implicated in the early phases [200]. This leads to albuminuria, a measure of kidney damage. To date, there are no studies of the actions of adiponectin on the glomerular endothelial glycocalyx. However, there is evidence that adiponectin may have effects on this structure. Sharma *et al.* demonstrated that knockout of adiponectin in mice caused albuminuria [113]. Secondly, albuminuria was reduced in diabetic rats overexpressing adiponectin in comparison to wild type diabetic rats [139]. These findings imply that adiponectin may regulate GFB and, due to the importance of the endothelial glycocalyx in regulating albuminuria, it is crucial to determine whether adiponectin affects this structure.

Modification of the glycocalyx is an attractive therapeutic target for treatment of several types of vascular diseases. In fact, the restoration of the glycocalyx has been achieved and shown to be effective in the myocardial vascular endothelium by hydrocortisone [67], and blood vessels by metformin [197]. More importantly for this study, it has been demonstrated by our group that the glycocalyx can be also restored in GEnC by $VEGFA_{165}b$ [43], VEGFC [156] and by Ang-1 [148]. In this chapter, adiponectin was tested *in vitro,* to assess whether its mechanism of action (the restorative effect) was through the glycocalyx.

The degradation of the glycocalyx leads to releasing of its components into the plasma. Several components mediate this disruption in inflammatory states such as TNF-α [35] and salt and aldosterone [61] and has proven to be a very useful model for studying the regulation of this structure *in vitro*. For example, the increase in TNF-α leads to the activation of MMPs that are known to cleave proteoglycans directly from the endothelial membrane. Therefore, this model allows for the study of the shedding of glycocalyx components such as GAG and proteoglycans as previously demonstrated by our group [35, 61]. As mentioned in the main introduction, GAG predominantly comprise the sulphated HS, CS, DS and KS, and also non-sulphated HA. Examples of proteoglycans are glypicans and syndecans. Syndecans, specifically, are of great interest due to their crucial role in development, cell proliferation and differentiation and wound healing [201]. The ectodomain of syndecans is constantly shed in cultured cells as a result of normal turnover. However, shedding is increased in pathophysiological conditions [201]. Hence syndecan shedding may be used as an index of glycocalyx dysfunction. Of the 4 main syndecans, SDC1 and SDC4 are prominent in the glomerulus. Using a custom designed TLDA, which enabled analysis of various glycocalyx components and regulatory enzymes,

our group has identified that SDC4 was mostly highly expressed in CiGEnC and freshly isolated human GEnC [35]. It was also shown to be the most significant syndecan altered by an external stimulus (TNF-α) suggesting a particular relationship between them [35]. The process of shedding itself is highly regulated and involves the direct action of enzymes which are members of the MMP family and commonly described as sheddases [201]. Preliminary data has suggested that of the MMPs, that differ widely in structure and function, MMP2 and MMP9 play an important role in the response to TNF-α [201]. MMP activity is hard to detect in healthy subjects but not in pathophysiological conditions such as wound healing [201]. Therefore, GAGs, SDC4 and MMP2/9 were measured to assess glycocalyx damage and whether this can be repaired by adiponectin.

The aim of this chapter is to delineate the role of adiponectin as a modifier of the endothelial glycocalyx in GEnC by

a- showing that adiponectin protects from TNF-α-induced, MMP-mediated damage of glycocalyx SDC4, in CiGEnC

b- translating the work done in cultured cells to *ex-vivo* glomeruli in a db/db mouse model.

5.2 Methods

5.2.1 qPCR validation of primers

Primers for SDC4, TNF-α, MMP2 and MMP9 were validated as explained in Chapter 3, section 3.2.1. Figure 5.1 shows the standard curves for these genes. They all show a good fit into the line with an R^2 between 0.90 and 1.10. This confirms that all the primers were validated and are specific for the gene of interest.

5.2.2 Alcian blue assay

An Alcian blue colorimetric assay was used to quantify the number of sulphated GAG shed into the cultured media from the surface of CiGEnC. The dye used is a tetracationic structure that carries isothiouronium groups (positively charged) that bind to the sulphate groups (negatively charged) present on GAG side chains. Briefly, the cells were grown to confluency and then thermoswitched for 3-5 days. The media was then changed to SFM for a minimum of 2h. TNF-α (10ng/ml) was applied to the cells for 1 or 2h (as for previously optimised laboratory protocols). The media was then harvested and centrifuged at 800g for 3min to remove cellular debris. The supernatant was added to a freshly prepared solution of 0.4% Alcian blue (Sigma-Aldrich, A5268) in 0.5M sodium acetate (Sigma-Aldrich, S2889), 30mM magnesium chloride hexahydrate (Sigma-Aldrich, m2670) and 2.8% of sulphuric acid (Sigma-Aldrich, 339741). The acidity (pH~2) minimises interference from non-glycosaminoglycan molecules. Absorbance at 490nm was measured after 15min incubation. The formation of complexes between GAGs in the cell supernatant and Alcian blue causes a reduction in absorbance. The linear relation between GAG mass and decreased absorption of 490nm by Alcian blue solution was used to quantify supernatant GAG content, referenced to known concentrations (0 to 500µg/ml) of CS (considered as a

main GAG) standards (chondroitin sulphate sodium salt from shark cartilage, C4384-1G Sigma-Aldrich).

5.2.3 TNF-α, HG and gAd stimulation

CiGEnC were treated with TNF-α or HG as previously described (10ng/ml and 25mM respectively) [35, 177]. A time course of TNF-α was done with times 1, 2, 4 and 24h. As for HG, the time points were 2,7 and 14D. SFM was used for the TNF-α treatments in the short-term exposure, while full media was used for HG treatments due to longer time points. To examine the effect of gAd in TNF-α environment, a co-treatment group is suggested in which TNF-α at 10ng/ml and gAd at 2.5μg/ml are premixed in SFM first then added to the cells.

5.2.4 Wild type, lean and diabetic mouse source

The batch of mice in Chapter 3 section 3.2.2.2 (lean and db/db), was used in the section 5.3.2 for various mRNA gene expression. Whilst the wild type mice from section 2.9.3 was used as littermate controls for the db/db glomeruli whenever applicable.

5.2.4.1 Adipocyte RNA Extraction

As mentioned in Chapter 2 section 2.9.4, the adipose tissue was also harvested from both wild type and db/db mice and RNA was extracted according to the protocol outline in section 2.9.4.2

5.2.4.2 Ex-vivo glomeruli treatments

After glomeruli were sieved from db/db and wt/wt mice as mentioned in Chapter 3 section 3.2.2.3, they were treated with gAd at 2.5μg/ml (in SFM) for 2h in a water bath at

37°C and subsequently RNA and protein were extracted as detailed in Chapter 3 sections 3.2.3 and 3.2.4.

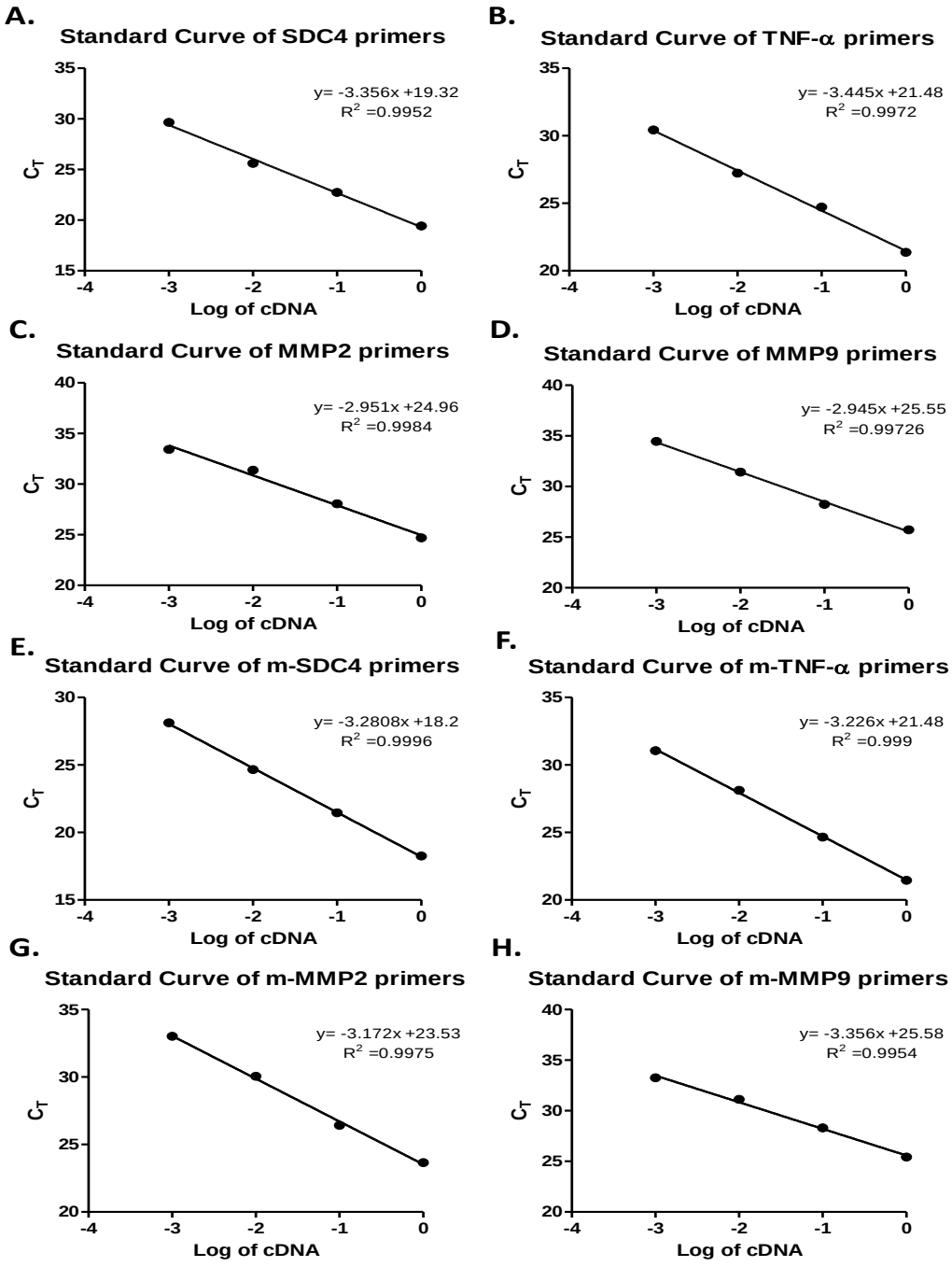

Figure 5.1 Standard curve of PCR amplification efficiency for certain human and mice primers

The initial cDNA concentration (1µg) was used and then serial dilutions of 1in 10 were performed. Standard curves were plotted as C_T values vs. log of cDNA. R^2 is the correlation coefficient that define the fitness of the curves (0.90-1.10 is considered a best fit). y is the formula of the line in the form of y=ax+b

5.2.5 MMP2 and MMP9 knockdown in CiGEnC

The knockdown of MMP2 and MMP9 was carried in the same way as AdipoR1/AdipoR2, as detailed in Chapter 4 section 4.2

5.3 Results

5.3.1 Adiponectin and TNF-α mRNA expression in db/db mice

In diabetes, there is an imbalance in the production of pro-inflammatory and anti-inflammatory molecules, due to excess levels of glucose [68]. Therefore, to understand the relationship between adipokines in diabetes, the mRNA levels of adiponectin (an anti-inflammatory adipokine) and TNF-α (a pro-inflammatory adipokine) were measured in the adipocytes of diabetic mice. Figure 5.2A showed that adiponectin mRNA was downregulated significantly in adipocytes of db/db mice. In contrast, TNF-α mRNA was significantly upregulated (4-fold). Therefore, this suggests that in diabetes the levels of these 2 adipokines are inversely proportional to one another.

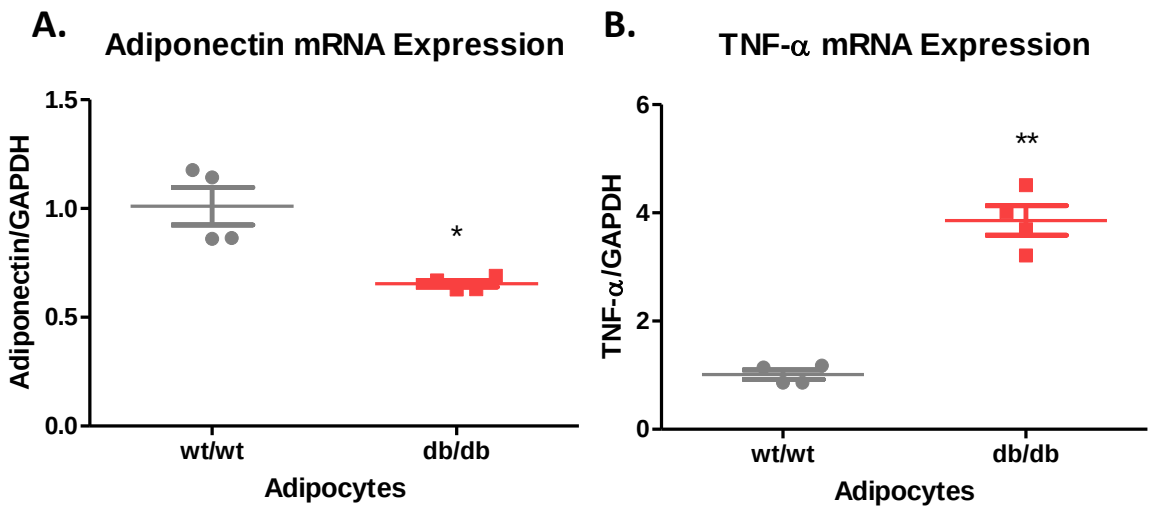

Figure 5.2 Adiponectin but not TNF-α mRNA is downregulated in adipocytes of db/db mice

qPCR analysis showing the mRNA expression of adiponectin is decreased (**A**), while mRNA expression of TNF-α (**B**) is increased in db/db adipocytes. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the loading control. n=4, unpaired t test, *p<0.05, **p<0.01

5.3.2 Gene expression in db/db mouse renal cortex

In order to confirm that there is a dysfunctional glycocalyx in diabetes, several genes known to be important in regulating the glycocalyx were measured. Figure 5.3 shows that the mRNA expression of TNF-α, SDC4, MMP2 and MMP9 mRNA were all increased in the kidney cortex of db/db mice when compared to lean controls.

