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The Physiological Role of Nrf2 in Diabetic Kidney Disease

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Résumé

La néphropathie diabétique (DN) est l'une des premières causes de maladie rénale en phase terminale (ESKD). L'ESKD est un important facteur de risque d'insuffisance cardiaque et d'accidents vasculaires cérébraux. La dysfonction du système rénine-angiotensine intrarénal (iRAS) est considérée comme étant l'une des principales causes du développement de la DN. Tous les composants du iRAS sont identifiés dans les cellules épithéliales des tubules rénaux proximaux (RPTCs), y compris l'angiotensinogène (Agt), le seul précurseur de toutes les angiotensines. Notre laboratoire a rapporté précédemment que la surexpression spécifique de l'Agt dans les RPTCs provoque l'hypertension, la protéinurie, la fibrose rénale, l'apoptose et des lésions rénales.

Nrf2 (Nuclear factor erythroid 2-related factor 2) est un facteur de transcription qui est exprimé de façon abondante dans les RPTCs et a été considéré comme étant un régulateur central de l'équilibre redox dans les réponses cytoprotectrices cellulaires. Le rôle de l'activation du Nrf2 dans la DN, toutefois, est controversé. L'objectif général de cette thèse est de comprendre le rôle physiologique du Nrf2 dans la DN et d'étudier le(s) mécanisme(s) moléculaire(s) de l'action de Nrf2.

Premièrement, nous avons démontré que la délétion génétique de Nrf2 ou l'inhibition pharmacologique de Nrf2 avec de la trigonelline chez les souris Akita diabétiques de type 1 régule à la hausse la voie Ace2/MasR et supprime l'expression de Agt/ACE dans les RPTCs, ce qui a pour effet d'atténuer l'hypertension systémique et les lésions rénales. Conformément, dans les cellules immoratalisées de tubule proximal de rat (IRPTC) en culture, la transfection de ARNsi ou le traitement à la trigonelline empêche la régulation positive de Agt/ACE induite par le HG, avec une baisse subséquente de l'expression des gènes Ace2/MasR. Ces données identifient un nouveau mécanisme dans lequel l'activation de Nrf2 stimule l'expression et l'activation des gènes du iRAS, menant au développement de l'hypertension et de la néphropathie dans le diabète.

Deuxièmement, nous avons généré des souris Nrf2 transgéniques qui surexprime spécifiquement Nrf2 dans les RPTCs (souris $Nrf2^{RPTC}$ Tg), sous le contôle du promoteur KAP (kidney specific androgen-regulated protein). Nous avons ensuite croisé les souris $Nrf2^{RPTC}$ Tg avec les

souris Akita $Nrf2^{-/-}$ pour générer des souris Akita $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg. Nous avons trouvé que la surexpression de Nrf2 dans les RPTCs des souris Akita $Nrf2^{-/-}$ augmentait significativement l'expression du gène SGLT2, entraînant une élévation du glucose sanguin, du taux de filtration glomérulaire, du rapport albumine/créatinine urinaire et de la fibrose tubulo-interstitielle. Dans les cellules tubulaires proximales humaines immortalisées (HK2), le traitement à l'oltipraz ou la transfection de l'ADNc du NRF2 stimule l'expression de l'ARNm du SGLT2 et l'activité de son promoteur. De plus, des tests de retard sur gel et d'immunoprécipitation de chromatine ont montrés que NRF2 se lie au NRF2-RE du promoteur du SGLT2. En outre, une expression plus élevée de NRF2 et SGLT2 est observée dans les RPTCs de reins de patients diabétiques que dans les reins de patients non diabétiques. Ces données ont établi un nouveau mécanisme de la régulation du NRF2 sur l'expression et l'activation du gène SGLT2, menant à une exacerbation du glucose sanguin, de l'hyperfiltration et des lésions rénales dans le diabète.

En somme, cette thèse a démontré que le stress oxidatif (hyperglycémie) induisait l'activation du Nrf2 qui stimulait le iRAS et l'expression de SGLT2, contribuant ainsi à la progression de la DN. Ces études suggèrent que le Nrf2 pourrait être une cible thérapeutique potentielle dans le traitement de la DN et pourront fournir de valabless données pré-cliniques pour les essais cliniques en cours avec le bardoxolone méthyle (un activateur de Nrf2).

Mots-clés : Nrf2, stress oxidatif, néphropathie diabétique, système rénine-angiotensine intrarénal, SGLT2

Abstract

Diabetic nephropathy (DN) is one of the leading causes of end-stage kidney disease (ESKD). ESKD is a major risk factor for heart failure and stroke. Dysfunction of intrarenal renin-angiotensin system (iRAS) is considered as one of the main reasons that caused the DN. All components of the iRAS are identified in the renal proximal tubule cells (RPTCs), including angiotensinogen (Agt), the sole precursor of all angiotensins. Our lab has previously reported that specific overexpression of Agt in RPTCs induces hypertension, proteinuria, kidney fibrosis, apoptosis and kidney injury.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that abundantly expresses in RPTCs and has been considered as a master regulator of redox balance in cellular cytoprotective responses. The role of Nrf2 activation in DN, however, is not clear. The overall aim of this study is to understand the physiological role of Nrf2 in DN and investigate the molecular mechanism(s) of Nrf2 action.

First, we have demonstrated that genetic deletion of Nrf2 or pharmacological blockade of Nrf2 with trigonelline in type 1 diabetic Akita mice effectively upregulates Ace2/MasR and suppresses Agt/ACE expression in isolated RPTCs, resulting in attenuation of systemic hypertension and kidney injury. Consistently, in cultured IRPTCs, Nrf2 siRNA transfection or trigonelline treatment prevents high glucose-induced upregulation of Agt/ACE with downregulation of Ace2/MasR gene expression. These data identified a novel mechanism in which Nrf2 activation stimulates iRAS gene expression and activation, leading to the development of hypertension and nephropathy in diabetes.

Second, we have generated Nrf2 transgenic mice under the kidney specific androgen-regulated protein (KAP) promoter which specifically overexpress Nrf2 in RPTCs ($Nrf2^{RPTC}$ Tg mice). We further crossbred the $Nrf2^{RPTC}$ Tg mice with Akita $Nrf2^{-/-}$ mice to generate Akita $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg mice. We have found that overexpression of Nrf2 in RPTCs of Akita $Nrf2^{-/-}$ mice significantly unregulated sodium-glucose transporter-2 (SGLT2) expression, resulting in elevation of blood glucose, glomerular filtration rate, albumin-creatinine ratio and tubulointerstitial fibrosis. In

immortalized human proximal tubular cells (HK2), oltipraz treatment or NRF2 cDNA transfection stimulated SGLT2 mRNA expression and its promoter activity. Furthermore, NRF2 bound to NRF2-RE of SGLT2 promoter were identified by gel mobility shift assay and chromatin immunoprecipitation assay. Moreover, human diabetic kidneys exhibited higher expression of NRF2 and SGLT2 in RPTCs than non-diabetic kidneys. These data established a novel mechanism of NRF2's regulation on SGLT2, leading to exacerbation of blood glucose, hyperfiltration and kidney injury in diabetes. In summary, this study documented that activation of Nrf2 in hyperglycemia contributed to the progression of DN via regulation of iRAS and SGLT2, suggesting that Nrf2 might be a potential therapeutic target in the treatment of DN.

Keywords: Nrf2, oxidative stress, diabetic nephropathy, intrarenal renin-angiotensin system, SGLT2

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List of Acronyms and Abbreviations

ACE	Angiotensin converting enzymes
ACEi	ACE inhibitors
Ace2	Angiotensin-converting enzyme-2
ACR	Albumin/creatinine ratio
AGEs	Advanced glycosylation end products
Agt	Angiotensinogen
Ang II	Angiotensin II
Ang 1-7	Angiotensin 1-7
AQP2	Aquaporin 2
AT1R	Angiotensin II type 1 receptor
AT2R	Angiotensin II type 2 receptor
ARBs	Angiotensin receptor blockers
ARE	Antioxidant response element
BW	Body weight
BM	Bardoxolone methyl
Cat	Catalase
CKD	Chronic kidney disease
DM	Diabetes mellitus
DN	Diabetic nephropathy
ECM	Extracellular matrix
eGFR	Estimated glomerular filtration rate
ESKD	Endstage kidney disease
EMSA	Electrophorretic mobility shift assay
FBG	Fasting blood glucose
FN1	Fibronectin 1
GBM	Glomerular basement membrane
GDM	Gestational diabetes mellitus
GSK3	Glycogen synthase kinase 3
GFR	Glomerular filtration rate
GLUT	Glucose transporter family
GST	Glutathione s-transferase

HG	High glucose
HO-1	Heme oxygenase-1
hnRNP F	Heterogenous nuclear ribonucleoprotein F
hnRNP K	Heterogenous nuclear ribonucleoprotein K
iRAS	Intrarenal renin-angiotensin system
Keap 1	Kelch-like ECH-associated protein 1
KW	Kidney weight
Masson	Masson's trichrome staining
MCP-1	Monocyte chemotactic protein 1
NF-Kb	Nuclear transcription factor κappa B
NHE3	Sodium–hydrogen exchanger 3
NO	Nitric oxide
NQO-1	NAD(P)H: quinone oxidoreductase1
Nrf1	Nuclear respiratory factor 1
Nrf2	Nuclear factor erythroid 2-related factor 2
Nrf2-RE	Nrf2-response elements
PAS	Periodic acid Schiff
PKC	Protein kinase C
PTCs	Proximal tubular cells
P62	Protein 62/SQSM 1 (Sequestosome 1)
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
RPTCs	Renal proximal tubular cells
SBP	Systolic blood pressure
SiRNA	Small interfering RNA
SGLT1	Glucose co-transporter 1
SGLT2	Glucose co-transporter 2
SGLT2i	Glucose co-transporter 2 inhibitors
STZ	Streptozotocin
TL	Tibia length
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TGF	Tubule glomerular feedback
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α

To my Mom

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Chapter 1 – Introduction

1.1 Diabetes Mellitus

1.1.1 Prevalence of diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia due to impairment of insulin secretion, insulin resistance, or both. Uncontrolled diabetes will lead to kidney failure, heart disease, blindness, nerve damage and more. This serious, long-term disease has a major impact on the lives and well-being of individuals, families and societies worldwide, and has become the leading cause of global deaths [1]. The number of people with diabetes has risen from 285 million in 2009 to 425 million in 2017 and has been estimated to reach up to 578 million (10.2%) by 2030 and 700 million (10.9%) by 2045. Compared to the low-income countries (4.0%), high-income countries were predicted a higher prevalence (10.4%) in 2045. For age and sex differences, people between 65–79 years account for 19.9% (111.2 million) and 9.0%, 9.6% in women and men, respectively [2].

1.1.2 Type 1 diabetes mellitus (T1DM)

Insulin is a hormone secreted by pancreatic β-cells and targets on reducing blood glucose level. T1DM, accounting for nearly 10% of all diabetes, is associated with insulin deficiency caused by pancreatic β-cell loss and resulting in hyperglycemia. It can occur at any age, though most of the cases start between 5-7 years of age and at or near puberty [3]. The incidence of T1DM is slightly more common in boys and men, and highly variable among different ethnic populations. For example, the overall incidence of T1DM is 0,1/100 000 per year in the Zunyi region in China, while more than 40/100 000 per year was reported in Finland [4].