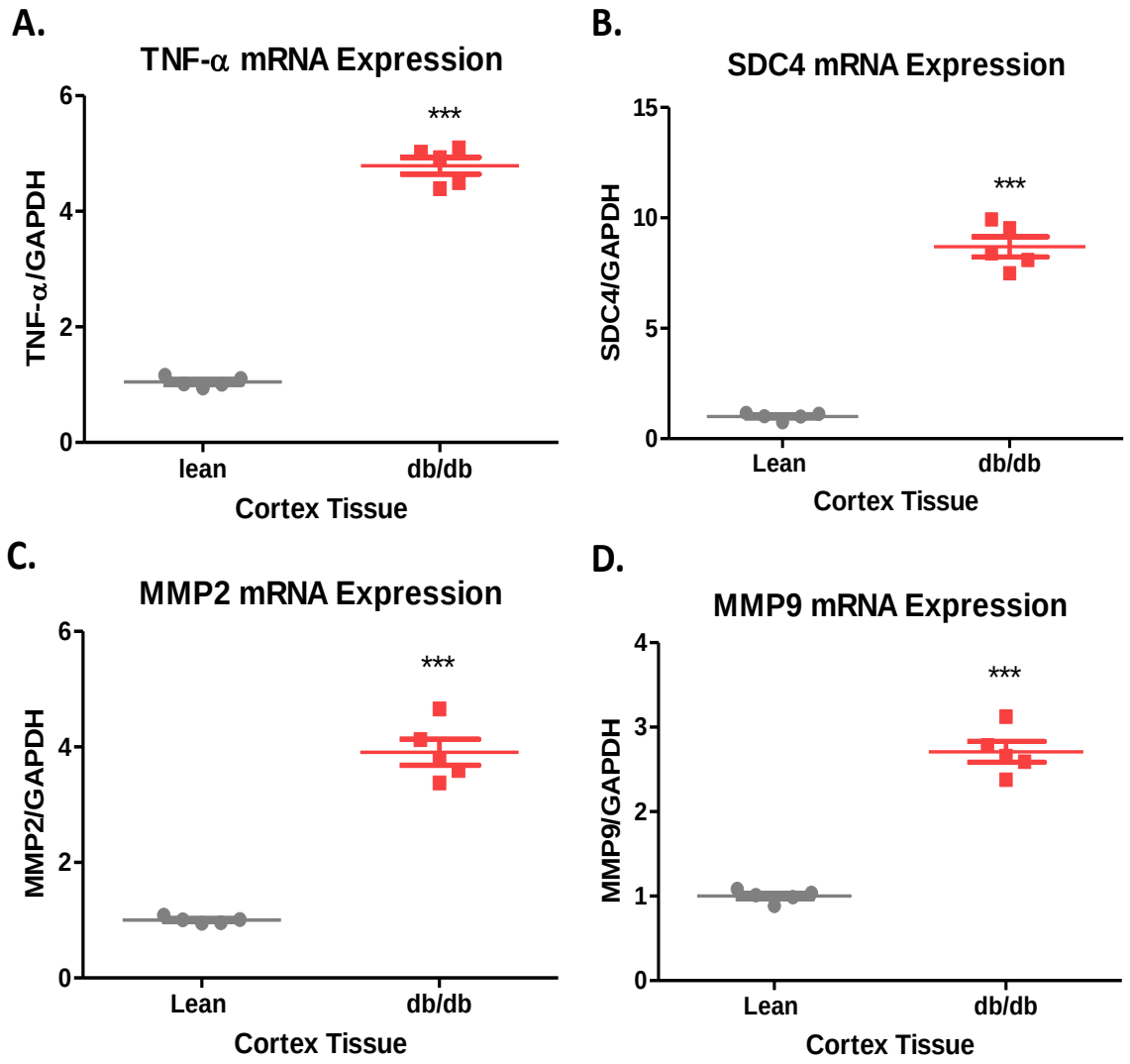

Figure 5.3 Messenger RNA expression of glycocalyx-related genes is upregulated in cortex tissue of db/db mice

qPCR analysis showing the mRNA expression of TNF-α (**A**), SDC4 (**B**), MMP2 (**C**) and MMP9 (**D**) are increased in cortex tissue of db/db mice. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the loading control. n=5, unpaired t test, ***p<0.001

5.3.3 Gene expression associated with glycocalyx dysfunction in db/db mouse glomeruli

Similarly, the expression of the genes mentioned above were also measured in sieved glomeruli from db/db mice. The results were comparable to those seen in the cortex; Figure 5.4 shows the mRNA expression of TNF-α, SDC4, MMP2 and MMP9 mRNA were all

upregulated in the glomeruli of db/db mice when compared to wild type controls. This suggests that the glomeruli expressing high levels of MMP2 and MMP9 also expressed high levels of glomerular SDC4 mRNA. These data would be consistent with the possibility that SDC4 mRNA upregulation is a consequence of increased MMPs.

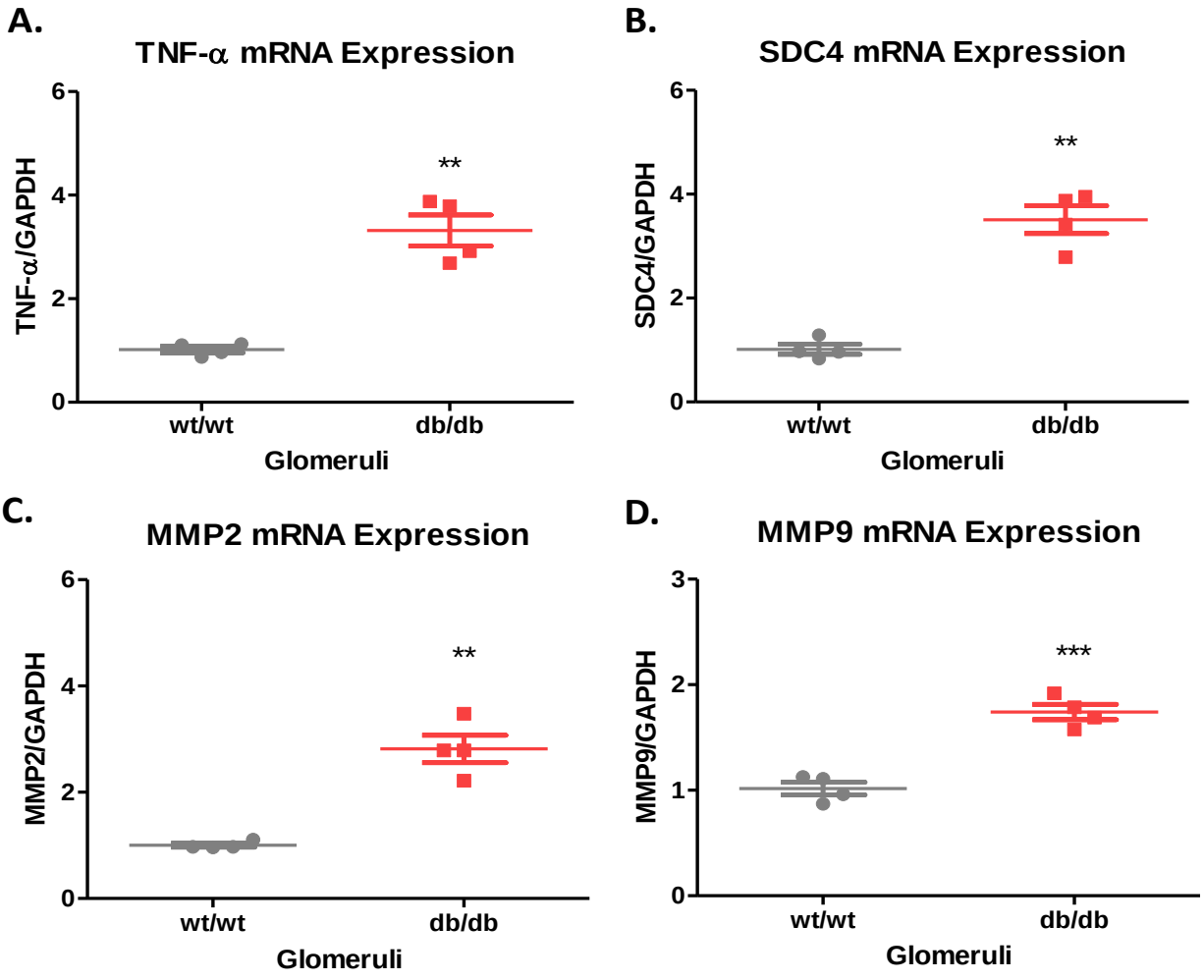

Figure 5.4 Messenger RNA expression of glycocalyx-related genes is upregulated in glomeruli of db/db mice

qPCR analysis showing the mRNA expression of TNF-α (**A**), SDC4 (**B**), MMP2 (**C**) and MMP9 (**D**) are increased in glomeruli of db/db mice. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the loading control. n=4, unpaired t test, ***p<0.001

5.3.4 TNF-α but not HG upregulates SDC4 mRNA expression

After demonstrating the upregulation of the genes associated with glycocalyx dysfunction in a diabetic animal model, the components of a diabetic milieu were then tested separately in cultured cells. Hence, the effect of TNF-α and HG on the mRNA expression of SDC4 was investigated using qPCR. TNF-α significantly increased the mRNA for proteoglycan SDC4 after just 1h by 2-fold. It was increased 3-fold by 2h and 4h). By 24h, SDC4 mRNA levels returned towards baseline (Figure 5.5A). In contrast, HG did not significantly change the mRNA levels of SDC4 at any of the time points tested although there was a trend towards an increase over time (Figure 5.5B).

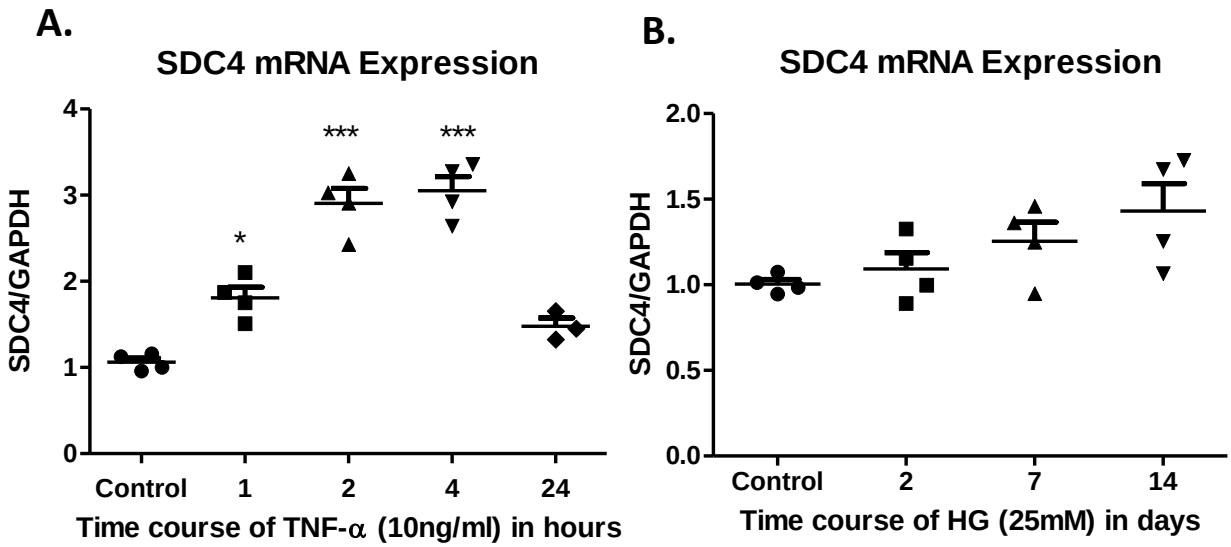

Figure 5.5 TNF-α but not HG increased SDC4 mRNA expression in CiGEnC

A: qPCR analysis of time-course for TNF-α showing an increase in SDC4 mRNA levels after 1,2, 4 and 24h. **B**: qPCR analysis of time-course for HG (25mM) showing no changes in SDC4 mRNA levels. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. One-way ANOVA, *p<0.05, ** p<0.01, ***p <0.001 post hoc analysis (Bonferroni).