1.1.2.1 The progression of T1DM

Patients with T1DM are very sensitive and responsive to insulin, especially in the early stages. One study on anatomy of the pancreas has suggested that patients with T1DM only have

∼10% of normal pancreatic β-cell mass left [5]. Without sufficient insulin, the body cannot take up glucose and ends up in polyphagia, polydipsia, and polyuria, the classic symptoms of diabetes. In addition, the body turns to break down fat as fuel, resulting in an increased level of ketones and eventually ketoacidosis, a common complication of T1DM [6]. The underlying mechanism of T1DM is not completely understood. It is currently believed that the cause of T1DM is a combined effect of both genetic and environmental factors. Genome-wide association studies and meta-analyses showed identified more than 50 genetic risk loci T1DM [7]. Among them, the human leucocyte antigen complex on chromosome 6 is responsible for 40–50% of the genetic risk of T1DM development. The insulin gene polymorphisms on chromosome 11, the cytotoxic T lymphocyte-associated antigen-4 gene (CTLA-4) on chromosome 2 and other genetic loci also contribute to genetic susceptibility for T1DM [8]. For the environmental factors, viruses (rubella, coxsackievirus B or enteroviruses), diet and gut microbiota have been reported to be involved in the pathogenesis of T1DM. Furthermore, other factors related to the immune system also have an impact on the β cells function [8, 9].

1.1.2.2 Treatment of T1DM

For the care of T1DM, maintaining the insulin level by preserving endogenous insulin secretion or insulin therapy is the main therapeutic goal [10]. Pancreatic islets transplantation and β-cell replacement therapy can partially restore the insulin secretion and improve glycemic control [11]. Improvement of lifestyle with low-carbohydrate diet and less psychological stress may also have positive effect on T1DM [12].

An earlier clinical trial from seven centres in Europe reported that Vitamin D supplementation was associated with a decreased risk of Type I diabetes without indication of heterogeneity in 820 patients and 2335 subjects [13]. This is also supported by another clinical trial that children who regularly took the recommended dose of vitamin D had a reduced risk of type 1 diabetes, compared to children whose vitamin D intake was less [14]. More therapeutic strategies for the management of T1DM are still ongoing.

1.1.3 Type 2 diabetes mellitus (T2DM)

T2DM is the most common type of diabetes (90%) and associated with hyperglycemia, insulin resistance, and insulin insufficiency [15]. It is often considered as a disease of middle-aged and older people, but recent data showed that T2DM has become more prevalent among adolescents and young adults due to the increasing obesity at younger age [16]. There seems to be no overall difference of the T2DM prevalence between men and women, though some statistics have reported small changes between two sexes in different regions. For instance, females are more prone to youth T2DM while males are more vulnerable to midlife T2DM [15]. Furthermore, compared to non-diabetic subjects, women trend to have greater increases of cardiovascular risk, myocardial infarction and stroke mortality than men [17].

1.1.3.1 The progression of T2DM

T2DM affects many organs. In the post absorptive condition, most of the glucose production is derived from the liver by glycogenolysis and gluconeogenesis, and a small part (15%) is produced from the kidney by gluconeogenesis. Half of the produced glucose (50%) is taken up by the brain and nervous system via glucose transporter 1 (GLUT-1), regardless of the presence or absence of insulin. While about 25% of glucose is utilized by the liver plus gastrointestinal tissues independent of insulin level, the remaining 25% of glucose is taken up by muscle and fat in an insulin-dependent pathway. Both the muscle and adipose tissue could use either glucose or ketone free fatty acids as fuel, according to the insulin level. With low insulin level, ketone and free fatty acid are used for energy, whereas the cells prefer to use glucose when exposed to high insulin level. As a result, blood glucose is lowered either by metabolism or storage as glycogen in the muscle and fat in the adipose tissue [18].

Patients with diabetes can be assessed by the measurement of glycated hemoglobin (HbA1c), fasting plasma glucose, and postprandial plasma glucose [19]. In the progression of T2DM, both fasting and postprandial hyperglycemia are directly correlated to the risk of diabetes and contribute to the HbA1c level [20].

In fasting state, glucose is produced by gluconeogenesis (formation of glucose from pyruvate or other 3-4 carbon compounds) and glycogenolysis (breakdown of glycogen to glucose).

As discussed, liver is the major source of gluconeogenesis in human and plays critical role maintaining constant blood glucose concentration. During starvation, glucagon is secreted by pancreatic alpha cells to increase hepatic gluconeogenesis and glycogenolysis, thereby increase circulating blood glucose to maintain glucose homeostasis. In T2DM patients, enhanced glucagon and reduced insulin action leads to increased hepatic gluconeogenesis, glycogenolysis and reduced glucose uptake by peripheral tissue, resulting in the development of hyperglycemia [21, 22]. Indeed, a significant number of patients present with fasting hyperglycemia first due to increased hepatic glucose production. One of most commonly used T2DM drugs, metformin, has been shown to decreased hepatic glucose production, probably by increased AMP mediated inhibition of fructose-1-6-bisphosphatase [23].

In the postprandial state, insulin is secreted from pancreatic islet β-cells and targets on reducing the high blood glucose level (via stimulating glucose uptake by peripheral tissues) and suppressing glucose production (by inhibiting hepatic gluconeogenesis and glycogenolysis) [21]. However, in certain conditions such as accumulation of oxidative stress, glycation, and advanced glycation end products (AGEs), early insulin release is decreased at the postprandial state, resulting in an inefficient glucose uptake by peripheral tissues, abnormal glucose production including glycogenolysis and gluconeogenesis [24-26]. The sustained hyperglycemia results in persistent secretion of insulin, impairment of islet β-cell cells and insulin insufficiency as well as insulin resistance.

As both insulin insufficiency and insulin resistance are present in the T2DM, it is difficult to determine which of these abnormalities is the primary defect [27]. The insulin resistance is a condition where insulin-targeting-cells no longer respond to insulin effectively, which happens in many organs including muscle, adipose tissue and liver [28]. Although insulin resistance has been considered one of the major characters of T2DM, it also occurs in T1DM [29]. The underlying mechanism of insulin resistance is thought to be due to genetic defects in a few individuals, and in most cases, due to being overweight, particularly central or visceral obesity [30, 31].

Risk factors for the progression of T2DM include genetics and family history, pre and post-natal environmental factors, such as low birth weight, obesity, inactivity, gestational diabetes,

and advancing age [32]. Many genes and pathways dependent or independent of the insulin action, have been studied to understand the molecular mechanisms of T2DM. For example, hyperglycemia-induced mitochondrial oxidative dysfunction might be one of the initial causes of the T2DM progression [33]. Moreover, NF-κB (nuclear factor κB)-mediated inflammation and its downstream cytokines, such as TNF-α and IL-6, are also involved in the progression of T2DM [34]. Also, tumor necrosis factor-alpha (TNF-α) mediated inflammation have also been reported as one of the major reasons for insulin resistance and adipose tissue inflammation [35]. Genome-wide screening yielded more candidate genes that may be responsible for the T2DM progression such as the transcription factor TCF7L2a in the Wnt pathway and the zinc transporter 8 (ZNT8) in the solute carrier family [32].

1.1.3.2 Treatment of T2DM

People with T2DM may develop symptoms gradually for several years without notice. Long-term diabetes without proper control would cause serve complications including heart and kidney failure, blindness, stroke and more. For the care for T2DM, lifestyle changes to lose weight and blood glucose by insulin, metformin, sulfonylureas or in combination are the widely used approaches. Depending on the patient's background, other medications can be used to lower the risks of complications. For example, people who have both T2DM and high blood pressure might receive anti-hypertensive therapy, such as renin-angiotensin-system blockers [36]. Furthermore, the new class drug, SGLT2 inhibitors, have yielded promising results for the treatment of T2DM [37].

1.1.4 Gestational diabetes mellitus (GDM)

GDM is the third type of diabetes in which pregnant women without diabetes history develop hyperglycemia during pregnancy. Approximately 7% of all pregnancies in different population are affected [38]. Along with the epidemics of obesity, prevalence of GDM is expected to increase [39]. GDM is associated with T2DM. Women with GDM develop hyperglycemia, insulin insufficiency and insulin resistance. Although most of the GDM resolve after delivery, untreated GDM can impact the health of the fetus or mother. Moreover, women with a history of GDM and their children, are at a higher risk of developing T2DM later on [40].

The underlying mechanisms of GDM remain unknown. Similar to T2DM, obesity, genetics, β cell dysfunction and insulin resistance are considered the reasons of GDM [41]. Other pathways including placental transport, gut microbiome and oxidative stress have been suggested to participate in the progression of GDM [42]. Treatment of GDM includes dietary education, blood glucose monitoring and control. Recently, Vitamin D has been reported to be associated with glucose metabolism in GDM [43].

Vitamin D is a group of fat-soluble steroids that could be synthesized in the body or supplied by the food. It is responsible for multiple physiological processes such as calcium and phosphate absorption in intestine and maintaining balance of bone skeletal calcium level. As discussed in 1.1.2.2, Vitamin D has been considered beneficial in the therapy of T1DM. Vitamin D deficiency is associated with a higher risk of GDM [44]. However, whether vitamin D deficiency contributes to the development of GDM is still not clear. Although administration of Vitamin D has shown to improve the insulin sensitivity and glucose tolerance in rats, no large clinical trial with various Vitamin D supplementation on human patients with GDM has been reported [45, 46]. A relevant report by Soheily khah S, et al. showed that supplementation of Vitamin D on pregnant but non-diabetic women increased the insulin level at a lower dose, whereas fasting blood glucose and calcium levels were not changed [47]. Hence, more clinical studies are needed to determine whether supplementation of vitamin D can prevent GDM.

1.1.5 Complications of DM

Diabetes is often accompanied by serious complications, which have been early termed by microvascular complications (refer to long-term complications that mainly affect small blood vessels, such as nephropathy, retinopathy and neuropathy) and macrovascular complications (cardiovascular disease and stroke) (Figure 1). In addition, other complications such as diabetic foot syndrome, dental disease, infection and inflammation are also common in diabetes [48]. Complications of DM are often responsible for significant morbidity and mortality. Along with the

prevalence of DM, number of people with diabetic complications also increase and has become a heavy burden for quality life and health services [49].

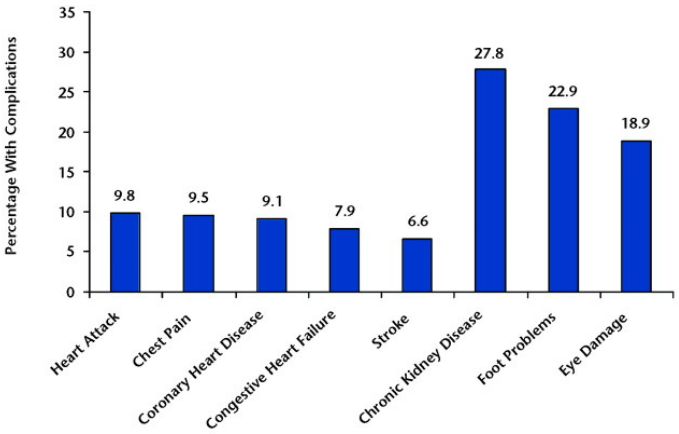


Figure 1. – Prevalence of diabetic complications among people with diabetes (1999-2004) from National Health and Nutrition Examination Survey [48].

1.1.5.1 Diabetic microvascular complications

Diabetic nephropathy (DN) is the leading cause of end-stage kidney disease (ESKD), defined by proteinuria > 500 mg in 24 hours with a subsequent decline in glomerular filtration rate on the base of diabetes. Chronic proteinuria is preceded by micro-albuminuria, which is defined as urinary albumin excretion of 30-299 mg/24 hours [50]. DN occurs at a relatively late stage of diabetes. It is associated with increased blood pressure, kidney hypertrophy and kidney injury. The etiology of DN is still poorly understood. Many underlying mechanisms have been suggested to contribute to the progression of DN. For the treatment of DN, in addition to the glycemic control, patients benefit from the treatment with ACE inhibitors or angiotensin II receptor type 1 blockers. Moreover, in the recent years, the new class drugs such as dipeptidyl peptidase-4 inhibitors (DPP-4), glucagon-like peptide-1 receptor agonists (GLP-1RAs) and SGLT2 inhibitors have exhibited multiple protective effects in the treatment of DN [48, 51, 52].

Diabetic retinopathy (DR) is referred to eye problems, resulting in vision loss and blindness in people with diabetes. It is one of the most common microvascular diabetic complications and the leading cause of blindness globally [53]. The prevalence of DR varies depending on the duration of the patients with diabetes. One analysis showed that approximately 25% of patients with T1DM develop DR after 5 years, almost 60% by 10 years and 80% at 15 years [54]. Hence,

early eye examination and proper glycemic control at an early stage are indispensable in prevention of the DR progression [55].

A few underlying mechanisms including the polyol pathway, advanced glycation end products (AGEs) accumulation, the protein kinase C (PKC) pathway and the hexosamine pathway, have been suggested to be involved in the progression of DR [56, 57]. Hyperglycemia-induced inflammation is also a major factor for DR. For example, chemokines such as monocyte chemotactic protein-1 (MCP-1) is involved in the pathogenesis of DR and mutation of MCP-1 protects diabetic mice from retinal vascular damage [58]. Lastly, although still in debate, the retinal neuro degeneration in diabetes, an early event during the progression of DR, might be a factor that responsible for DR development [59].

Diabetic neuropathy refers to various types of nerve damage induced by hyperglycemia. More than half of the individuals with diabetes eventually develop neuropathy [60]. Risk factors of diabetic neuropathy includes age, duration of disease, hypertension and higher BMI as well as alcohol consumption [48]. Patients with diabetic neuropathy may suffer from motor changes like weakness or sensory symptoms including numbness, tingling, or pain. Similar to DR, the mechanisms of diabetic neuropathy are related to persistent hyperglycemia and its downstream metabolic disorders such as increased flux of the polyol pathway, AGEs production, activation of PKC and oxidative stress [61].

1.1.5.2 Diabetic macro-vascular complications

Cardiovascular disease (CVD) is the primary cause of death among all people with diabetes. The mortality rates due to heart disease are 2 to 4 times higher in patients with diabetes compared with those without diabetes [48]. Multiple factors are thought to contribute to the progression of heart failure, including abnormal glucose metabolism in the heart, hypertension, hyperlipidemia and premature atherosclerosis as well as insulin resistance. For instance, cross-linking of AGEs with proteins such as collagens contribute to ventricular stiffness and subsequent diabetic cardiomyopathy. In addition, the β-linked N-acetylglucosamine (O-GlcNAc), a key Ca^{2+} handling protein and an important mediator of cardiac function, is modified by high glucose and impairs the cardiac contractility. These factors lead to the structural and functional

abnormalities of the heart and increase the risk of stroke as well as stroke-related mortality [50, 62, 63].

To date, diabetic patients with risk of CVD are treated with strict glycemic control, administration of blood pressure-lowering agents, lipid-lowering therapy with statins and/or antiplatelet agents [62]. Particularly, a recent clinical trial has shown that the new class of agent, SGLT2 inhibitors, have achieved a promising effect in the care of patients with heart failure [64].

Apart from the diabetic complications mentioned above, there are some acute complications of diabetes, such as diabetic ketoacidosis, the hyperglycemic hyperosmolar state, and hypoglycemia. Although preventable, they still account for a part of the high morbidity and mortality in diabetes. Therefore, understanding the mechanisms of diabetes and diabetic complications are crucial and indispensable.

1.2 Diabetic Nephropathy (DN)

1.2.1 Renal physiology

The kidneys play a fundamental role in maintaining homeostasis of body fluid. Firstly, the kidneys filter large quantities of blood, reabsorb substances that the body needs, and form urine with the metabolic waste. Secondly, the kidneys maintain fluid and electrolyte balance including pH and osmolality. Thirdly, the kidneys secrete a variety of hormones, including renin, erythropoietin and calcitriol, which relate to the regulation of blood pressure, production of red blood cells and bone health, respectively [65].

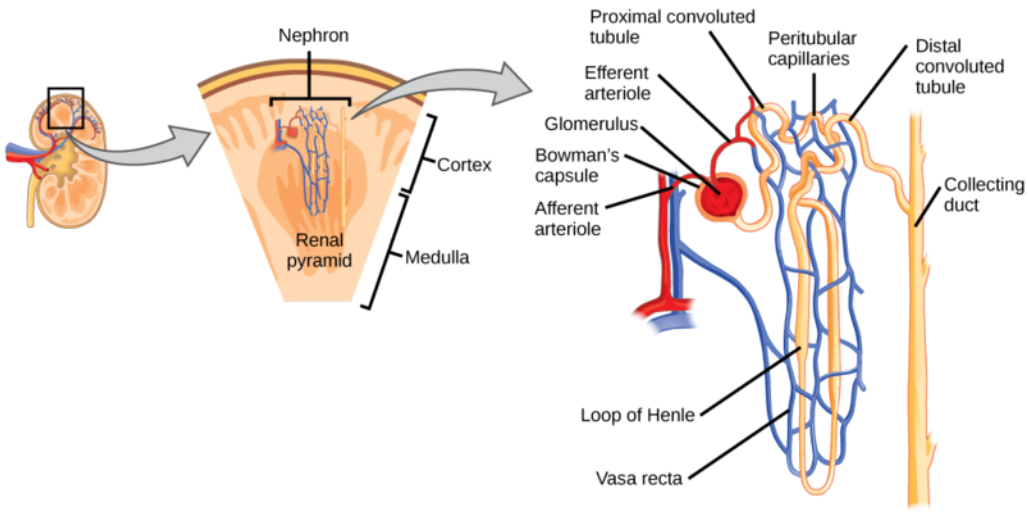


Figure 2. – The structure of nephron [66].

The functional unit of kidney is nephron (Figure 2). A healthy human kidney contains around 1.8 million nephrons which lies up both in the renal cortex and medulla. Each nephron contains a renal corpuscle, proximal tubule, loop of Henle, distal tubule and collecting duct. Blood is filtered by the glomerulus, and necessary substances such as glucose, amino acids, and sodium, are reabsorbed by the renal tubular cells, especially at the proximal tubular cells. The remaining fluid which consists of water, metabolic waste and toxins is excreted as urine [67, 68].

1.2.2 The renal corpuscle

The renal corpuscle is composed of the glomerulus and Bowman's capsule. As shown in Figure 3, blood from the afferent arteriole is filtered by the glomerulus. The filtration contains three layers: fenestrated endothelium, glomerular basement membrane (GBM) and the podocytes. Small molecules such as water, glucose, sodium ion, amino acids and urea can pass freely into Bowman's space, but not large protein. The filtrate in the Bowman's capsule is similar to blood plasma except large proteins, which enter into proximal convoluted tubule for reabsorption [69].

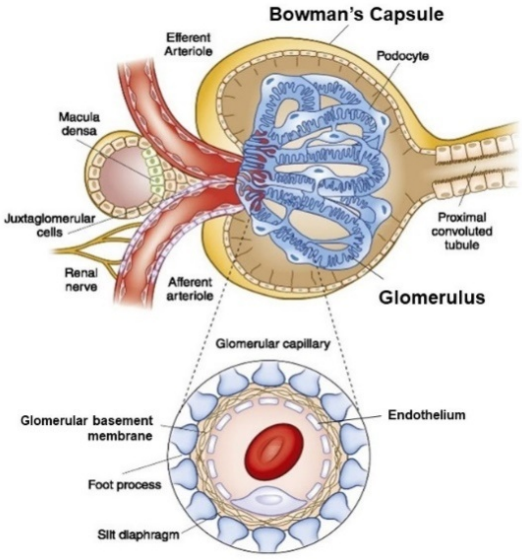


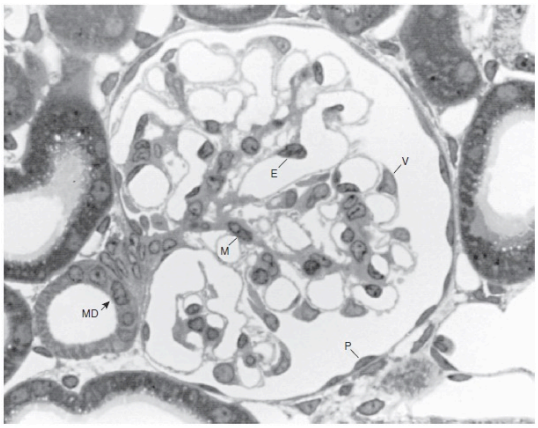
Figure 3. – Diagram of the renal corpuscle structure [69].

1.2.2.1 The glomerulus

Located within Bowman's capsule, the glomerulus is a tuft of capillaries and responsible for the blood filtration. In this filtration, small molecules with a radius smaller than 20 Å such as water, glucose, sodium ion, amino acids and urea can pass freely into Bowman's space, but not molecules larger than 42 Å. Larger protein like serum albumin, with molecular radius of 35.5 Å, is

usually poorly filtered. In addition to a size-selection, the less negative or more positive charge of the protein have more permeability to the filtration barrier [70].

a.



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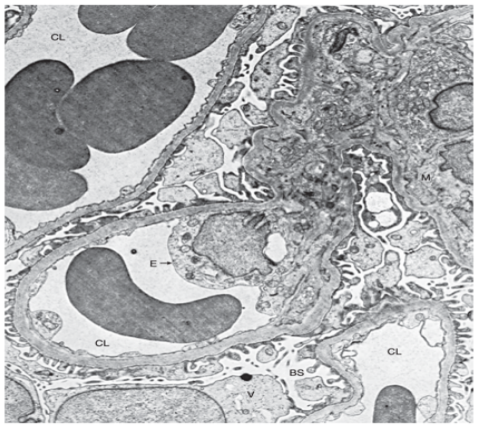


Figure 4. – Normal rat glomerulus by light micrograph from rat (4a) and human (4b). Mesangial cell (M), endothelial cell (E), visceral epithelial cell (V), and parietal epithelial cel l/podocyte (P). MD, macula densa, capillary loops (CL), Bowman's space (BS) [68].

The first filtration layer is the fenestrated endothelium, which consists of endothelial cells and lies adjacent to the mesangium, away from the urinary space (Fig. 4). The endothelial cells contribute to the charge-selective properties of the glomerular filtration due to their negatively charged glycocalyx. The endothelial cells also synthesize nitric oxide (NO) and endothelin1, which are important for the regulation of vasoconstriction [68].

The second layer of the filtration, GBM, is a fibrous and stratified lattice with heterogeneous pores, formed by the membrane fusion of endothelial cells and podocytes. Type IV collagens and laminins are the most important components in the constitution of the GBM [71]. Approximately 30-40% of the cells inside glomerulus are mesangial cells, as shown in Figure 4. Mesangial cells are surrounded by matrix material called mesangial matrix, which contains sulfated glycosaminoglycans, large amount of fibronectin, laminin and various collagens. The mesangial cells possess multiple functions including structural support, regulation of glomerular filtration, generation and metabolism of the mesangial matrix as well as response to glomerular injury [72]. Besides mesangial cells, epithelial cells are also present in the renal corpuscle. The

parietal epithelial cells line up inside of the Bowman's capsule, whereas the visceral epithelial cells reside outside of the GBM, namely podocyte.

1.2.2.2 The podocytes

Podocytes are the largest, octopus-like cells in the glomerulus. Their cell bodies develop foot processes, wrapping around the glomerular capillaries. As shown in Figure 5 [73], neighboring podocytes are physically adjoined through their foot processes by unique intercellular junctions, slit diaphragm (SD). The function of SD has been recognized as essential in the retention of large protein in the plasma. In the SD, Nephrin and podocin are expressed and indispensable in maintaining the glomerular filtration and overall kidney function [70]. Structural changes of the SD and foot processes effacement are associated with proteinuria and contribute to many kidney diseases.