5.3.5 TNF-α but not HG upregulates MMP2 and MMP9 mRNA expression

Having established that a key component of the glycocalyx (SDC4) was upregulated in response to TNF-α exposure, I next investigated potential mechanisms of glycocalyx loss. We have previously shown that MMP2 and MMP9 cause the shedding of SDC4 by cleaving the ectodomain near the cell surface [201]. TNF-α treatment at 10ng/ml for 2h and 4h significantly upregulated MMP2 mRNA by 2.9-fold (Figure 5.6A). MMP9 mRNA levels were also upregulated at 2h by 2-fold and 4h by 2.7-fold (***$p<0.001$). Similar to the results for SDC4, HG did not change the expression of MMP2 and MMP9 even after 14 days of HG treatment (Figure 5.6C and D). Therefore, the HG experiments were discontinued and only further TNF-α treatments were conducted.

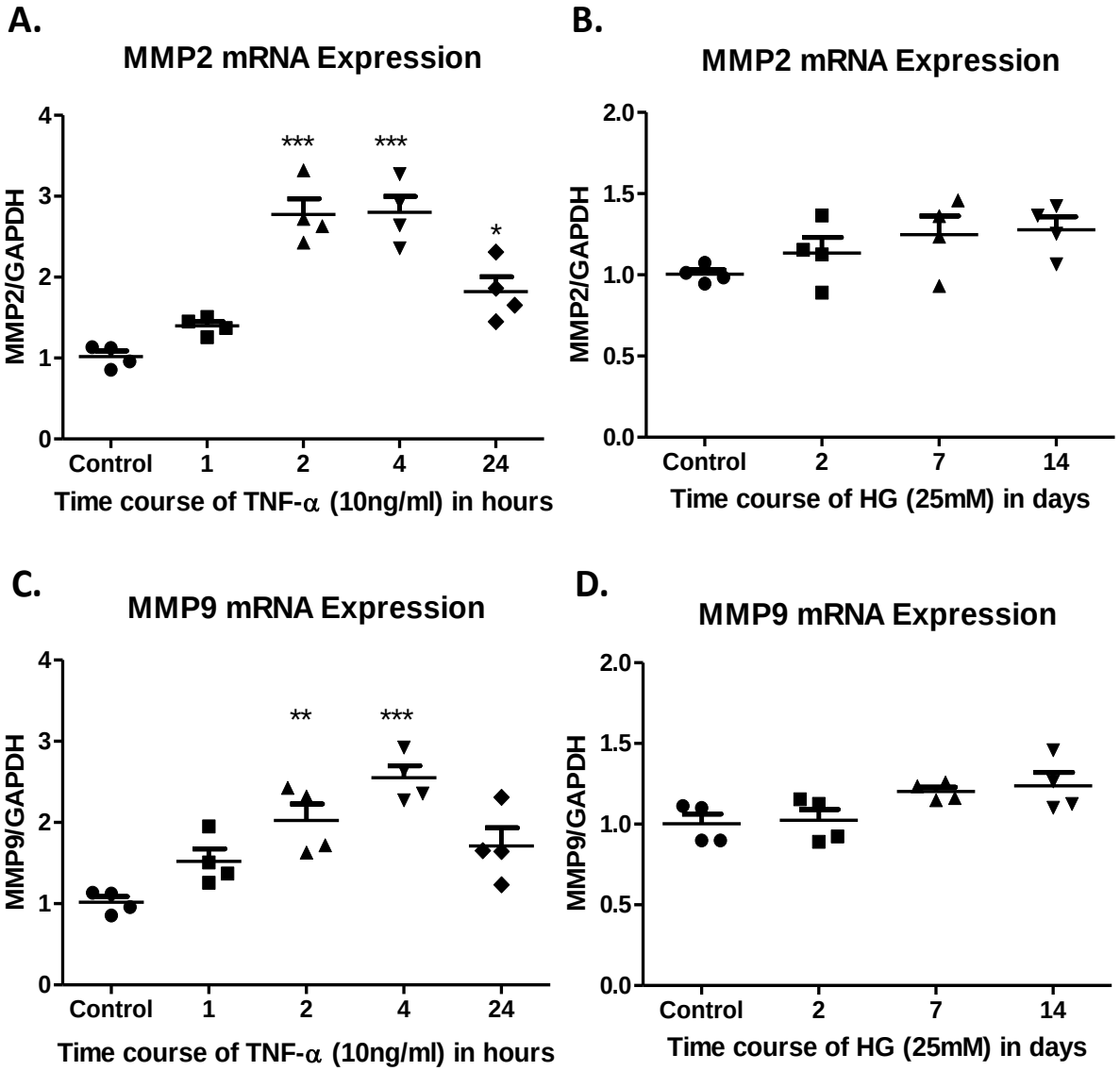

Figure 5.6 TNF-α but not HG increased MMP2 and MMP9 MRNA expression in CiGEnC

A and **C**: MMP2 (**A**) and MMP9 (**C**) mRNA is increased after 2h and 4h exposure to TNF-α. **B** and **D**: MMP2 (**B**) and MMP9 (**D**) mRNA is not changed during long exposure to HG. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. One-way ANOVA, *p<0.05, ** p<0.01, *** p <0.001 post hoc analysis (Bonferroni).

5.3.6 Alcian blue colorimetric binding assay: protocol optimization

It has been previously demonstrated by our group that human CiGEnC express an endothelial glycocalyx that contributes to barrier properties [30, 40]. To investigate whether TNF-α treatment induces the shedding of glycocalyx GAG, an Alcian blue colorimetric assay was used. First, a standard curve with known CS concentration was carried out (figure 5.7A).

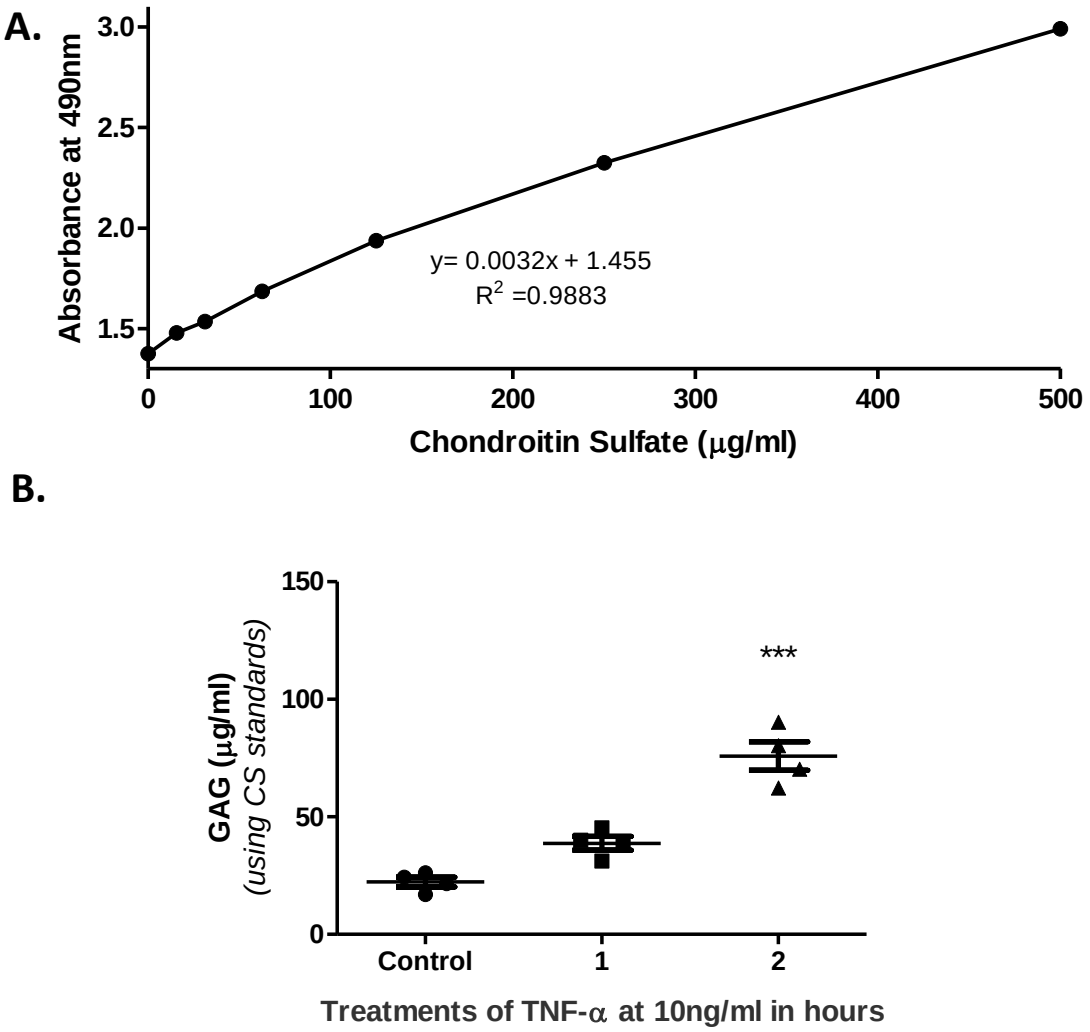

Figure 5.7 Alcian Blue Assay: Standard curves and GAG concentration measurement in TNF-α treatments in CiGEnC

A: Standard curve of Chondroitin Sulfate (CS). Serial dilutions from 0ug/ml- 500ug/ml. This is acquired using the Alcian blue assay **B**: Supernatant GAG concentration of TNF-α treated CiGEnC for 1h and 2h (***p<0.001) (unpaired t test, ***p<0.001, n=4). GAG concentration derived from the CS standard curve by extrapolation.

Concentrations ranging from 0-500μg/ml was achieved by serial dilutions of the highest concentration (500μg/ml). TNF-α is known to increase GAG concentrations in the media. Therefore, TNF-α was used to verify that the assay was working. Supernatant GAG concentration was calculated from the CS standard curve. The GAG concentration was 22.25±2.009μg/ml from control non-treated cells and increased with 1h TNF-α to

38.73±2.911µg/ml and became significantly higher with 2h TNF-α to 75.83±6.081µg/ml (Figure 5.7B). This shows an increase of GAG shedding into the media after 2h of TNF-α application.

5.3.7 Adiponectin decreases shedding of GAG in CiGEnC

The protective effect of gAd treatment on the release of GAG from the surface of CiGEnC was then examined. Co-treatment of TNF-α with gAd for 2h resulted in a significant decrease in the TNF-α induced shedding (36.04±3.083µg/ml compared to TNF-α only treated cells (85.54±9.985µg/ml)) (figure 5.8).

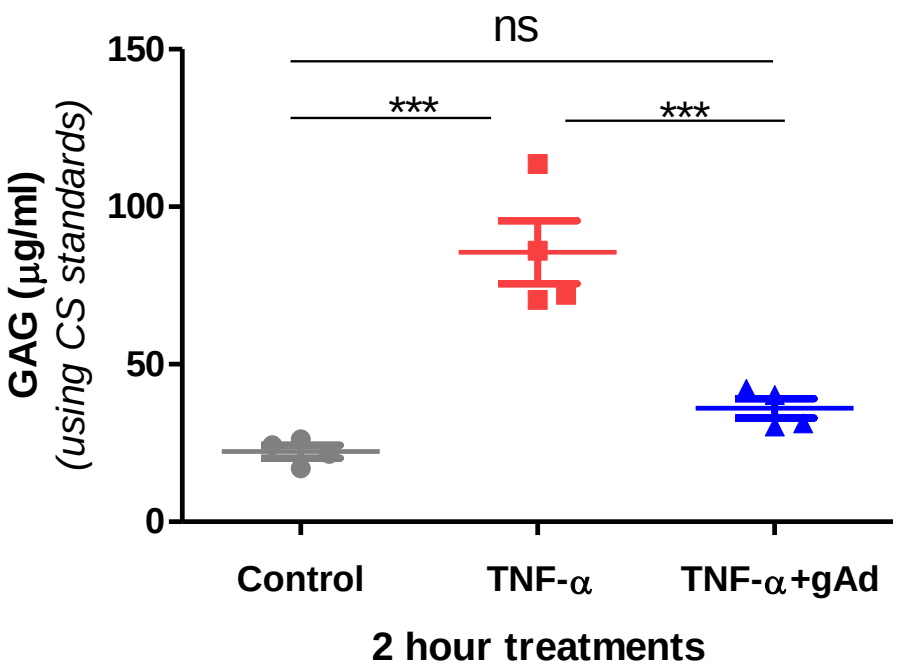

Figure 5.8 Adiponectin decreased GAG shedding after TNF-α treatments

Supernatant GAG concentration of TNF-α treated CiGEnC with or without the effect of gAd for 2h. One-way ANOVA, ***p < 0.001, *post hoc* analysis (Bonferroni), n=4.