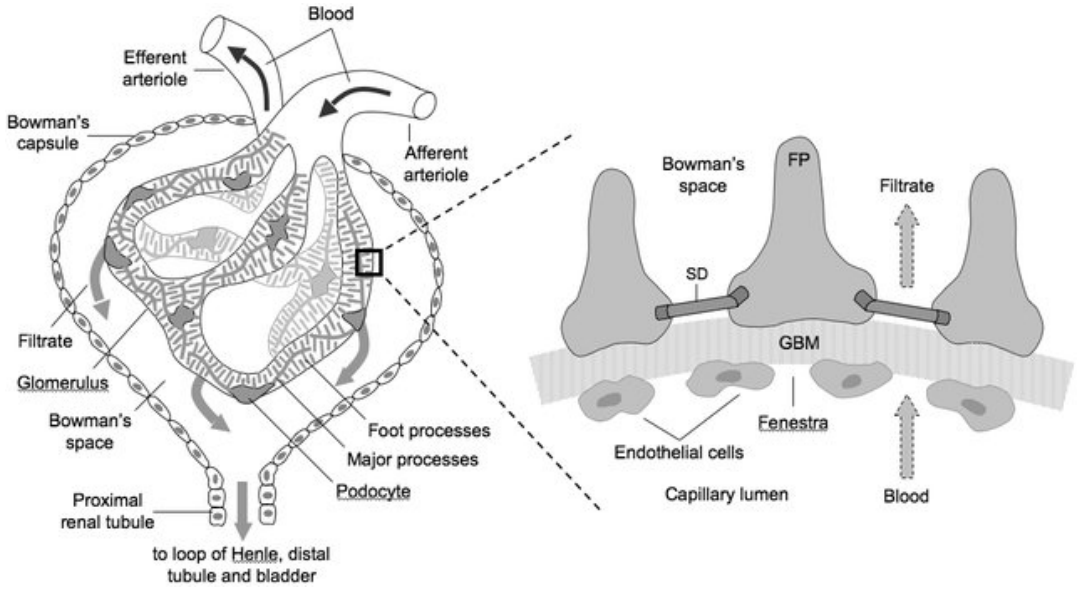


Figure 5. – Diagrams structure of podocytes. FP: foot processes; SD: slit diaphragms.

1.2.2 The renal tubules

1.2.3.1 The renal proximal tubules

The renal proximal tubule begins from the renal pole of the Bowman's capsule till the beginning of loop of Henle. The length of the proximal tubule is approximately 14 mm in humans and 8 mm in rats [74]. Three morphologically distinct segments, S1, S2, and S3, have been identified. The initial portion of the proximal tubule, S1 segment, lies in the cortex and consists of most of the convolute part, while the S2 and S3 segments represent the rest of the convolute tubule and the straight portion, respectively. As shown in the right panel of Figure 6, the renal proximal tubule cells (RPTCs) in S1 segment are characterised by tall brush border, long mitochondria and well-developed Golgis. Cells in the S2 segment are similar to that the S1 segment but with shorter and smaller mitochondria. Cells in the S3 segment, in contrast, possess small mitochondria but with longest brush border microvilli and prominent peroxisomes [68, 75].

The RPTCs possess a fundamental role in the kidney reabsorption. As shown in Table 1, many factors are reabsorbed by the RPTCs including glucose, amino acids, water and electrolytes such as Na^+, HCO_3^-, Cl^-, K^+ and Ca^{2+} [76]. Each of them has crucial functions in the body. For instance, change of sodium transport in RPTCs can directly affect extracellular fluid volume and subsequently blood pressure. Multiple sodium transporters including the Na^+/K^+ ATPase, Na^+/H^+ antiporter 3 and Na^+/HCO_3^- exchanger, participate in the regulation of fluid balance and blood pressure [77]. Importantly, the RPTCs also secrete more waste substances such as ammonium and creatinine [78]. Taken together, the proximal tubule plays a key role in kidney function.

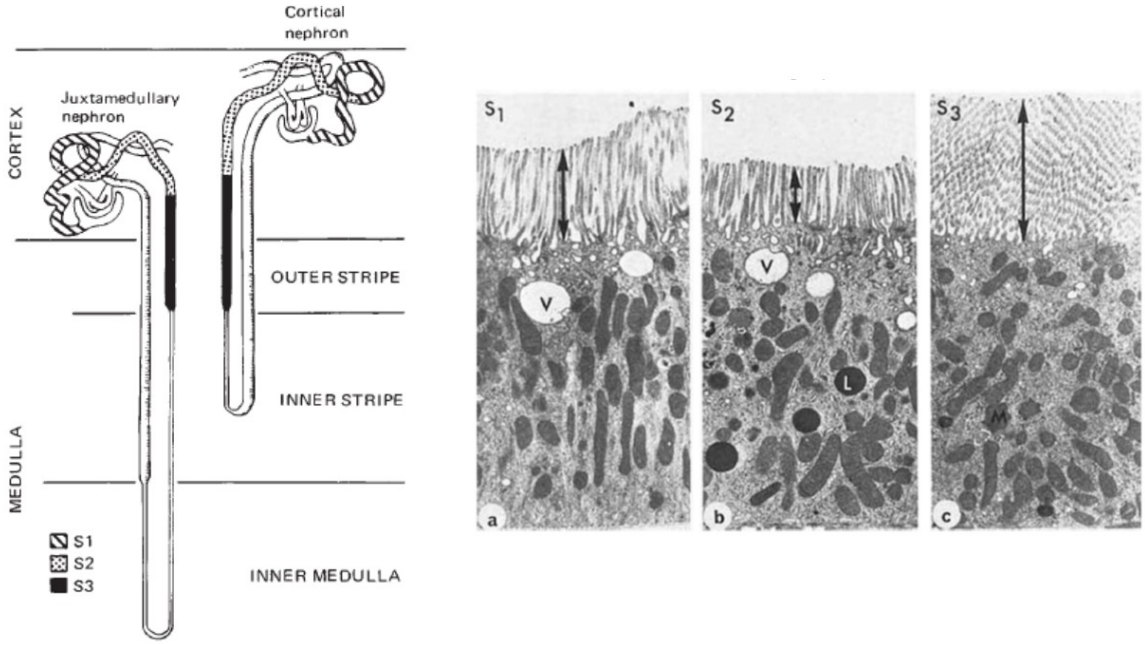


Figure 6. – Renal proximal tubule. Left panel: the locations of S1, S2 and S3 segment of proximal tubule [75]. Right panel: the electron micrographs of S1, S2, and S3 segments in rat (a to c, magnification, X10,600). Tall brush border, big mitochondria and numerous vesicles (V) are observed in the S1 and S2 segment, whereas brush border microvilli are longest in S3. Lysosomes (L) and microbodies (M) are most numerous in S2 and S3 cells, respectively [68].

1.2.3.2 The other renal tubules

As shown in Table 1, the loop of Henle, distal tubule and connecting tubule are also important parts in the kidneys and participate the reabsorption [79]. The loop of Henle is a segment that starts from the end of proximal tubule and consists of the descending limb and the ascending limb. The primary function of the loop of Henle is to create a concentrated urine for excretion. The distal tubule is the portion between the loop of Henle and collecting tubule. Unlike proximal tubules, distal tubules do not have brush border. The main function of distal tubules is their regulation on potassium, sodium, calcium and pH. The last part of the nephron is the collecting duct system. This section mediates the electrolyte and fluid balance, which is regulated by antidiuretic hormone. In the presence of antidiuretic hormone, water is reabsorbed into the body, whereas without the antidiuretic hormone, water ends up passing through the duct and excreted as urine [68].

Substance	PCT	Loop of Henle	DCT	Collecting ducts
Glucose	100% reabsorbed; secondary active transport with Na^+			
Proteins, amino acids	100% reabsorbed; symport with Na^+			
Vitamines	Reabsorbed			
Urea	50% reabsorbed by diffusion; also secreted	Secretion, diffusion in descending limb		Reabsorption in medullary collecting ducts; diffusion
Sodium	65% actively reabsorbed	25% reabsorbed	5% reabsorbed; active	5% reabsorbed, stimulated by aldosterone; active
Chloride	Reabsorbed, symport with Na^+, diffusion	Reabsorbed	Reabsorbed; diffusion	Reabsorbed; symport
Water	67% reabsorbed osmotically with solutes	15% reabsorbed; osmosis	8% reabsorbed if ADH; osmosis	Variable amounts reabsorbed, controlled by ADH, osmosis
Bicarbonate	80–90% symport reabsorption with Na^+	Reabsorbed, symport with Na^+		Reabsorbed antiport with Cl^-
H^+	Secreted; diffusion		Secreted; active	Secreted; active
NH_4+	Secreted; diffusion		Secreted; diffusion	Secreted; diffusion
HCO_3^-	Reabsorbed; diffusion	Reabsorbed; diffusion in ascending limb	Reabsorbed; diffusion	Reabsorbed; antiport with Na^+
Potassium	65% reabsorbed; diffusion	20% reabsorbed	Secreted; active	Secretion controlled by aldosterone; active
Calcium	Reabsorbed; diffusion	Reabsorbed		Reabsorbed if parathyroid hormone present; active
Phosphate	85% reabsorbed, inhibited by parathyroid hormone, diffusion		Reabsorbed; diffusion	

Table 1. – Substances secreted or reabsorbed in the renal tubules and their locations. ADH: antidiuretic hormone [79].

1.2.4 Diabetic nephropathy (DN)

DN is one of the most common diabetic complications and the leading cause of ESKD. It is classically characterized by the appearance of albuminuria, the reduction of renal function (decreased glomerular filtration rate and creatinine clearance) and development of hypertension. Morphologically, DN is accompanied by excessive deposition of extracellular matrix proteins, thickening of GBM, glomerular hypertrophy, tubule dilation and tubulointerstitial fibrosis. The progression of DN is classified in five stages based on the albuminuria and glomerular filtration rate (GFR). Stage 1 develops in the early diabetes with a hyperfiltration and an above-normal GFR. Normal GFR is maintained in the Stage 2, which can be accompanied by small amounts of microalbuminuria up to 30 mg/24hrs. The microalbuminuria increases to 30-300 mg/24hrs in stage 3 with a drop of GFR down to 60 ml/min/1.73 m^2. GFR falls to a level less than 30 ml/min/1.73 m^2 in stage 4 with higher albuminuria. In the end stage of the DN, the kidney losses most of its function with a GFR level less than 15 ml/min/1.73 m^2 [80, 81].

1.2.5 The pathogenesis of DN

1.2.5.1 Intrarenal renin-angiotensin system (iRAS) and DN

IRAS is a hormone system that regulates blood pressure, fluid and electrolyte balance. Activation of RAS is considered one of the most important pathological processes in the progression of DN. Beyond traditional systemic RAS, existence of local RAS in different organs such as heart and kidney have been identified [82]. Compared to systemic RAS, all components of the intrarenal RAS (iRAS) has been recognized in the RPTs [83]. For example, the concentration of angiotensin II (Ang II), one important component of RAS, has been reported 1000-time higher in proximal tubular and interstitial fluid than in systemic blood [84]. Hence, understanding iRAS and its function is necessary for the study of DN.

It is not fully understood that whether systemic RAS and intrarenal RAS could regulate each other. Our previous data showed that overexpression of Agt in RPTCs caused hypertension and renal injury without affecting the expression of liver Agt or circulating Agt, indicating that intrarenal RAS does not regulate systemic RAS and functions locally [85]. However, one study by

Matsusaka's group showed that liver specific KO of Agt markedly reduced the plasma angiotensinogen level, renal Agt and Ang II expression, implying that systemic RAS affects the intrarenal RAS. Moreover, this study also found that RPTC-specific KO of Agt had no detectable change in intrarenal Agt [86]. Concerns about the RPTC-specific KO in this study was addressed as only 50% of urinary Agt was reduced. Hence, a further study that knockout of Agt in mice nephron (Pax8-rtTA-Cre) and found that Nephron-KO-Agt significantly reduced BP and renal Agt expression. Unexpectedly, Agt mRNA expression in liver and plasma Agt were also decreased, suggesting that the iRAS participated the regulation of systemic RAS [87, 88].