5.3.8 Adiponectin decreases SDC4 mRNA and protein in TNF-α induced CiGEnC

After verifying that TNF-α treatments increased SDC4 mRNA, the co-treatment of gAd with TNF-α was applied to determine the effect of gAd on SDC4 mRNA. After 1h, the increase in SDC4 (2-fold) with TNF-α was not reduced significantly by gAd (1.8-fold) (Figure 5.9A). However, after 2h, the increase in SDC4 mRNA with TNF-α (2.5-fold) was significantly reduced when TNF-α was co-treated with gAd (1.3-fold) (Figure 5.9B). It is worth mentioning that gAd treatment alone did not alter the SDC4 mRNA expression. Similarly, the expression of SDC4 was evaluated by Western blot under different conditions. There was an increase in SDC4 expression with TNF-α within 1h and 2h (Figure 5.9A and B). However, this increase was only reduced when gAd was co-treated with TNF-α for 2h and not 1h (Figure 5.9B). Likewise, gAd did not change the SDC4 surface expression on its own for either 1h or 2h.

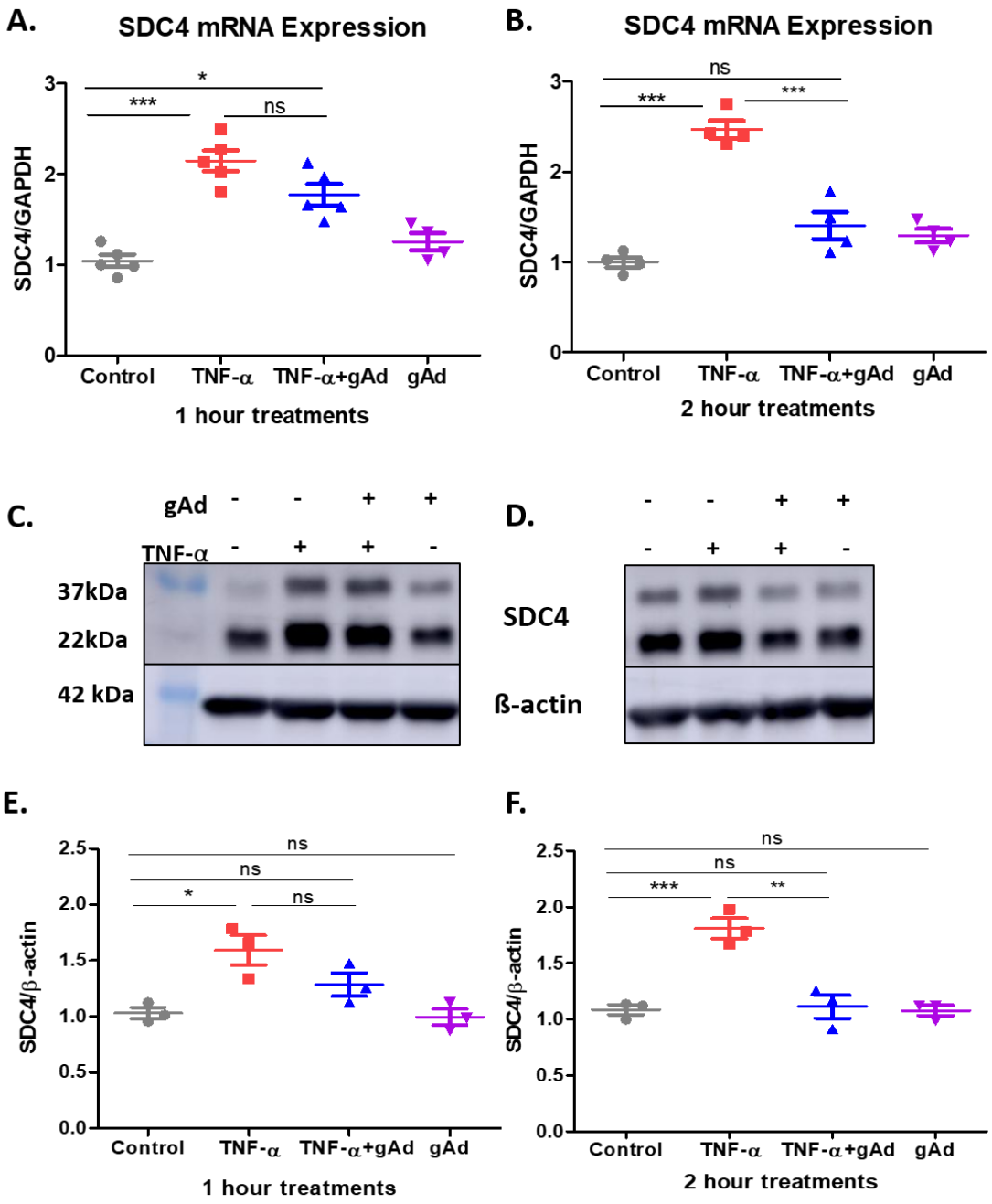

Figure 5.9 Adiponectin restored SDC4 mRNA and protein expression after TNF-α treatments in CiGEnC

A and **B**: qPCR analysis of SDC4 mRNA levels in CiGEnC treated with TNF-α and/or gAd for 1h (**A**) and 2h (**B**). Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. **C** and **D**: Representative western blot analysis of SDC4 protein levels in CiGEnC treated with TNF-α and/or gAd for 1h (**C**) and 2h (**D**). SDC4 has 2 bands; glycosylated form: 37kDa and unglycosylated form: 22kDa. Western blot analysis was done on the two bands. **E** and **F**: Densitometry was performed on 3 representative blots from 3 independent repeats (n=3) showing levels of protein of interest normalised to β-actin loading control. Data represent means ±SEM, one-way ANOVA, *p<0.05 ** p <0.01 *** p < 0.001. *post hoc* analysis (Bonferroni).

5.3.9 Adiponectin decreases SDC4 mRNA in db/db isolated glomeruli

To confirm the physiological relevance of the results above, in a diabetic animal model, ex-vivo glomeruli from wt/wt and db/db mice were isolated as explained in the materials and methods. In the untreated db/db glomeruli there was an increase in SDC4 (3.5-fold). However, in the gAd treated db/db glomeruli, the increase in SDC4 mRNA was significantly reduced (1.5-fold) (Figure 5.10). Treatment of gAd on wt/wt glomeruli did not alter SDC4 mRNA expression.

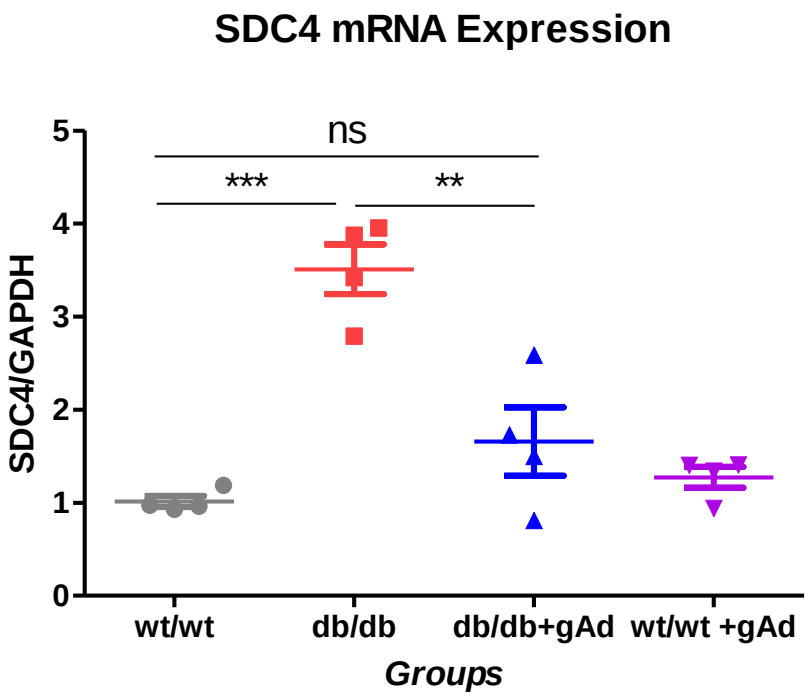

Figure 5.10 Adiponectin restores SDC4 mRNA expression in db/db glomeruli

qPCR analysis of SDC4 mRNA in ex-vivo sieved glomeruli in db/db mice treated with gAd for 2h. The db/db group with gAd showed less SDC4 expression than without gAd. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. One-way ANOVA, n=4 **p<0.01, ** p < 0.001, *post hoc* analysis (Bonferroni).

5.3.10 Adiponectin protects from TNF-α induced MMP2 and MMP9 mRNA upregulation in CiGEnC

It was shown earlier that MMP2 and MMP9 were upregulated in diabetic glomeruli and in TNF-α treated CiGEnC. To investigate whether gAd reduced the expression of these mediators, qPCR was carried out. The increase of MMP2 mRNA caused by TNF-α was ameliorated when gAd was co-treated with TNF-α (Figure 5.11A). In contrast, the increase in MMP9 mRNA by TNF-α was not significantly affected by gAd as seen in Figure 5.11B.

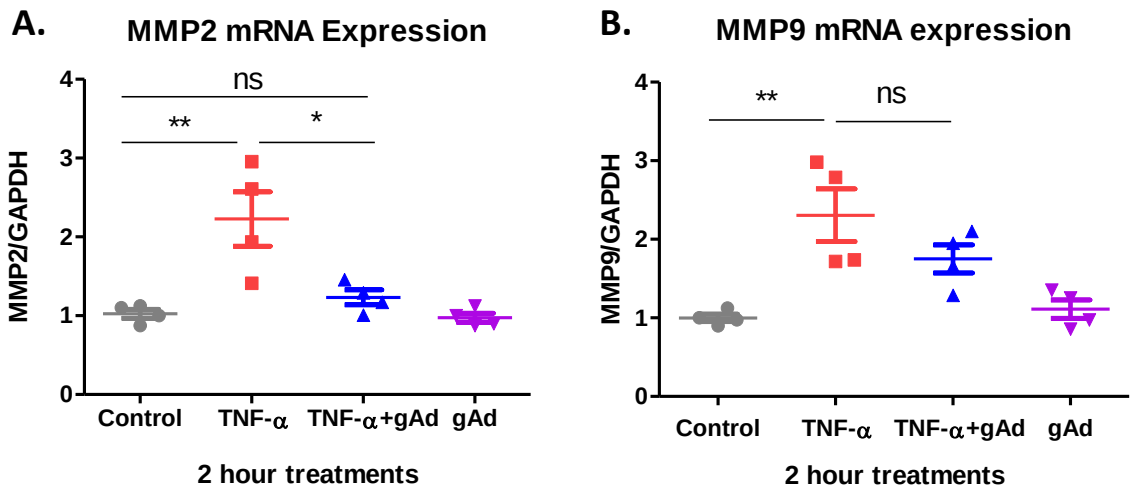

Figure 5.11 Adiponectin restored MMP2 but not MMP9 mRNA expression after TNF-α treatments

A and **B**: qPCR analysis of MMP2 (**A**) and MMP9 (**B**) mRNA levels in CiGEnC treated with TNF-α and/or gAd. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. One-way ANOVA, *p<0.05, ** p < 0.01, *** p <0.001 *post hoc* analysis (Bonferroni).

5.3.11 MMP9 knockdown in CiGEnC

SDC4 shedding was shown to be mediated by MMP9 in a previous study within our group [35]. Therefore, I aimed to confirm whether gAd acted through the same mechanism.

First, I attempted to knockdown MMP9 gene in CiGEnC. Unfortunately, the knockdown itself was not successful. As seen in Figure 5.12, the 2 constructs remained unchanged compared to the scrambled control.

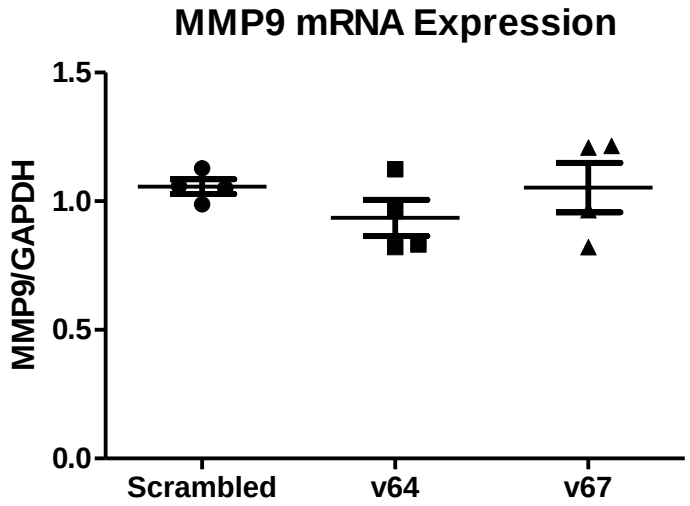

Figure 5.12 Knockdown analysis of MMP9 in CiGEnC

qPCR data analysis showing insignificant KD of MMP9 in CiGEnC using 2 different shRNA constructs. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. one-way ANOVA, ns $p>0.05$ *post hoc* analysis (Bonferroni).

5.3.12 MMP2 knockdown in CiGEnC

Data from above suggests that MMP2 expression is altered by adiponectin (figure 5.11). Therefore, I knocked down MMP2 in CiGEnC using shRNA to determine the relation of MMP2 with SDC4 shedding and gAd effects. The knockdown was confirmed by qPCR. The 3 different constructs showed a significant decrease in MMP2 mRNA with almost 60-80% (Figure 5.13A). A Western blot analysis was also carried out to confirm the mRNA results (Figure 5.13B). Densitometry showed a significant downregulation using all shRNA constructs (based on 3 different repeats). (Figure 5.13C).

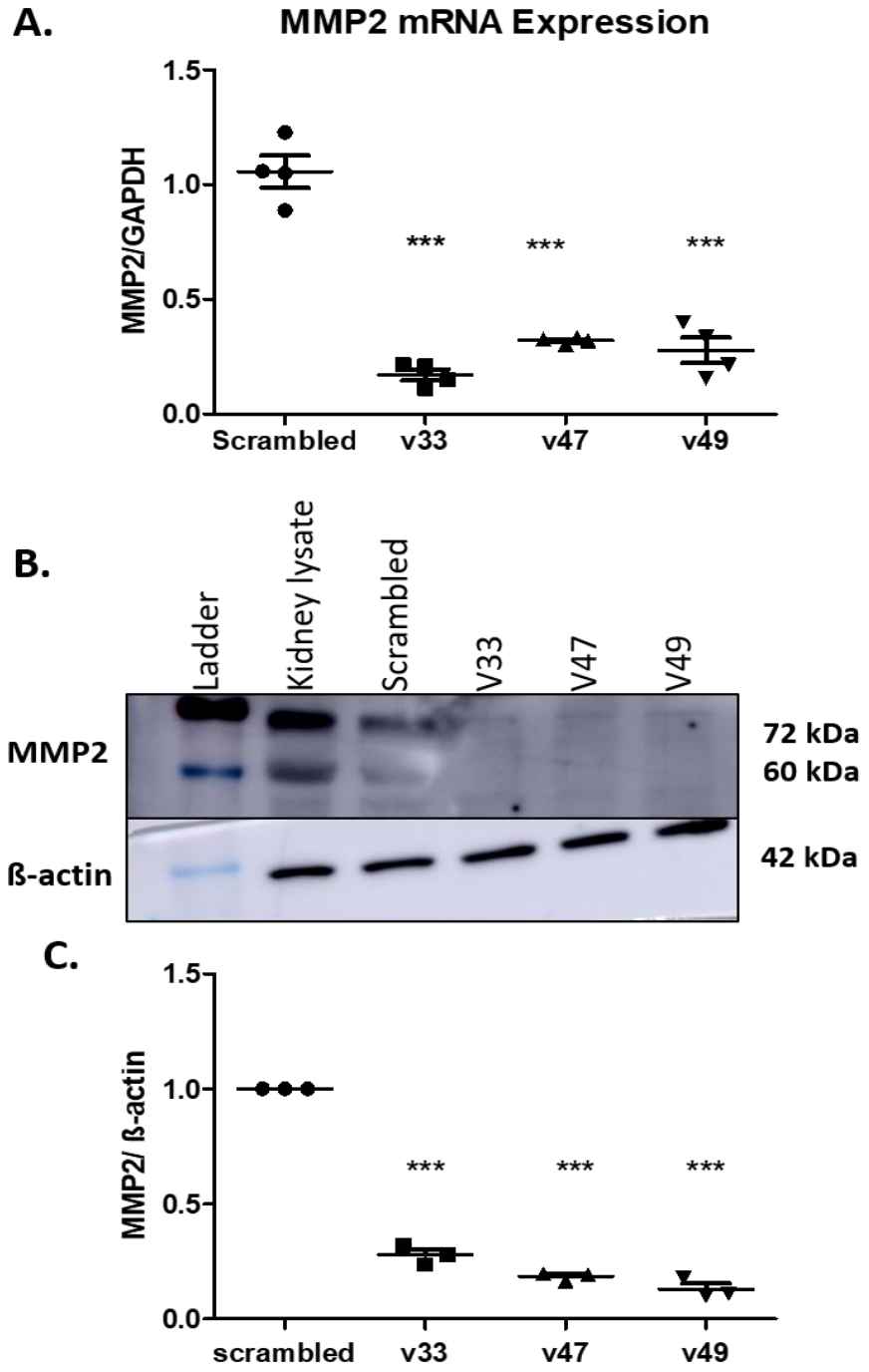

Figure 5.13 Knockdown of MMP2 in CiGEnC is confirmed at mRNA and protein levels

A: qPCR data analysis highlighting the decreased expression of MMP2 mRNA in CiGEnC using 3 different shRNA constructs. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. One-way ANOVA, *** p <0.001 *post hoc* analysis (Bonferroni). **B**: Representative western blot demonstrating the knockdown extent of MMP2 in all shRNA constructs **D:** Densitometry for MMP2 was calculated for the two bands and showed significant knockdown of MMP2 protein expression. Data normalised to β-actin loading control, dots represent means ±SEM, one-way ANOVA, *** p < 0.001. *post hoc* analysis (Bonferroni)

5.3.13 Effect of TNF-α on MMP2 and SDC4 mRNA expression in MMP2 knockdown CiGEnC

To confirm that the activity of MMP2 caused SDC4 shedding following TNF-α exposure, mRNA expression of SDC4 was repeated in the MMP2 knockdown CiGEnC. Scrambled control and knockdown were treated with TNF-α for 2h and MMP2 and SDC4 mRNA were analysed. Figure 5.14A showed that as expected, the MMP2 mRNA expression was reduced by 60% in TNF-α stimulated MMP2 knockdown CiGEnC. Interestingly SDC4 levels were significantly reduced by 50% in both MMP2 knockdown unstimulated and TNF-α stimulated CiGEnC. This suggests that the actions of TNF-α on SDC4 are MMP2 dependent (Figure 5.14B). However, there might be other MMPs involved in this process.

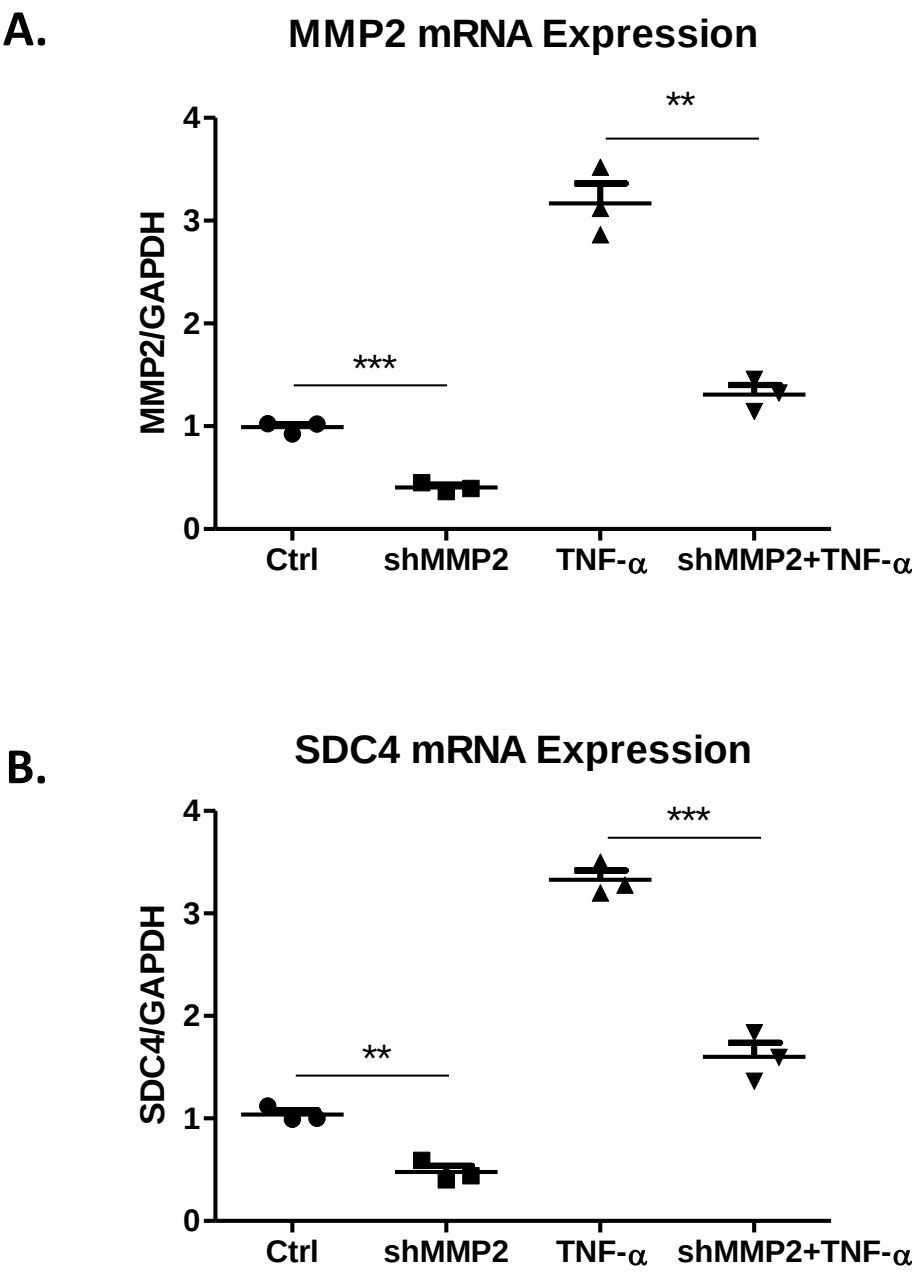

Figure 5.14 TNF-α effect on MMP2 and SDC4 mRNA expression in MMP2 knockdown CiGEnC

A: qPCR data analysis highlighting the decreased expression of MMP2 mRNA in unstimulated and in TNF-α stimulated cells in MMP2 knockdown. **B**: SDC4 mRNA is decreased in unstimulated cells and in TNF-α stimulated cells. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. n=3, unpaired t test, * p< 0.05, ** p < 0.01, ***p<0.001

5.3.14 Adiponectin did not attenuate SDC4 mRNA expression in TNF-α induced MMP2 knockdown CiGEnC

After establishing that SDC4 mRNA levels were reduced by TNF-α and MMP2, the protective effect of gAd was examined in MMP2 knockdown CiGEnC. As seen in Figure 5.15, even though there was still a residual effect of TNF-α on SDC4 levels in MMP2 knockdown, it was not altered by adiponectin suggesting there may be other mechanisms involved other than MMP2.

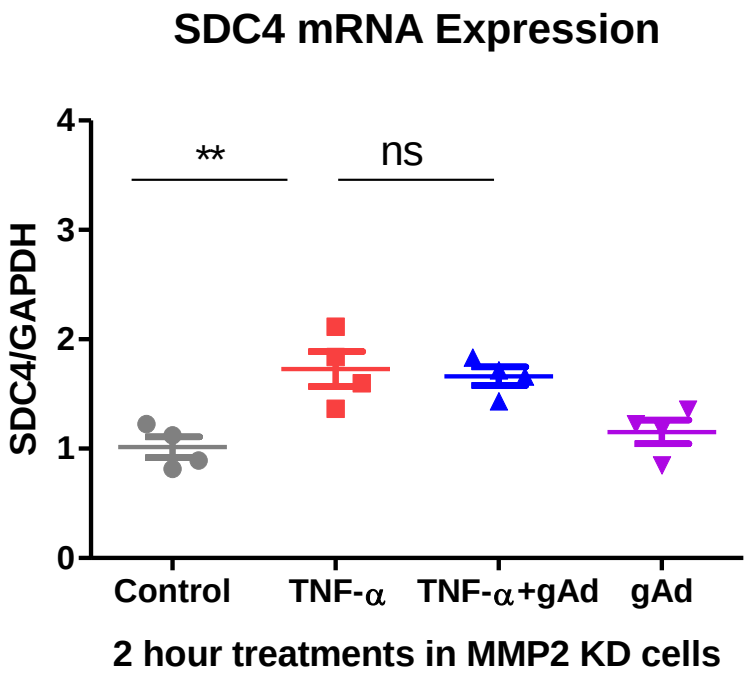

Figure 5.15 Adiponectin did not attenuate SDC4 mRNA expression in TNF-α induced MMP2 knockdown CiGEnC

qPCR analysis of SDC4 mRNA levels in MMP2 KD treated with TNF-α and/or gAd. MMP2 KD cells exhibited less SDC4 mRNA expression. Data are plotted as the mean $2^{-(\Delta\Delta CT)}$ of each triplicate with mean. GAPDH used as the housekeeping gene control. One-way ANOVA, ** $p < 0.01$, *** $p < 0.001$ *post hoc* analysis (Bonferroni)

5.4 Discussion

As explained in the main introduction, the over-secretion of pro-inflammatory adipokines (such as TNF-α,) and hyposecretion of anti-inflammatory adipokines (adiponectin, leptin) may be a major mechanism involved in lifestyle-related diseases, including T2DM, hypertension and atherosclerosis [68]. In fact, studies have shown less adiponectin plasma levels in obese [158] and diabetes type 2 human subjects [151] and in a diabetic animal model (db/db mice) [202]. As expected, our results showed a similar pattern; adiponectin mRNA expression was downregulated in db/db adipocytes. TNF-α has been shown to be dysregulated and linked to type 2 diabetes [78, 198]. It has also been shown to be an indicator of endothelial dysfunction and an upregulation was observed in db/db mice hearts, suggesting coronary endothelial dysfunction [198, 203]. Similarly, we showed that TNF-α mRNA was increased in db/db adipocytes as well as in the glomerulus. TNF-α-induced expression of adhesion molecules has been shown to be attenuated by adiponectin treatment in human aortic endothelial cells (HAECs) [74].