For the iRNA, as shown in Figure 7, angiotensinogen (Agt), the solo precursor of RAS, is cleaved into angiotensin I (Ang I) by renin. Ang I is further cleaved into angiotensin II by angiotensin-converting enzyme (ACE), or angiotensin 1-7 (Ang 1-7) by the angiotensin-converting enzyme 2 (Ace 2). Although derived from the same precursor, Ang II and Ang 1-7 exert very different, rather opposite and counter-balancing effect. It is considered that the iRAS is equipped with two arms, the principle pressor arm Agt/Ang II/ ACE/AT1R and the depressor arm of Ace2/MasR/Ang 1-7, in a counter-balanced way [89, 90].

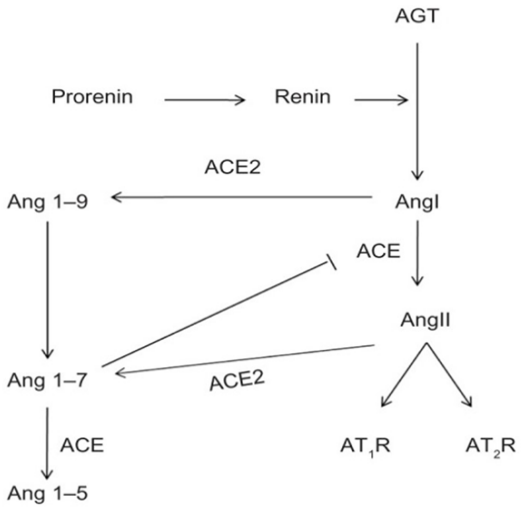


Figure 7. – The intrarenal RAS system. Renin hydrolyzes Agt to Ang I which is converted to Ang II by ACE or Ang1-7 by Ace2. Ang I could be also cleaved into Ang 1-7 and Ang 1-5 via ACE. Ang II exerts its action by binding to AT_1R and AT_2R. Ace2 also catalyzes the conversion of Ang II to Ang 1–7. Agt: angiotensinogen; Ang I: angiotensin I, Ang II: angiotensin II; ACE: angiotensin converting enzyme, PTC: proximal tubule cells, AT_1R: angiotensin II Types I receptors, AT_2R: angiotensin II Types 2 receptors [91].

a) The pressor arm Agt/ACE/Ang II/AT1R

The Agt is a peptide of 485 amino acids and expresses in renal proximal tubule cells at a high level. Our group has demonstrated that high glucose can dramatically stimulate Agt expression via p38 mitogen-activated protein kinase (p38 MAPK) pathway in rat RPTCs [92]. Specifically, overexpression of Agt in RPTCs causes hypertension, proteinuria, kidney fibrosis, apoptosis and kidney injury [85, 93]. In diabetic condition, high glucose-induced Agt and ACE result in significant elevation of Ang II level [94]. Ang II plays a key role the progression of DN via binding to its receptor type I and II (AT_1R and AT_2R) [91]. These two receptors share a sequence identity about 30%, possessing similar affinity for the Ang II but exerting different functions. Through binding to AT_1R, Ang II induces a variety of harmful effects such as vasoconstriction, hypertension, fibrosis, cardiac and renal damage. Multiple pathways are responsible for this effect, mainly including: i) coupling with G protein in vascular smooth muscle cells, and activating IP3-mediated rise in intracellular calcium levels and ultimately causing vasocontraction [95]; ii) increasing the sodium reabsorption via Na^+/H^+ exchanger (NHE3) and resulting in water retention, vasoconstriction and augment of arterial blood pressure [96]; iii) stimulating of transforming growth factor (TGF-β1), a powerful fibrotic gene that results in expansion of mesangial matrix, glomerulosclerosis and interstitial fibrosis [97]; iv) activating the MCP-1 and NF-Kb mediated inflammation; v) triggering the oxidative stress which contributes to the mitochondrial and cellular damage [98]. Infusion of Ang II in mice caused glomerulosclerosis, tubulointerstitial fibrosis, albuminuria, increased systolic blood pressure, cardiac hypertrophy and kidney damage [99, 100]. Blockade of AT_1R (ARBs) and ACE inhibitors have been major therapeutic approaches in the treatment of DN and hypertension. In contrast, activation of AT_2R has been protective via preventing sodium (Na^+) retention and lowering BP in the Ang II infusion model [101]. Beneficial effects of AT_2R activation have been also reported in diabetic mice model and diabetes-induced cardiovascular and renal disease [102, 103]. As shown in the Table 2, AT_2R activation counteracts most effects of AT_1R by inhibiting cell proliferation and differentiation, promoting vasodilation, and reducing inflammation and oxidative stress [104]. In diabetic Akita mice, activation of AT_2R with C_{21} (an agonist of AT_2R) significantly attenuated diabetes–induced mesangial expansion,

glomerulosclerosis, albuminuria. It also inhibited expression of various proteins implicated in oxidative stress, inflammation and fibrosis [105]. Furthermore, knockout of AT_2R in diabetic mice accelerates development of hypertension, renal hypertrophy, tubular apoptosis and extracellular matrix (ECM) protein accumulation as well as increased GFR. This effect is partially mediated via heightened oxidative stress and ACE/ACE2 ratio in RPTCs [106].

b) The depressor arm Ace2/Ang 1-7/MasR

As shown in Figure 8, Ace2/Ang 1-7/MasR axis exerts a counter-balanced effect against Ang II. Multiple studies have shown the protective effect of Ang 1-7 in DN. For example, our group found that Akita mice treated with Ang 1-7 agonist significantly attenuated systolic blood pressure, hypertension, fibrosis and kidney damage via reducing renal oxidative stress [107]. Ang 1-7 can also blunt the Ang II-induced activation of inflammation and Ang II–stimulated phosphorylation of MAP kinases [108]. Ace2, homologue of ACE, exerts 60% sequence similarity of ACE but resists the ACE inhibitors. Multiple studies suggested that Ace2 rather protects mice from diabetes and maybe considered as an attractive new target in the treatment of DN and other diabetic complications [109]. For instance, Ace2 knockout mice yield proteinuria and overexpression of Ace2 did not develop cardiac hypertrophy with Ang II infusion [110].

The MasR receptor (MasR) belongs to the G protein-coupled receptor family and considered as the receptor of the Ace2/Ang 1-7 axis of iRAS. Studies have shown that MasR participates in several physiological processes including reno- and cardio- protection as well as regulation of the central nervous system [111]. In human mesangial cells, Ang 1–7 increased phosphorylation of p38 and ERK1/ERK2 through MasR and antagonist of MasR (but neither the antagonists of AT_1R nor the AT_2R) could block this effect [13]. One of our earlier studies showed that in diabetic Akita mice, Ang 1–7 administration normalized systemic hypertension, attenuated glomerular injury, tubulointerstitial fibrosis and decreased oxidative stress, whereas co-administration of A779 (an antagonist of MasR) effectively reversed most of the effects of Ang 1–7, indicating the effect of Ang 1-7 in kidney is MasR dependent [107].

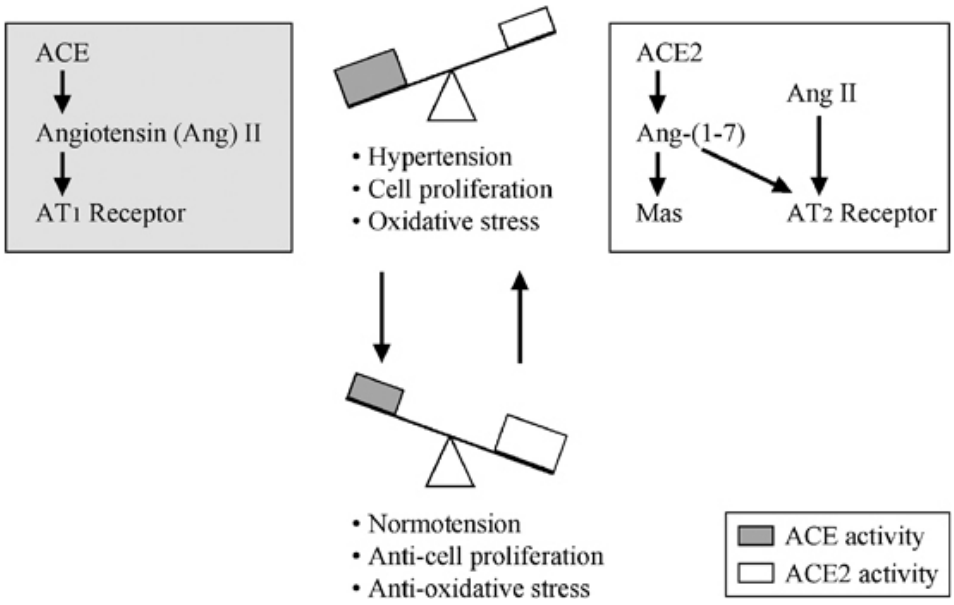


Figure 8. – The counter-balanced effect between Ang II/ ACE/ AT_1R and Ace2/Ang 1–7/MasR in iRAS system [89].

AT_1 Receptor	AT_2 Receptor	MAS Receptor
Vasoconstriction	Vasodilation	Vasodilation
Proliferative	Anti-proliferative	Antiproliferative
Profibrotic	Anti-oxidative stress	Antifibrotic
Sodium and fluid retention	Anti-apoptotic	Anti-hypertrophy
Increase intracellular calcium	eNOS phosphorylation and NO generation	Anti-inflammative
Aldosteron production	NO/cGMP activation	Improve insulin action
Increase glomerular pressure and glomerular damage	Prostaglandins and ceramides production	Improve lipid metabolism
NOX activation and ROS generation	Inhibit NF-kB activation and inflammation	Diuresis/Natriuresis
Decrease insulin sensitivity	Protection from vascular famage	
Induce apoptosis		
Activation of transcription factor (AP-1, STAT, CERB, NF-kB)		
Activation of protein kinase (PKC, PTC, MAPK, JAK, JUN)		

Table 2. – Role of angiotensin receptors in the kidneys and vasculature

1.2.5.2 Oxidative stress and DN

A large body of evidence indicates that oxidative stress is one of the major pathways that responsible for the progression of DN [112]. Oxidative stress refers to an imbalance between excessive reactive oxygen species (ROS) and the capability of effective antioxidant response. The ROS are a family of free radicals including superoxide anion (O_2^-), hydroxyl racial ($HO^{\cdot}$), hydrogen peroxide (H_2O_2), nitric oxide (NO) and other oxygen species (RO2 •, RO•, O_2, and O_3) [113]. It has been well established that the ROS in diabetes derives from several overlapped pathways, including the polyol pathway, AGE-RAGE ligand binding, PKC activation and hexosamine pathway flux [114, 115].

In polyol pathway, the aldose reductase can reduce toxic aldehydes to inactive alcohols, whereas in diabetes, excess glucose was reduced to sorbitol. The co-factor of aldose reductase, nicotinamide adenine dinucleotide phosphate (NADPH), is consumed. The NADPH provides reducing equivalents for a variety of bioreaction and allows the regeneration of glutathione (GSH, reduced state) from glutathione disulfide (oxidative state), a process that increases the intracellular oxidative stress [114].

Advanced glycation end products (AGEs) are composed of glycated proteins or lipids when exposed to glucose. In diabetes, the number of AGEs is markedly increased in both extracellular matrix and cells. Through binding to its receptor (RAGE), AGE causes a series of damage, including causing the production of inflammatory cytokines, oxidative stress and growth factors [116].

The protein kinase C (PKC) has 11 isoforms and 9 of them can be induced by diacylglycerol (DAG), a molecule formed by excess glyceraldehyde-3-phosphate in the environment of hyperglycemia. Activation of PKC pathway leads to a variety of damage effects including increase of the vasoconstriction in renal vessels, mesangial expansion, albuminuria, GFR, TGF-β1, inflammation, decrease of NO production and vascular permeability [117]. For example, an earlier study has shown that in rat glomeruli, the PKC activator can stimulate production of reactive oxygen stress via cyclic AMP pathway [118].

In diabetic condition, hyperglycemia drives excess glucose into the hexosamine metabolic pathway, which converts the fructose-6 phosphate to glucosamine-6 phosphate. Increased glucosamine-6 phosphate results in modification of many genes including Sp1, which in turn increases the expression of transforming growth factor-β (TGF-β1) and causes extracellular matrix accumulation [114].

To understand the major pathways that contribute to the DN, a review by M. Brownlee proposed that the overproduction of superoxide by the mitochondrial electron transport chain (ETC) acts in the upstream of all these mechanisms and stimulates these aberrant glucose metabolisms. From the Brownlee theory, in diabetic condition, excess glucose uptake by susceptible cells would lead to an increase of pyruvate production, which is then oxidized by the citric acid cycle in mitochondria. This process subsequently leads to accumulation of electron donors (NADH and FADH2) into the ETC and causes electron leakage and overproduction of superoxide. Furthermore, chronic hyperglycemia would impair the activity of mitochondrial ETC and cause the mitochondrial dysfunction, cellular damage and apoptosis [114]. Indeed, mitochondrial dysfunction is associated with a variety of metabolic diseases including diabetic nephropathy [119].

In Mitochondria, glucose is oxidized to CO_2 and water, yielding energy mostly in the form of ATP. As shown in Figure 9a, the mitochondrial respiratory chain complex contains four mitochondrial inner membrane-associated enzyme Complexes (I, II, III and IV), ATP synthase (also named Complexes V), the cytochrome c and the mobile electron carrier ubiquinone. The electrons transfer through Complexes I, III and IV pumping protons outwards into the intermembrane space, generating a proton gradient that drives ATP synthase. However, in diabetes, high glucose concentration provides TCA cycle with overwhelmed pyruvate, which is accompanied by marked increase in electron donors (NADH and $FADH_2$) and leads to subsequent increase of voltage gradient. Eventually, electron transfer inside complex III is inhibited and lead to increase of the lifetime of ubiquinone which reduces O_2 to O_2^-. The increased ROS is responsible for the damages of mitochondrial DNA, mitochondrial morphological change and reduction of the ATP synthesis [115, 120, 121]. Studies by Brownlee et al have shown that in endothelial cells, removal of the mitochondrial electron transport chain (ETC) completely blocked the effect of hyperglycemia on

ROS production. Moreover, removal of ETC completely abolished the hyperglycemia-induced activation of the polyol pathway, AGE formation, PKC and the hexosamine pathway. Similarly, these four pathways were also not activated when overexpression of UCP-1 or MnSOD, indicating the importance of mitochondria in generation of ROS in diabetic condition [114, 115].

Other pathways that involved in the mitochondria-derived ROS have been reported. As shown in Figure 9b, glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) participates in several pathways that contribute to the development of DN. Studies have shown that GAPDH is decreased in all kinds of diabetic animals and patients and this decrease was diminished when mitochondrial-derived ROS is supressed by either UCP-1 or MnSOD [115].

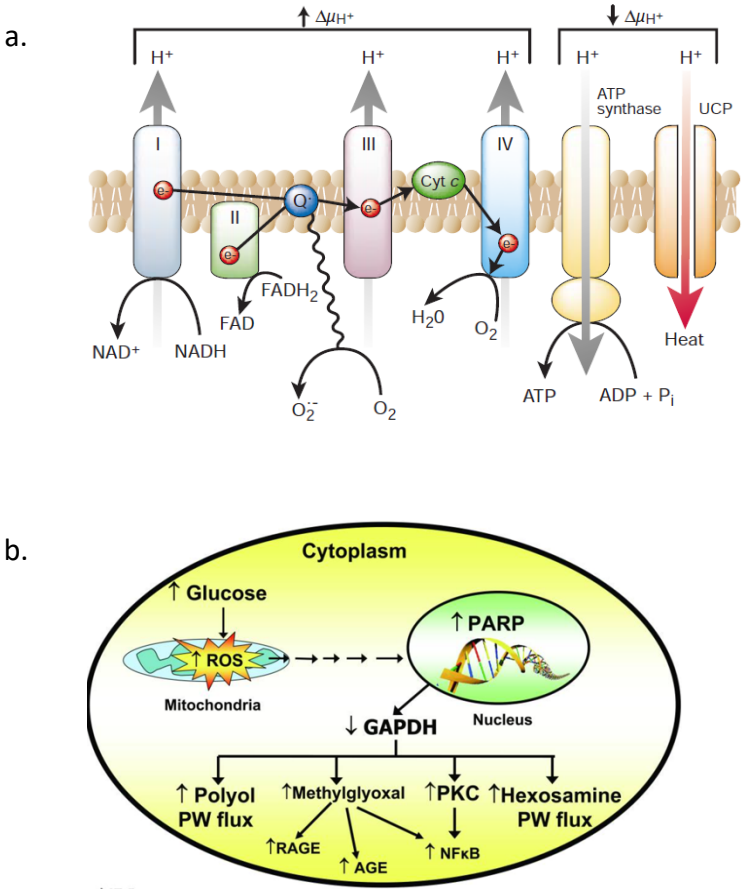


Figure 9. – a) Production of superoxide by the mitochondrial electron-transport chain. Increased hyperglycaemia-derived electron donors from the TCA cycle (NADH and FADH2) generate a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half-life of free-radical intermediates of coenzyme Q (ubiquinone), which reduce O^2 to O_2^-; Cyt c: cytochrome c; Q: the mobile electron carrier ubiquinone.

b), schemes of the unifying mechanism of hyperglycemia-induced cellular damage; GAPDH: glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase [115, 120].

In the development of DN, ROS can be also produced by other pathways, such as NADPH oxidases (Nox). The Nox are multi-subunit enzymes that transfers one electron to oxygen from NADPH and reduce O_2 to O_2^- (Figure 10).

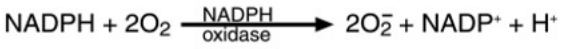


Figure 10. – Relations between O_2 and NADPH with NADPH oxidase. Molecular oxygen is reduced by NADPH oxidase to produce superoxide anion.

As shown in Table 3 [122], seven members of the Nox family have been identified, namely, Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2. These members are different from subunits, tissue expression and pathophysiological functions. Among them, Nox2 (gp91phox) was the first identified and most well-studied isoform. It contains a few core subunits and widely expresses in the immune defense system, heart and brain as well as renal tubular cells [122]. A study by You's group has shown that knockout of Nox2 did not significantly change the body weight, mesangial matrix, tubulointerstitial disease, and transforming growth factor-β, suggesting that lack of Nox2 does not protect against diabetic kidney disease in type 1 diabetes [123]. The Nox1 has similar structures with Nox2 and primarily expresses in colon epithelium, vascular smooth muscle cells, endothelial cells, heart and lung as well as renal proximal tubule cells. In early diabetic kidney induced by STZ, Nox1 knockout did not significantly change the proteinuria, inflammation or fibrosis markers, rather attenuated the glomerular volume and mesangial matrix area compared to WT-STZ, probably via reducing the phosphorylation of p38 mitogen-activated protein kinase pathway [124]. The Nox3 shares ~56% amino acid identity with Nox2 and mainly expresses in the inner ear. Low levels of Nox3 have been reported in other tissues fetal spleen, fetal kidney, skull bone, and brain. But function of Nox3 is not clear and still needs further study to determine [125].

The augment ROS causes multiple damage to the kidney including: i) affecting all the layers of the glomerular filtration barrier; ii) increase of extracellular matrix deposition; iii) increase of hypoxia; iv) inflammatory responses; v) tubulointerstitial fibrosis. Therefore, studies of antioxidants such as vitamin E, SOD, Nrf2 and catalase, have been ongoing for the therapy of DN [112, 126].

Nox isozyme	*Subunits/regulators*	*Tissue expression/ROS type*	*Main features*
Nox1	$p22^{phox}$, NOXO1, NOXA1, Rac1 (canonical), or $p22^{phox}$, $p47^{phox}$, NOXA1, Rac1 (hybrid)	Heart, lungs, blood vessels, brain. Superoxide anion.	Depending on the cell type can utilize varied cytosolic subunits (canonical or hybrid). The canonical system is constitutively active. Involved in necrosis, hypertrophy, migration, and tissue growth.
Nox2	$p22^{phox}$, $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac1, or Rac2	Innate immune cells, heart, lungs, blood vessels, brain. Superoxide anion.	Prototype Nox. Tightly regulated. Involved in immune defense, angiogenesis, neurodegenerative disorders, and necrosis.
Nox3	$p22^{phox}$, NOXO1, NOXA1 (p47/p67), Rac1	Inner ear (cochlear and vestibular sensory epithelia). Superoxide anion.	Constitutively active. Involved in balance, otoconia biosynthesis, and potentially hearing loss.
Nox4	$p22^{phox}$, Poldip2	Heart, lungs, blood vessels, kidney, prostate. Hydrogen peroxide (?).	Involved in differentiation, migration, growth, and survival.
Nox5	No subunit identified to date. Regulated by Ca^{2+}	Cardiovascular system, spleen lymph nodes, testes. Superoxide anion.	EF hand domains. Involved in inflammatory gene expression, growth, and proliferation.
Duox1	DUOXA1 (for maturation)	Thyroid gland, airway epithelium, cerebellum, testis, prostate. Hydrogen peroxide.	EF hand domains and N-terminal peroxidase-like domain. Involved in thyroid function.
Duox2	DUOXA2 (for maturation)	Thyroid gland, airway epithelium, gastrointestinal epithelia, uterus, gallbladder. Hydrogen peroxide.	EF hand domains and N-terminal peroxidase-like domain. Involved in thyroid hormone synthesis.

Table 3. – Seven members of the Nox family of proteins [122].

1.2.5.3 Autophagy and DN

Autophagy is a fundamental cellular process that removes unnecessary or dysfunctional components. It has been earlier described as a stress adaptive response for nutrient starvation to provide nutrients and maintain cellular homeostasis. Later studies have demonstrated that autophagy plays an important role in the development of several diseases including diabetes and diabetic complications [127, 128]. Autophagy is commonly described as three major types, namely macroautophagy, microautophagy and chaperone-mediated autophagy. Among them, the macroautophagy, referred to autophagy, is the most well-studied type of autophagy. It begins with an isolation membrane, namely phagophore, which is likely derived from lipid bilayer in the endoplasmic reticulum (ER) and/or the trans-Golgi and endosomes. The phagophore engulfs cellular components that need to be degraded, forming a double membrane known as autophagosome, which then travels to the lysosome for degradation [128, 129].

Dysregulated autophagy has been reported to participate in the progression of DN. For example, in STZ-induced diabetic model, autophagy was inhibited in the proximal tubule cells and insulin treatment could reverse inhibition of autophagy [130]. In high fat diet-induced obese mice and patients with type 2 diabetes or obesity, autophagy was also suppressed via hyperactivation of mTORC1 pathway [131]. Furthermore, dietary restriction (a daily food restriction by 40%) in diabetic rats attenuated progression of DN via suppression of inflammation and regulation of autophagy [132].

Overexpression of TGF-β1 promotes autophagy has been reported in a few studies. In cultured mouse mesangial cells, treatment with TGF-β1 resulted in induction of autophagy protein LC3 while suppressing caspase 3 activation [133]. In human renal proximal tubular cells, presence of TGF-β1 induced accumulation of autophagosomes and LC3 in a time and dose dependent manner via generation of ROS [134]. Furthermore, in Koester's study, overexpression of TGF-β1 in mice renal tubular cells induced nephrons degeneration and tubular cells decomposition by autophagy, which eventually caused interstitial proliferation, tubular autophagy, and fibrosis [135]. Thus, whether TGF-β1 driven autophagy has protective or deleterious effects on kidney is still unknown [136]. Although DN is usually accompanied with impaired autophagy and enhanced TGF-β1 level, whether TGF-β1 can drive autophagy in diabetic

kidney still needs further clarification. However, implied by Koester's study, it might be possible that TGF- β1 can also increase of autophagy in DN but not persistent, which subsequently leads to widely spread of kidney fibrosis.