Both adiponectin and TNF-α are affected in diabetes. Analysis of qPCR data confirmed the alteration of glomerular gene expression in db/db mice. This work was performed using RNA because a much smaller quantity of sieved glomeruli is needed compared to protein extraction. However, measuring RNA has obvious limitations and so discoveries should be validated at the protein level. The genes that I have studied are key components of the glycocalyx, specifically SDC4. In db/db kidney lysates and sieved glomeruli, SDC4 mRNA levels were shown to be increased up to 8-fold and 4-fold respectively. Similarly, SDC4 mRNA levels were increased in cardiomyocytes of diabetes-induced rats [204]. According to the literature, there are some candidate sheddases for SDC4 shedding, including MMP2

and MMP9 and several of the ADAM family [201]. The gene expression changes that I have shown suggest that the increases in MMP2 and MMP9 may contribute to the glomerular SDC4 mRNA upregulation. Likewise, the *in vivo* results were similar to the work done in cultured GEnC; Similarly, I also found that SDC4, MMP2 and MMP9 mRNA are increased in response to TNF-α treatments (one of the components in a diabetic environment). This observation was based on earlier work done by Ramnath *et al.* had suggested that SDC4 mRNA in CiGEnC was increased by TNF-α and it is mediated by MMP9. This translates to a role of SDC4 in glycocalyx integrity. Similar results were observed in cardiomyocytes [205], smooth muscles [206] and HUVEC [207] where an upregulation of SDC4 mRNA levels was seen in response to TNF-α. Hyperglycemic conditions, on the other hand, had no effect on SDC4, MMP2 and MMP9 mRNA. This was also validated in a similar study in GEnC where SDC4 levels remain unaffected in high glucose conditions [208]. However, further studies are needed to verify the link between shedding of proteoglycans and diabetes.

An important role of the glycocalyx is to preserve the endothelial function as demonstrated by a number of studies on enzymes *in vitro* [40] and *in vivo* [209]. Other than SDC4, we also know that GAG are a part of glycocalyx components. Several papers published by our group have underlined the importance of the integrity of the glycocalyx and how shedding of SDC4 and GAG can affect this function. For example, it has been demonstrated that enzymatic removal of GAG increased macromolecular passage in CiGEnC measured using trans epithelial electrical resistance (TEER) [40]. GAG can be shed in response to different stimuli such as reactive oxygen species (ROS) [30], TNF-α [35, 53] and hyperglycemia [177]. In fact, as demonstrated by Padberg *et al.* the endothelial glycocalyx damage during CKD is mainly associated to shedding of glycocalyx components

[210]. The effect of TNF-α in CiGEnC *in vitro* has been demonstrated by Ramnath *et al.* showing that TNF-α increased GAG release in the media [35]. In this study, I confirmed that sulphated GAGs are shed in response to TNF-α. This was a time-dependent effect (after 2h but not 1h). One of the limitations using the Alcian blue assay is that the treatments must be done in SFM because the complete medium contains GAG which masks the effect of our treatments. For this reason, no higher time points were done except 1h and 2h which showed significant changes in SDC4 levels. The absence of serum in the media can cause the cells to behave in a different way; therefore, GAG shedding might be altered. Therefore, due to the long exposure of HG to the cells, SFM conditions were not achieved and GAG release was not measured in the HG conditions. However, it has been proven by Singh *et al.* that a decreased synthesis of GAG and increased permeability was a result of high glucose conditions in CiGEnC [177].

Given that glycocalyx components are being released and genes being dysregulated, I thought adiponectin could be a candidate for modification and even restoration strategies of the glycocalyx in the future. The effect of adiponectin was first investigated to see if it can inhibit the release of sulphated GAGs caused by TNF-α in the media to protect the glycocalyx. The result was promising; gAd attenuated the release of GAG in the medium induced by TNF-α. This is the first time that gAd has been shown to inhibit the release of GAG caused by an external stimulus. Secondly, adiponectin treatments attenuated the increase in SDC4 mRNA and protein levels in response to TNF-α in CiGEnC and in *ex-vivo* db/db mice glomeruli. Therefore, I have shown that gAd had a beneficial effect on GEnC in decreasing SDC4 level during stressful conditions induced by TNF-α and in db/db glomeruli. However, the mechanism of action of adiponectin in inhibiting the release of GAGs or decreasing SDC4 levels is yet unclear. The fact that adiponectin can protect the

glycocalyx may provide an explanation for its ability to decrease albuminuria in diabetes [137, 211]

Since MMPs control SDC4 levels, I also decided to check adiponectin effects on MMPs mRNA expression in TNF-α stimulated cells. So far, little is known about the direct effect of adiponectin on MMP in disease states. However, it is known that gelatinases (MMP2 and MMP9) in general, are highly expressed in fatty regions and atherosclerotic plaques compared to normal regions of the vessel [212]. Furthermore, it was shown that women with metabolic syndrome (MS) had increased activity of circulating MMP2 accompanied with lower levels of adiponectin [58]. Hence, an inverse relationship between adiponectin and MMPs might exist and it would be possible to make interventions to avoid future complications. Therefore, we investigated how increases in MMPs levels in an inflammatory state (TNF-α) can be reduced in GEnC by adiponectin. Indeed, gAd was shown to be effective by reducing MMP2 levels but not MMP9. In hyperlipidemia subjects, adiponectin also resulted in decreased MMP2 and MMP9 activity [213]. To date, MMP2 and adiponectin has never been correlated in kidney diseases. Therefore, our study would be the first to correlate MMP2/9 and adiponectin in TNF-α induced GEnC. To further increase the knowledge about the MMP2/9 complex with adiponectin, knockdown of MMP2 was carried out using shRNA. Firstly, successfully silencing the MMP2 gene, I was able to show that SDC4 mRNA was regulated by MMP2 levels in which SDC4 shedding was mediated by MMP2. Secondly, the results showed that gAd had no effect on TNF-α induced shedding of SDC4 in MMP2 knockdown CiGEnC. This did not exclude a role for MMP9 in the actions of TNF-α on SDC4 mRNA effect, or in fact other MMPs in the increase in SDC4 mRNA. Finally, the question about the effect of gAd treatment on SDC4 levels in MMP2 knockdown was not fully addressed. The next stage

will be to investigate MMP2 protein levels and activity in the absence or presence of both TNF-α and adiponectin.

5.5 Conclusion

In this results chapter, I wanted to pinpoint that modifying the glycocalyx by adiponectin would be a critical step in altering disease states. I saw changes in important genes in a diabetic animal model. I was then able to use cell culture (CiGEnC) models and show that increases in the genes were only mirrored by TNF-α but not by HG. Furthermore, adiponectin blocked the rises of gene expression in isolated db/db glomeruli as well as the TNF-α effects in CiGEnC. Finally, MMPs might be involved in this pathway but I need to ensure this by attempting more experiments addressing the MMP involvement in the SDC4 shedding, such as measuring their activity and their protein expression. Therefore, adiponectin can be considered as a tool in changing the constituents of components of the glycocalyx. However, it was not enough to label adiponectin as marker for albuminuria due to incomplete data and a future work (discussed in the last chapter) should be done in order to support these findings.

Chapter 6 Overall Discussion and Conclusions

6.1 Achievement of objectives

The role of adipocytes as an endocrine organ that secretes several adipokines, specifically adiponectin, has become of increasing interest over the past two decades in the race to discover new therapeutic targets for diabetes and metabolic syndrome [75]. Adiponectin has important metabolic effects on glucose and lipid metabolism, both directly, in an tissue-specific manner, or indirectly, by amelioration of insulin sensitivity [214].

There is increasing evidence for adiponectin being renoprotective and so understanding the mechanism of action of this adipokine in the kidney may lead to new therapeutic strategies for the treatment of DN. The main aim of this project was to determine whether adiponectin acts on GEnC and whether it can protect the glycocalyx against inflammatory mediators implicated in diabetes.

Data from this thesis provides strong evidence that adiponectin contributes to GFB maintenance through direct actions on GEnC and its glycocalyx in experimental diabetes.

6.2 Summary of aims of chapter 3

The main aim of chapter 3 was to determine the levels of components of the adiponectin system in GEnC under normal and experimental diabetes conditions.

In summary the results showed that adiponectin is not secreted by either GEnC or podocytes under normal conditions (Table 5). However, adiponectin receptors are present on GEnC and podocytes and their expression are altered in diabetic conditions. Importantly, mRNA expression of AdipoR1 and AdipoR2 in CiGEnC was significantly

decreased under hyperglycemic conditions as summarized in Table 6. Similarly, renal cortex expression was also decreased in a type 2 diabetic animal model (db/db). The presence of AdipoR1 and AdipoR2 on GEnC and the fact that their levels are altered by high glucose suggests that adiponectin and its' receptors may be involved in DN. Also, the low expression of adiponectin in the db/db mice confirms other studies; hypoadiponectinemia exists in obesity and diabetes [87, 142].

After establishing that expression levels of the adiponectin receptors were altered in diabetes, it was then important to understand how adiponectin exerts its actions in cultured and *in vivo* GEnC by initiation of different signalling pathways through these receptors.

Chapter 3 summary Results	Unstimulated cells (Basal level) Human				Sieved Glomeruli (controls)	
	CiGEnC		**CiPod**		**Human**	**Mouse**
	mRNA	protein	mRNA	protein	mRNA	protein
Adiponectin	-	-	-	-	-	-
AdipoR1	++	++	++	++	++	++
AdipoR2	+	+	+	+	+	+

Table 5 Summary of results of Chapter 3-Part 1

A table showing the differences between adiponectin and receptors in CiGEnC, CiPod and sieved glomeruli at the mRNA and protein level. (- means absence, + means presence)

Chapter 3 summary Results	Stimulation of cells with HG		Diabetic glomeruli	
	CiGEnC	**CiPod**	**Human**	**Mouse**
AdipoR1	Downregulation was significant as early as 24h and persisted to 14 days (***p<0.001)	Downregulation was evident after long term exposure (4 and 7 days) (***p<0.001)	mRNA expression was decreased (ns due to low n number)	mRNA and protein expression were significantly decreased (**p<0.01)
AdipoR2	Downregulation was significant after 7 and 14 days (**p<0.01)	Downregulation was significant after 4 and 7 days (***p<0.001)	mRNA expression was decreased (ns due to low n number)	mRNA and protein expression were significantly decreased (*p<0.05)

Table 6 Summary of results of Chapter 3-Part 2

A table showing the effects of HG on AdipoR1 and AdipoR2 in CiGEnC and CiPod and diabetes in glomeruli. (*p< 0.05, **p<0.01, ***p<0.001)

6.3 Summary of aims of chapter 4

Chapter 4 focused on identifying key adiponectin signalling pathways in cultured and *in vivo* GEnC (1) and determining whether these are mediated by AdipoR1 or AdipoR2 (2). In fact, adiponectin resulted in the phosphorylation of several protein kinases that are crucial in cellular metabolism. Firstly, the phosphorylation of AMPK-α was significantly increased in CiGEnC and human and mouse sieved glomeruli as summarized in Table 7. This pathway is a major stress pathway activated by an increased AMP:ATP ratio. That is, most of the anabolic processes such as gluconeogenesis and lipid synthesis are inactivated

while major catabolic pathways such as glucose uptake into cells and fatty acid oxidation are increased [114]. The latter is due to increased phosphorylation of ACC by AMPK. During diabetes, the AMPK pathway is impaired due to excess glucose and insulin resistance and hence, there is less phosphorylation of AMPK-α. Treatment with adiponectin in experimental diabetes such as HG and in db/db glomeruli preserved the phosphorylation of AMPK-α, highlighting the potent activity of adiponectin in these conditions (Table 8). Other pathways were also activated by gAd, such as the Akt and p38 MAPK pathways, under normo-glycemic conditions (Table 7). However, their role was not clear in a diabetic environment, and hence they were not further investigated in isolated glomeruli.

The second objective was to determine which receptor was most likely to be mediating the actions of adiponectin in GEnC. After the successful knockdown of AdipoR1 in CiGEnC, the phosphorylation of AMPK-α was again re-analysed (Table 7). The results showed that AdipoR1 but not AdipoR2 is the main receptor contributing to the activation of p-AMPKα in response to treatment of GEnC by adiponectin. Hence, this suggests that gAd acts on GEnC via AdipoR1 to initiate a series of downstream signalling to protect the integrity of the cells.

Chapter 4 summary Results	Different phosphorylation pattern of the studied proteins in different cell types/glomeruli					
	CiGEnC with gAd	**AdipoR1 knockdown with gAd**	**AdipoR2 knockdown with gAd**	**CiPod with gAd**	**Human glomeruli with gAd**	**Mouse glomeruli with gAd**
Time point in min	30'	30'	30'	60'	30'	60'
p-AMPK-α	Increased *	ns	Increased **	Increased **	Increased *	Increased **
p-ACC	Increased **	ns	ns	Increased **	ND	ND
p-Akt	Increased *	ND	ND	ND	ND	ND
p-p38 MAPK	Increased **	ND	ND	ND	ND	ND

Table 7 Summary of results of Chapter 4-Part 1

A table showing the different phosphorylation profile in the presence of gAd in cultured cells and knockdown cells (ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ND: Not Determined)

Chapter 4 summary Results	Different phosphorylation pattern of the studied proteins in CiGEnC				db/db mouse glomruli
	HG (48h)	HG (48h) + 30' gAd	TNF-α (2h)	TNF-α + gAd (2h)	1h gAd
p-AMPK-α	Decreased ns	Increased **	Decreased **	Increased ***	Increased **
p-ACC	Decreased *	Increased **	Decreased **	Increased ***	ND
p-Akt	Increased **	Increased **	ND	ND	ND
p-p38MAPK	Decreased ns	Increased *	ND	ND	ND

Table 8 Summary of results of Chapter 4-Part 2

A table showing the level of phosphorylation of key protein kinases under diabetic conditions (TNF-α or HG) in the presence or absence of gAd in cultured cells and isolated glomeruli (ns $p>0.05$, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ND: Not Determined)

6.4 Summary of aims of chapter 5

The first two chapters demonstrated that adiponectin signals in GEnC via activation of AdipoR1. Due to the importance of the endothelial glycocalyx in regulating the biology of these cells, then the last aim was to determine whether adiponectin can directly protect the components of this structure *in vitro.* To test this, CiGEnC were cultured under diabetic conditions. TNF-α is a well characterised diabetic inflammatory mediator that can damage the glomerular endothelial glycocalyx [35]. Adding adiponectin in such conditions

allowed us to understand the beneficial effects actions of this adipokine on glycocalyx components. Firstly, adiponectin was able to enhance the glycocalyx of CiGEnC *in vitro* by decreasing the shedding of sulphated GAGs in the presence of TNF-α. Secondly, the effects of gAd on SDC4 expression was also promising. SDC4 expression (at both the mRNA and protein level) was upregulated in TNF-α induced conditions. Importantly, this was reversed back to almost normal levels when gAd was administered as summarized in Table 5. Interestingly, gAd also attenuated increases in MMP2 but not MMP9 mRNA in TNF-α induced media. Finally, knockdown of MMP2 resulted in less SDC4 shedding in TNF-α conditions suggesting a direct relationship between MMP2 and SDC4. Future work should be done to fully understand the link between the adiponectin and the SDC4-MMP2 pathway. Further evidence for a link between adiponectin and SDC4 was achieved by *ex vivo* treatment of sieved glomeruli with gAd. Importantly, diabetic sieved glomeruli treated with gAd showed less SDC4 mRNA expression than untreated diabetic glomeruli (Table 9). This showed one of most important regulators of the endothelial glycocalyx was protected by adiponectin treatment in diabetes. Future work should be focused on determining the effect of adiponectin on other glycocalyx components. Table 9 shows the prominent results of chapter 5.

Chapter 5 summary Results	Maintenance of the glycocalyx					
	CiGEnC			Mouse Glomeruli		
	Control	TNF-α	TNF-α + gAd	Control	db/db	db/db + gAd
SDC4	basal	Increased ***	Decreased **	basal	Increased ***	Decreased **
MMP2	basal	Increased ***	Decreased **	basal	Increased ***	ND
MMP9	basal	Increased ***	Decreased ns	basal	Increased ***	ND

Table 9 Summary of results of chapter 5

A table showing the protective effects of gAd in maintaining the glycocalyx when disturbed in CiGEnC or in diabetic glomeruli.

6.5 Conclusions from the study

This project has highlighted significant beneficial effects of adiponectin on the cell biology of GEnC. A summary of the findings from this work are as follows:

1- Adiponectin is not expressed in cultured GEnC or sieved glomeruli.

2- AdipoRs are expressed in CiGEnC and their levels are regulated under experimental diabetic conditions and in experimental animal models of diabetes.

3- Cultured GEnC are responsive to gAd, which activates several key signalling pathways including AMPK, ACC, Akt and p38 MAPK pathways.

4- Adiponectin activates the AMPK pathway in sieved human and mouse glomeruli.

5- The activation of AMPK is a result of downstream signalling through AdipoR1 but not AdipoR2.

6- HG and TNF-α conditions decreased AMPK and ACC signalling but gAd restored their activation.

7- Diabetic mouse glomeruli exhibit less AMPK signalling but gAd restored this activation

8- TNF-α but not HG conditions increased the expression of SDC4, MMP2 and MMP9

9- Adiponectin protected from the shedding of sulphated GAGs caused by TNF-α conditions.

10- Adiponectin decreased compensatory upregulation of glycocalyx component, SDC4, and its mediator MMP2 caused by TNF-α induction.

11- In diabetic glomeruli, gAd protected from the increase of SDC4 mRNA expression.

A schematic diagram that reflects all the main conclusions and results are shown in this below diagram.

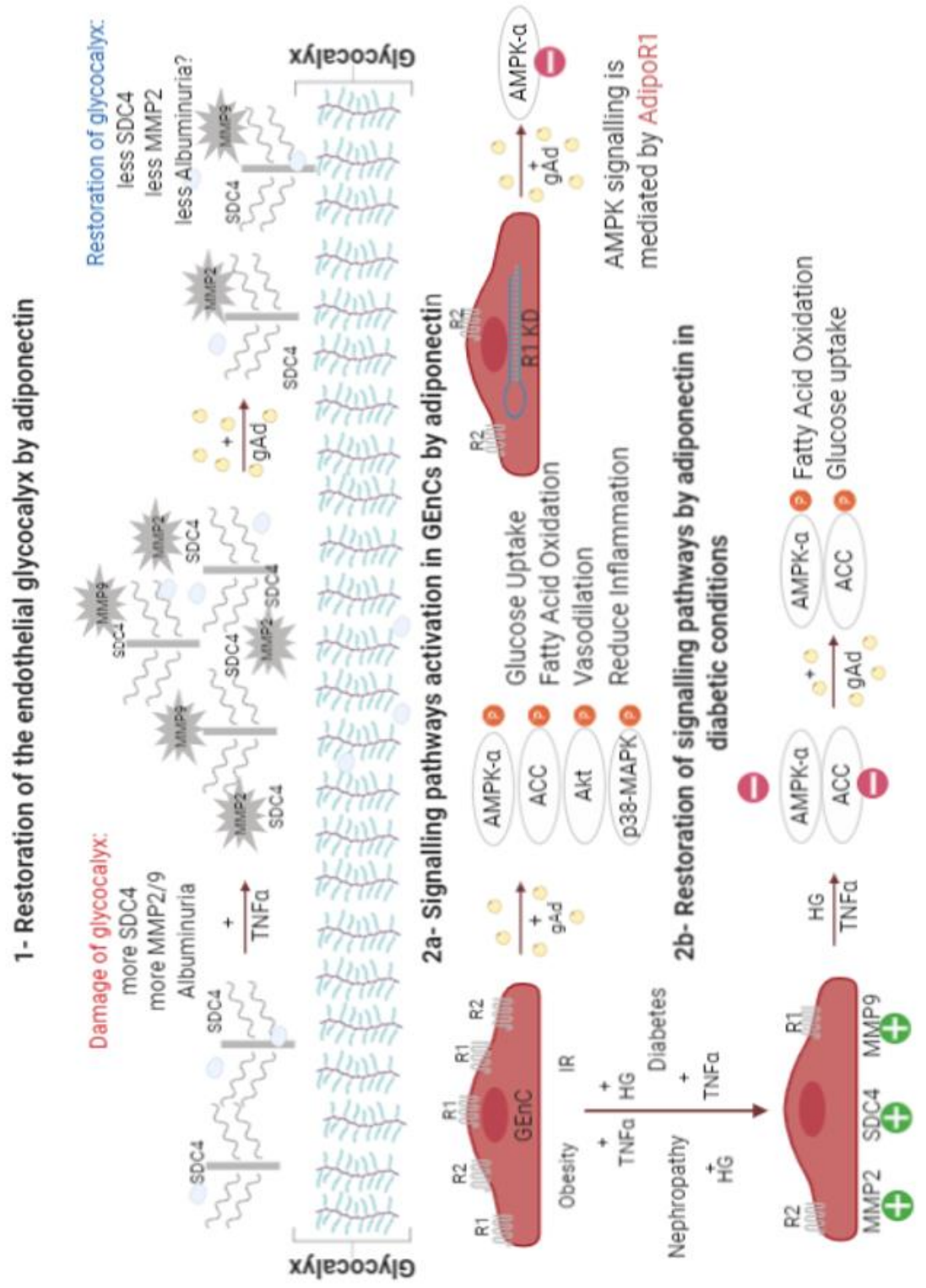

Figure 6.1 A schematic diagram summarizing the main findings of the project

A schematic diagram showing the main results of this project; the restoration of the glycocalyx after TNF-α induction in CiGEnC by gAd (1) and the activation of several pathways in CiGEnC such as AMPK, ACC, Akt and p38 MAPK pathway and that it is mediated by AdipoR1 (2a). AdipoR1 and AdipoR2 levels are decreased in obesity, diabetes, IR and nephropathy in GEnC. 2b: the effect of gAd in HG and TNF-α conditions on restoring p-AMPKα levels.

6.6 Limitations and future work

Despite accomplishing the goals of this project, several issues arose during this project. Firstly, the commercial production of the endothelial growth media used was paused for 4 months during the project. Therefore, time was taken up establishing the experimental system with another media (from EBM-2MV from Lonza media to MCDB 131 medium from Gibco #10372019) to match the conditions as the previously obtained data. However, all the results that was the outcome of CiGEnC treated with the Gibco media was excluded from this thesis, and experiments were repeated when the original media was available again.

Another limitation of this project was the lack of a control littermate for the db/db mice. Usually with a diabetic mice model (C57BLKsJ-db/db), the ideal littermate control would be the lean ones. However, in the comparison of glomeruli gene expression, I used wild type mice due to unavailability of the lean mice.

Other limitations include some of which are being addressed by future work. For example, the glycocalyx studies are missing essential IF staining. Although SDC4 mRNA and protein was detected, it would have added value if SDC4 staining pattern on CiGEnC with or without TNF-α/gAd was also checked. Along with the Alcian blue assay technique that detects GAGs released in the media, an HS ELISA on the supernatant media would also complete the profile of glycocalyx components (GAGs, HS and SDC4). As for the MMP2 knockdown studies, we only measured MMP2 mRNA expression levels, but it would have been clearer if I investigated the protein levels as well as activity of MMP2.

The effects of adiponectin on GEnC barrier properties was also part of the *in vitro* cellular work. The electrical cell-substrate impedance sensor system (ECIS) measures impedance

in real time. Any decrease in impedance means that water and small solute passage increases across the CiGEnC monolayer hence, there is an increase in permeability. The study was rather incomplete due to low repeat number and inconsistent results.

The work presented suggests a protective role for adiponectin in GEnC. The next stage would be to assess the effect of adiponectin on albuminuria in a T2D mouse model. This will be achieved by using the C57BLKS/J-db/db mouse as a model of type 2 diabetes [163]. By over-expressing adiponectin (using adenovirus) in mice it will be possible to determine whether adiponectin reduces albuminuria [215]. Lean mice treated with control adenovirus only will act as a non-diabetic control group. Adenoviral overexpression of adiponectin for 10 days has previously been sufficient to reduce albuminuria in adiponectin knockout mice [113]. Therefore, adenoviral vectors expressing adiponectin or control will be injected into the tail vein of db/db mice at 10 weeks of age. Serum adiponectin levels, blood glucose and albuminuria will be measured at baseline and at 3-day intervals for 2 weeks. After 2 weeks, animals will be sacrificed. Before sacrifice, blood and urine will be taken to measure glucose and albuminuria respectively. For every animal, once a stable anaesthesia is achieved, the abdominal aorta will be exposed with a midline laparotomy followed by abdominal aorta cannulation. At this point, one of the kidneys will be perfused with 4% glutaraldehyde and processed for light and electron microscopy (EM) analysis in order to detect changes in glycocalyx thickness. The other kidney will be used to study the albumin glomerular permeability coefficient (PS'alb). Using this novel technique [216] that was developed by Desideri *et al*. in our lab, it is possible to detect glomerular permeability changes associated with the reduction of the glycocalyx thickness. Finally, this will complete the profile about restoring the glycocalyx in *in vivo* studies, and not only *in vitro* studies as I have done on CiGEnC.

6.7 Clinical significance

One third of T2DM patients will develop kidney disease (DN), which is detected by albuminuria as a result of an impaired glomerular permeability [146]. Good glycaemic control and current treatments (insulin, metformin, ACE, ARB) can decelerate the progression of DN [217]. However, to date, there are still limited treatments that can efficiently save these patients from vascular complications. Therefore, innovative therapeutic advances are essential to target early endothelial dysfunction in diabetes.

The focus of this project was to investigate the beneficial effects of adiponectin to provide early protection of GEnC. This work identified the mode of action of adiponectin on GEnC under pathophysiological conditions (diabetic conditions); adiponectin protects the GEnC in HG conditions by activating major signalling pathways via AdipoR1.