The p62 is a key receptor of autophagy. Disrupted autophagy leads to accumulation of p62 in RPTCs in diabetic condition. Importantly, the accumulated p62 could activate Nrf2, a transcriptional factor that plays an important role in the development of DN. Through interaction with the Nrf2 suppressor (Keap 1), accumulated p62 expression in diabetes allows Nrf2 to translocate into nuclear and leads to a series of important gene expression [137, 138]. Taken together, restoration of autophagy might be a potential therapeutic target for DN.

1.2.5.4 Other pathways with DN

TGF-β1 is the central mediator of renal fibrosis. As shown in Figure 11, beyond PKC and hexosamine pathway, TGF-β1 signaling can be activated by many other pathways including ROS, Ang II and AGEs. Activated TGF-β1 can bind its receptor and cause multiple damages including: a) overexpression of fibrotic genes such as collagen I and fibronectin, resulting in mesangial matrix expansion, tubular cells atrophy, podocytes deformation and eventually renal fibrosis [139]; b) accumulation of inflammatory cells and fibroblasts and leading to inflammation; c) promoting autophagy and subsequently kidney fibrosis in both mesangial and tubular cells via overexpression of autophagy related genes [136, 140].

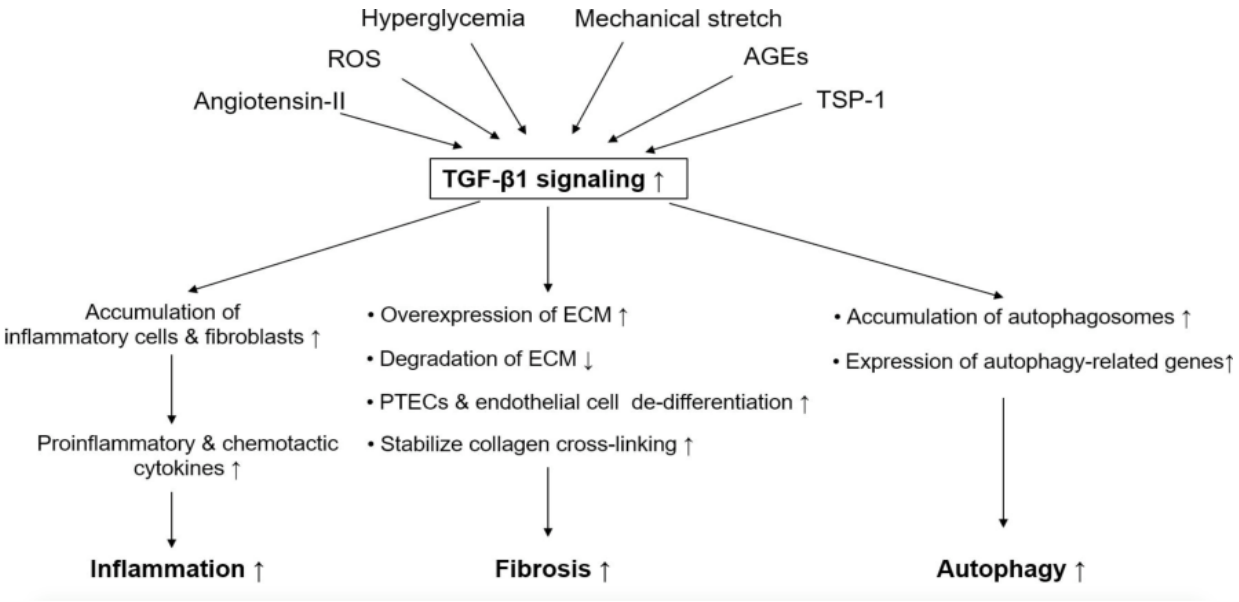


Figure 11. – Schematic diagram of pathological role of TGF-β1 signaling in diabetic kidney disease. Hyperglycemia, AngII, ROS, mechanical stretch, AGEs and thrombospondin-1 are able to active TGF-β1 signaling. TGF-β1 signaling plays an important role in mediating renal fibrosis, inflammation, and autophagy in proximal tubular epithelial cells in diabetic kidney disease. TGF-β, transforming growth factor-beta; ROS, reactive oxygen species; PTECs, proximal tubular epithelial cells; AGE, advanced glycation end products; TSP-1, thrombospondin-1; ECM, extracellular matrix [136].

In additional to the pathways that mentioned above, several other factors have also been reported to be significant in the progression of diabetic nephropathy. For example, Sirtuin-1 is a member of the Sirtuin family (Sirtuin 1-7) and contributes to cellular regulation via protein deacetylation. Sirtuin-1 is down regulated in diabetes [141] and overexpression of Sirtuin-1 in RPTCs ameliorated the glomerular injury, whereas deletion of Sirtuin-1 in RPTCs aggravated glomerular damage in diabetic mice [142]. FoxO3a is a member of FoxO subfamily of Forkhead transcription factors, a factor that is known to regulate longevity. It has been shown that FoxO3a protects cells from oxidative stress damage [143]. Catalase is a powerful enzyme and antioxidant factor that convert hydrogen peroxide into water and oxygen. Diabetes is associated with decreased level of catalase and overexpression of catalase in type 1 diabetic Akita mice significantly attenuated the hypertension and renal injury [144].

1.2.6 Treatment of DN

DN treatment varies depending on the patient's DN stage. Overall, therapeutic approaches of DN include glycaemic and blood pressure control, lipid lowering and low-carb low-fat diet as well as exercise [145]. ACE inhibitors and angiotensin II receptor blockers (ARBs) are used to treat high blood pressure. Insulin and other drugs can be used for the glycemia control. Furthermore, addition of PPAR-γ inhibitors such as rosiglitazone to metformin reduce albuminuria and blood pressure independent of glycemic control. Recently, a new class agent for DN treatment, the sodium-glucose cotransporter-2 (SGLT2) inhibitors, has yielded considerable protective effect against type 2 diabetic kidney and cardiovascular disease [64, 146]. Currently, monotherapy of SGLT2 inhibitors or combination with current DN agents such as ARBs or dipeptidyl peptidase-4 inhibitors are being tested [147].

1.3 Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2)

1.3.1. Pilot study of Nrf2

Nrf2 was firstly reported in the study of beta-globin locus control region in 1994 [148]. In this study, a hypersensitive site 2, located in the beta-globin locus control region, was addressed that confers high levels of expression in the beta-globin gene cluster. Within the hypersensitive site 2, a tandem repeat of the consensus sequence for the transcription factors, activating protein 1 (AP1) and nuclear factor erythroid 2 (NF-E2), are absolutely required for strong enhancer activity. Using this tandem repeat as a recognition site probe to screen a lambda gt11 cDNA expression library from K562 cell line, several DNA binding proteins were isolated. Among these clones, two of them had remarkable similarities with NF-E2. One was named NF-E2-related factor 1 (Nrf1), and the other was referred to NF-E2 related factor 2 (Nrf2) [149].

1.3.1.2 Nrf2 and ARE

Human antioxidant response element (hARE) is a unique cis-element that contains AP1 elements and a 'GC' box. HARE has been reported to be bound by multiple transcription factors including Jun, Fos and Fra [150]. This sequence is present in the promoter of human NAD(P)H:quinone oxidoreductase$_1$ (NQO_1), an enzyme that is responsible for detoxifying quinones and protecting cells against redox cycling and oxidative stress. Similar cis-element has also been identified in the promoters of rat NQO1, mouse and rat glutathione *S*-transferase (GST) [151].

The binding sequences of Nrf1 and Nrf2, interestingly, are very similar to the hARE. Nrf1 and Nrf2 were shortly proved to be stronger regulator of NQO1 by binding to the hARE sequence. As expected, Nrf2 also induces GST via binding with hARE sequence in the GST promoter [152]. Further study has showed that in Nrf2 deficient mice, several defense proteins responsible for electrophilic agents and oxidative stresses, including oxygenase-1 (HO-1), GSH level and stress protein A170, were profoundly impaired, indicating the importance of Nrf2 against oxidative stress [153].

1.3.2 The Nrf2/Keap 1 system

1.3.2.1 The molecular structure of Nrf2/Keap 1 system

Numerous studies have been performed to understand the role of Nrf2 against oxidative stress [154]. Itoh, K. et al. showed that the human and chicken Nrf2 peptide sequences are highly conserved and six highly conserved homology regions in Nrf2 were named Neh1 to Neh6 (Nrf2-ECH homology; ECH: erythroid cell-derived protein with CNC homology) [154, 155]. Importantly, domain Neh2 was found to negatively regulate Nrf2 activity by a cysteine-rich protein, namely Kelch-like ECH-associated protein1 (Keap 1) [154]. Hence, regulation of Nrf2 by Keap 1 under normal and oxidative stress condition was revealed.

As shown in Figure 12, the Neh1 contains the CNC (cap'n'collar) homology region and basic-leucine zipper (bZIP) domain, which are responsible for hetero-dimerization with small Maf, a protein that contributes to bZIP transcription factor binding. The Neh2 located in N-terminal, containing DLG and ETGE motifs which can specifically bind to Keap 1. The Neh6 is targeted for degradation of Nrf2 by the E3 ubiquitin ligase complex, whereas the Neh3–5 domains has been proved to function in transactivation by binding to various components of the transcriptional apparatus [156]. Wang, H. et al. group identified that retinoic X receptor alpha (RXRα), one member of nuclear receptor family, is an unrecognized repressor of Nrf2 and designated it as Neh 7 [157].

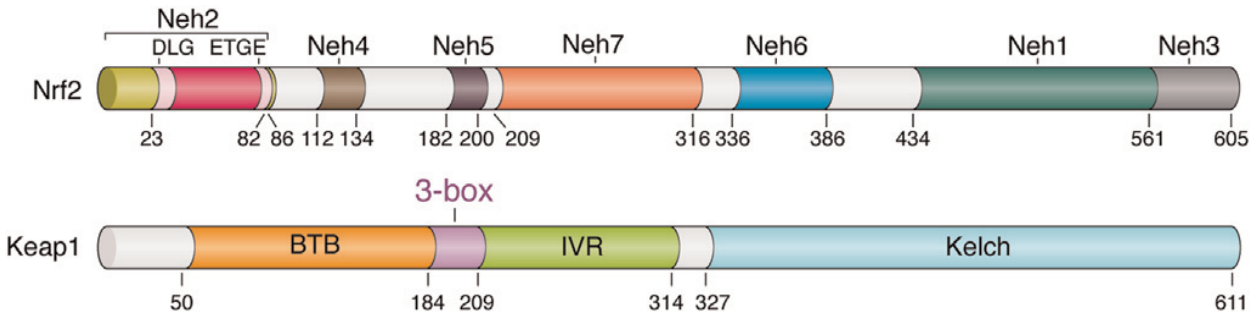


Figure 12. – Schematic representation of the regions of Nrf2 protein [156]

Keap 1 is a highly redox-sensitive member of the BTB-Kelch family for a Cul3-dependent ubiquitin ligase complex. The Kelch domain in the C-terminal of Keap 1 binds to the DLG and ETGE motifs in the Neh2 of Nrf2, followed by Cullin3-based E3-ligase complex induced degradation. Further studies have shown that cysteine residues Cys151, Cys273 and Cys288 in BTB domain of Keap 1 are responsible for stress sensing of Keap 1 and Nrf2 activation [158, 159].

Keap 1/Nrf2 system has been considered the major cytoprotective responses to oxidative and electrophilic stress. As shown in Figure 13, Nrf2 is kept in the cytoplasm by Keap 1 and degraded by E3-mediated ubiquitin-proteasome system in normal condition. However, in oxidative stress condition such as diabetes, cysteine residues of Keap 1 undergoes covalent modifications and results in releasing of Nrf2 and its translocation into the nucleus, where Nrf2 initiates transcription of downstream genes expression via binding to the ARE [156].