The glycocalyx is disturbed not only in DN but also other nephropathies such as glomerulosclerosis and ischaemia-reperfusion [218] as well as in renal transplant patients [219]. The glycocalyx is also damaged in other kidney related diseases including atherosclerosis [220] and sepsis. Therefore, restoring the glycocalyx reduces glomerular albumin leak.

6.8 Adiponectin and receptors as therapeutic targets for DN

Available data shows that adiponectin is renoprotective and, therefore, a rational approach for developing new therapeutics would be to find ways to enhance adiponectin effects by either increasing adiponectin plasma levels or by stimulating adiponectin receptors to increase adiponectin sensitivity [136]. This will lead to protective metabolic effects via downstream signalling pathways. In fact, several compounds are known to increase circulatory adiponectin in pathophysiological states as seen in Figure 6.2. Thiazolidinediones (TZD) are a class of heterocyclic compounds used as an anti-hyperglycemic drug for the treatment of T2DM [221]. Several studies have shown that TZDs directly upregulate adiponectin gene transcription through the activation of PPARγ in adipocytes, thereby promoting adipocyte differentiation. For example, rosiglitazone treatment greatly reduced albumin excretion rate AER in diabetics and this decrease has been associated with increased serum adiponectin levels [222] (Figure 6.2). TZD was used to treat mice with an ablated podocyte function, showing that recovery of injured podocytes was seen by increasing adiponectin serum levels [136]. Also, in adiponectin deficient ob/ob mice, rosiglitazone did not improve the glucose tolerance while in ob/ob mice there was a substantial improvement in glucose tolerance [222]. Therefore, this shows that significant glucose tolerance after treatment with rosiglitazone is totally dependent on the presence of adiponectin [223]. Hence, this supports the approach of promoting adiponectin therapeutically. Overall, TZD effects on DN might be directly related to upregulating adiponectin levels.

Sensitizing adiponectin receptors is also of therapeutic importance. The elucidation of the mechanisms in which these receptors become activated is of great importance to the future improvements of diabetes and its complications. In 2013, Okada *et al* discovered a small molecule as an AdipoR agonist and named it AdipoRon [224]. Primarily, AdipoRon was shown to bind directly to both receptors and thus activating AMPK in skeletal muscle and liver of WT mice. Furthermore, in obese mice, AdipoRon significantly reduced glucose and insulin levels and this effect was dependent on the presence of both receptors, since a double knockout mice did not have attenuate of hyperglycaemia and hyperinsulinemia [224]. Consistently, in a db/db mice model, AdipoRon administration greatly reduced glucose, insulin and lipid accumulation in just 2 weeks. Surprisingly, the effects of AdipoRon was as quick and potent as of that of adiponectin. There was also an up-regulation of phospho-AMPKα and PPARα in the treated db/db mice. In cultured GEnC and podocytes, AdipoRon treatments had equivalent results to that seen *in vivo*; there was an inhibition in lipid-induced endothelial dysfunction, one of the common mediators of diabetic nephropathy, through the activation of AMPK and PPARα signalling [224]. Taken all together, the protective role of AdipoRon against the development and progression of diabetic nephropathy appears to occur through a direct action on the renal cells, particularly in GEnC and podocytes.

In future years, AdipoR-activating compounds is expected to be an important aspect in therapeutic models for the treatment of diabetes and its complications, thus contributing to a healthy life expectancy.

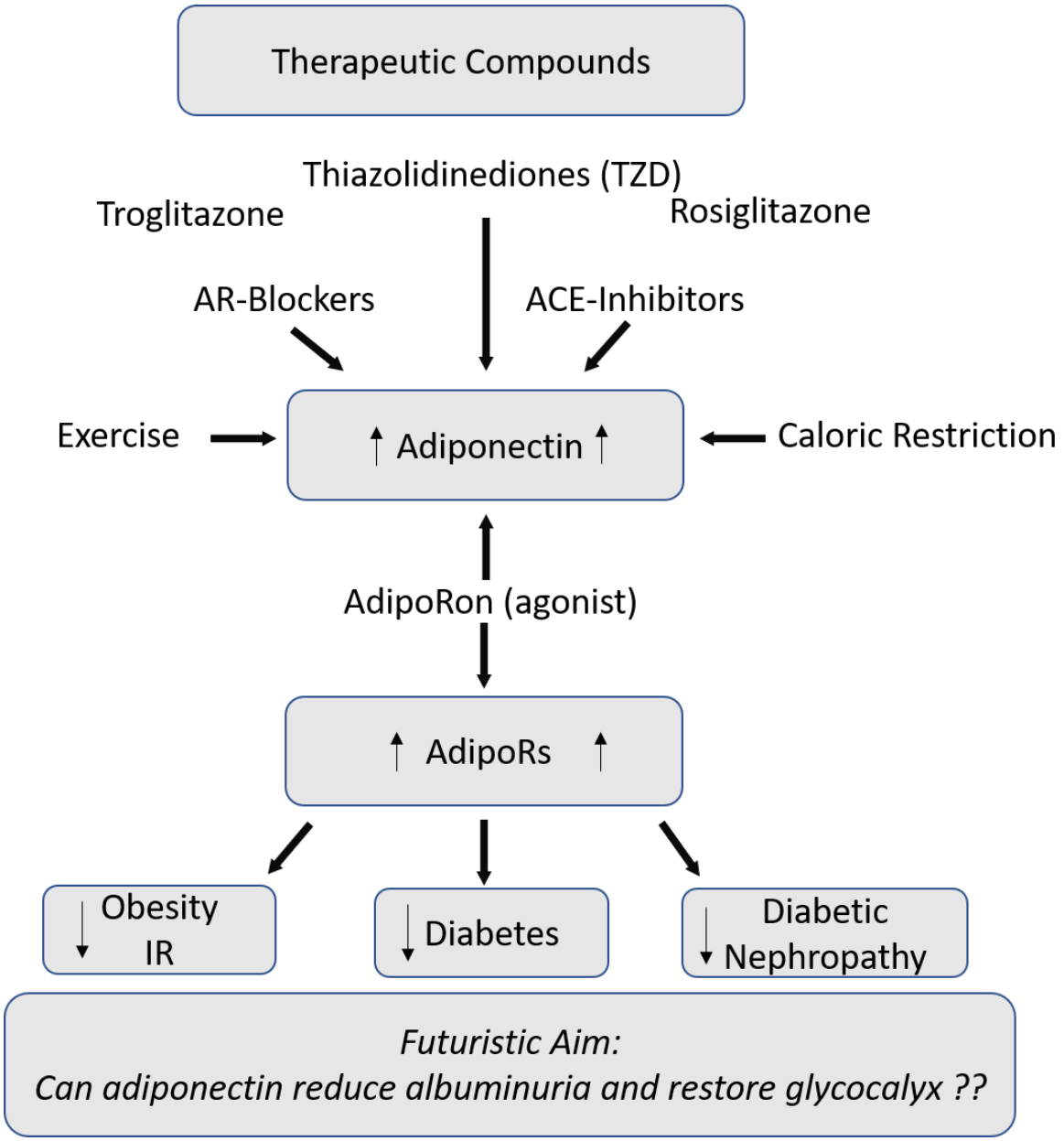

Figure 6.2 Schematic diagram of adiponectin sensitizers and downstream effects of increasing adiponectin levels.

Adiponectin can be sensitized by several compounds such as TZD, ARBs, ACE-I and factors such as exercise and caloric restriction. This will lead to increase in AdipoRs (also by AdipoRon). Increase in sensitivity might lead to decrease in Obesity, IR and diabetes with or without nephropathy.

Despite mentioning all the beneficial effects of adiponectin in different tissues or organs, there are a number of questions to address. Initially, adiponectin circulating levels are abundantly high and it accounts for 0.01% of total plasma proteins [136]. Therefore, there would be consequences in providing excess adiponectin. For example, adiponectin may

promote angiogenesis and adipogenesis associated with the growth of tumours and weight gain respectively [72]. Furthermore, infertility can result from chronically high adiponectin levels [225]. Finally, it has been suggested that circulating fAd indirectly inhibit bone mass by increasing insulin sensitivity [226]. These consequences will need to be addressed when establishing a strategy to upregulate adiponectin and its receptors.

Human trials where adiponectin is given as a treatment in diabetic patients has not been done yet or even approved. However, adiponectin research is increasing at a fast pace, and together with the work from this thesis suggest that targeting adiponectin pathways especially in GEnC may provide novel therapeutic targets for DN and other diabetic diseases.

Appendix

Media	Composition	Source
Endothelial cell media		
Complete Media	Endothelial cell basal medium MV2	Lonza EBM-2 CC-3156
	Supplement pack containing (FCS, hEGF, hydrocortisone, VEGF, hbFGF, R3-IGF, and ascorbic acid).	PromoCell C-39221
	5% Fetal Bovine Serum	GIBCO 10500
Serum free media (SFM)	Endothelial cell basal medium MV2 ONLY	Lonza EBM-2 CC-3156
Podocytes Media		
Complete Media	RPMI-1640 medium	Sigma #R8758
	10% Fetal Bovine Serum	Sigma #F7524
	1% penicillin/streptomycin antimicrobial agent	Sigma #P4333
Serum free media (SFM)	RPMI-1640 medium ONLY	Sigma #R8758

Protein Sample Buffer	
Reagents	**Volume**
dH_2O	3.4ml
0.5M Tris-HCl pH6.8	1ml
Glycerol	2ml
10% Sodium dodecyl sulphate (SDS)	1.6ml
Bromophenol blue	Sprinkling
β-Mercaptoethanol	0.4ml

1X Transfer buffer	
Reagents	**Volume**
Methanol	100ml
10X Transfer buffer	100ml
dH_2O	800ml

10X Transfer buffer	
Reagents	**Weight**
Trizma Base	30.3g
Glycine	144g
Dissolve in 1L dH_2O	

1X Running Buffer	
Reagents	**Weight**
Trizma Base	30.3g
Glycine	144g
SDS	10g
Dissolve in 1L dH2O	

Antibodies	Molecular Weight kDa	Dilution	Supplier
P-AMPKα (Thr172)	62	1:1000	CST #2535
Total AMPK-α(D5A2)	62	1:1000	CST #5831
Phospho-ACC (Ser79)	280	1:1000	CST #11818
Total ACC (C83B10)	280	1:1000	CST #3676
P-p38 MAPK (Thr180/Tyr182)	43	1:1000	CST #4511
Total p38 MAPK	43	1:1000	CST #9212
p-Akt (Ser473) (D9E)	62	1:1000	CST #4060
Total Akt	62	1:1000	CST #4685
anti-AdipoR1 (EPR6626)	44	1:1000	Abcam ab #126611
Anti- AdipoR2	43	1:1000	Thermofisher PA5-25029
Adiponectin (C45B10)	27	1:1000	CST #2789
Syndecan 4	37	1:1000	Thermofisher #36-3100
β-actin	42	1:5000	Sigma #A5441
MMP2	60	1:1000	Proteintech 10373-2-AP

Antigen	Dilution	Supplier
Rabbit IgG	1:10,000	Sigma A6667
Mouse IgG	1:10,000	Sigma A9044

Secondary (IF)	Dilution	Supplier
Alexa Fluor 488 Goat anti-mouse	1:500	Life technologies UK, #A11001
Alexa Fluor 488 Goat anti-rabbit	1:500	Life technologies UK, #A11008

Step	Temperature	Time	
Initial	95°C	10min	
Denaturation **Annealing & Extension**	95°C 60°C	15s 60s	45 cycles
Final	95°C	15s	
Hold	4°C	∞	

Human primers	**Forward sequence**	**Reverse Sequence**
β -actin	ATGTGGCCGAGGACTTTGATT	AGTGGGGTGGCTTTTAGGATG
Adiponectin	CAGGCCGTGATGGCAGAT	AGTCTCCAATCCCACACTGAAT
AdipoR1	ACAAGGTCTGGGAGGGACGT	ACAAGGTCTGGGAGGGACGT
AdipoR2	TGCAGCCATTATAGTCTCCCAG	GAATGATTCCACTCAGGCCTAG
Syndecan-4	CCTCCTAGAAGGCCGATACTT	AGGGCCGATCATGGAGTCTT
MMP2	GAGACCATGCGGAAGCCAAGATG	GGTGTGTAACCAATGATCCTGTATGT
MMP9	GCCCCAGGAGTCTGGAT AAGTTGG	GTCGAATCTCCAGACAC GCCCC
TNF-α		
Mouse Primers		
β-actin	CTGTCCCTGTATGCCTCTG	ATGTCACGCACGATTTCC
Adiponectin	GATGGCAGAGATGGCACTCC	CTTGCCAGTGCTGCCGTCAT
AdipoR1	ACGTTGGAGAGTCATCCCGTAT	TGTCCAGATGTTGCCAGTCTCTGTGTG
AdipoR2	GCCCAGCTTAGAGACACCTG	CTCTGTGTGGATGCGGAAGAT
Syndecan-4	CCCTTCCCTGAAGTGATTGA	AGTTCCTTGGGCTCTGAGG
MMP2	GCTATGTCCACTGTGGGTGGAAAT	GATCCCTTGATGTCATCATGGGATAAT AGACC
TNF-α	AGACCACGTGCGCTGTGAC	ACAGGTCTCCCATGCACAA

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