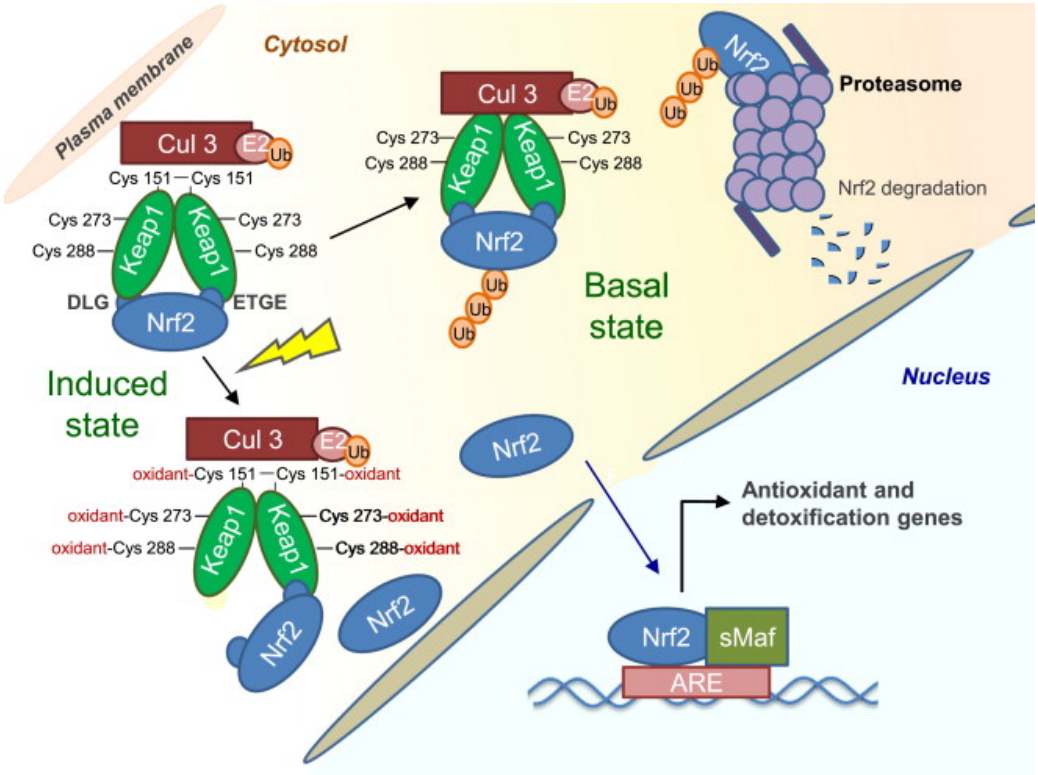


Figure 13. – Nrf2 signaling pathway. Cysteine residues critical for Keap 1 dimerization (C151) and redox sensing (C273, C288, C297) are indicated [160].

1.3.3. Activators of Nrf2/Keap 1 system

As Nrf2 is normally kept in the cytoplasm by Keap 1 for degradation, Keap 1 is the primary target for Nrf2 activation. Many chemicals which are electrophilic or reactive to thiol groups can induce covalent modifications of Keap 1 and result in Nrf2 activation.

One of the most well-known Nrf2 activators is Bardoxolone Methyl (BM), a compound that belongs to the synthetic oleanane triterpenoids class [161]. These compounds can react with nucleophiles containing –SH groups including cysteine and induce covalent change of Keap I. Treatment with BM significantly elevated Nrf2 and its downstream protein expression, whereas this effect is absent in Nrf2 deficient cells [162].

Oltipraz is a synthetic organosulfur compound that belongs to the dithiolethione family. It stimulates a battery of phase II detoxification enzymes and antioxidant genes including NQO1 and GSTs in vitro and in vivo [163, 164]. It has been demonstrated that the oltipraz can react with thiols and modify the cysteine residues inside the Keap 1 (Figure 14). One oltipraz analogue, 3H-1,2-dithiole-3-thione, induces intermolecular disulfide bonds at Cys273 and Cys288 between two Keap 1 molecules and results in Nrf2 activation [165]. Furthermore, oltipraz can generate radical superoxide and further leads to activation of Nrf2 [166]. Our previous data have shown that oltipraz can significantly stimulate Nrf2 expression both in mice and cell lines [144].

Several other bioactive nutrients can activate Nrf2. For example, tBHQ, a compound oxidized from butylated hydroxyanisole (BHA), is commonly used as an antioxidant food preservative. A study has shown that Cys151 of Keap 1 is a specific sensor for tBHQ. Mutants of C151 in Keap 1 diminished Nrf2 induction in cell culture and zebrafish. Sulforaphane, an isothiocyanate that is rich in broccoli, also activates Nrf2 by direct modifications of Keap 1 cysteines at Cys151 [167]. As shown in Figure 13 and 14, Cystein (Cys151) in the BTB domain of Keap 1 is the most critical and redox-sensitive cysteine residue. When exposed to oxidative stress, two molecules of Keap 1 form an intermolecular disulfide ‘Cys151–Cys151’, which allows Nrf2 to evade from Keap 1-mediated ubiquitination [160]. Cys273 and Cys288 have also been considered crucial for the Keap 1 and Nrf2 interaction. Mutation of either Cys273 or Cys288 to Ala inhibits Keap 1’s ability to direct constitutive ubiquitination of Nrf2. But mutation of these two cysteine

to Ser does not affect the stability of Keap 1 to Nrf2. Hence, the underlying mechanisms by which C273 and C288 mutations impact Keap 1 function are not fully understood [168].

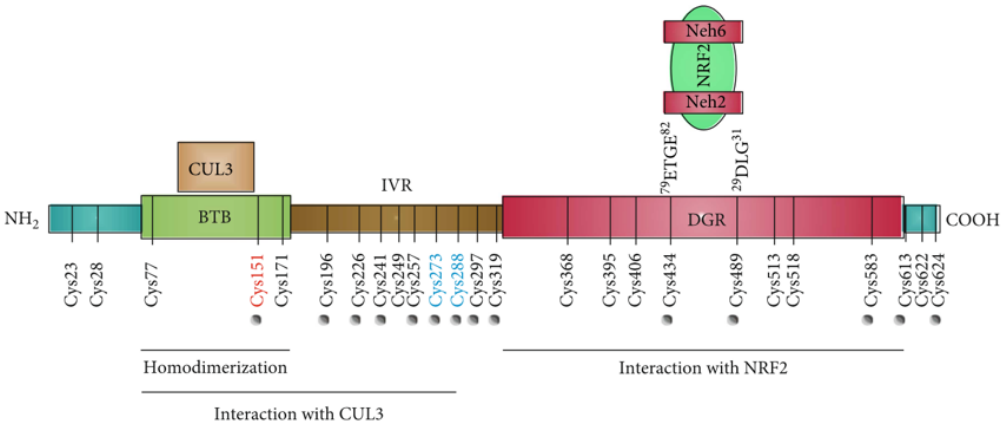


Figure 14. – Structure of the Keap 1 with the position of cysteine residues. The N-terminal BTB domain participates in homodimerization and binding to CUL3/RBX1. The C-terminal region, DGR (double glycine repeat) domain, contains a double glycine repeat called Kelch repeat that binds NRF2-Neh2 domain. The intervening region (IVR) contains redox-sensitive cysteine residues. Red and blue cysteine residues in Keap 1 are the most relevant for electrophile reactivity [169].

1.3.4 Role of Keap 1/Nrf2 in oxidative stress and toxicity

In normal condition, oxidants are constantly formed in responding to several physiological actions such as cell division, inflammation, autophagy, and stress response in a controlled manner. However, when exposed to stress, toxicities or disease, uncontrolled production of oxidants result in oxidative stress and impair multiple cellular functions [170]. Hence, a variety of antioxidants has been considered crucial in the therapy of several diseases. Among these antioxidants, the role of Nrf2 as an antioxidant factor has been widely supported and well established.

Targeted genes of Nrf2 have been summarised in three major groups by Q. Ma *et al.* [155]. First, Nrf2 induces drug metabolism and disposition genes such as cytochrome p450, NQO-1, aldehyde dehydrogenase and GST [171]. Second, Nrf2 induces catabolism of superoxide and peroxides through SOD, peroxiredoxin, glutathione antioxidant protein thioredoxin (Trx) [172, 173], synthesis of reducing factors glutamate-cysteine ligase (GCLC)and heme-oxygenase (HO-

1)[174]. Third, Nrf2 also mediates oxidant signaling such as p62 (related to autophagy) and Parkinson disease 7 (related to mitochondrial apoptosis) [155, 175].

1.3.5 Role of Nrf2 in diabetes and diabetic complications

1.3.5.1 Nrf2 expresses in different organs

From the study of Moi *et al.* group, Nrf2 is ubiquitously expressed in different tissues at different levels. As shown in Figure 15, Nrf2 highly expresses in muscle, kidney and lung in the adults [148]. Hence, Nrf2 has been proposed as a multi-organ protector due to its central role in protecting cells from ROS and electrophiles [176]. Moreover, studies support that Nrf2 participates in the regulation of several diseases including diabetes and diabetic complications [23].

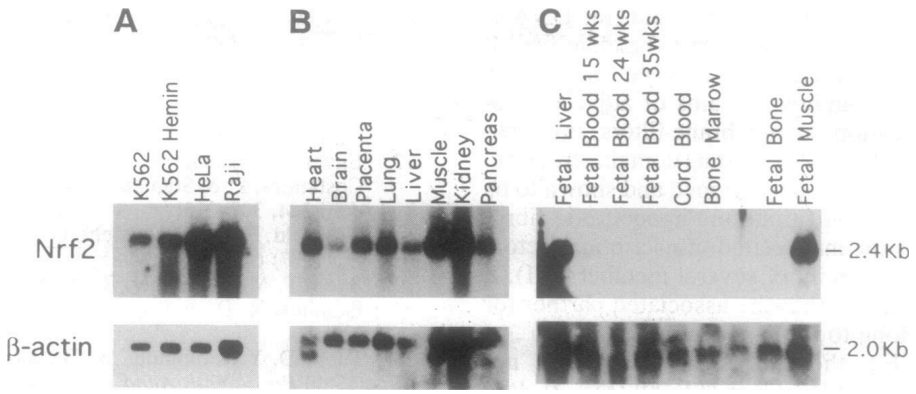


Figure 15. – Nrf2 mRNA expression by northern blot analysis in human cell lines and different organs [148].

1.3.5.2 Role of Nrf2 in diabetes

Oxidative damage has long been considered as one of major reasons in the progression of diabetes. Several studies have shown that activation of Nrf2 ameliorated diabetic damage in the pancreas, heart, kidney and skin [177, 178]. One of the significant studies by Uruno, et al., showed that genetic activation of Nrf2 by Keap 1 knockdown (Keap $1^{flox/-}$) markedly prevented onset of diabetes (Figure. 15) [179]. In this study, activation of Nrf2 significantly ameliorated blood glucose level and oral glucose tolerance test (OGTT) while improved insulin secretion and insulin

sensitivity in type 2 diabetic db/db mice (Figure.15). Oral administration of Nrf2 activator CDDO-Im (BM) attenuated diabetes in db/db mice. In gastrocnemius and soleus muscle (SkM), overexpression of Nrf2 ameliorated HFD-induced impairment of locomotor activity and protected mice from diet-induced obesity. Enhanced Nrf2 in brown adipose tissue increased the oxygen consumption, whereas overexpression of Nrf2 in liver suppressed gluconeogenesis via transcriptional repression of a variety of enzymes, including gluconeogenic enzyme glucose-6-phosphatase (G-6-P). Other studies have reported that activation of Nrf2 preserved *β*-cell mass, function and apoptosis. Furthermore, Nrf2 has also been shown to protect insulin sensitivity via reducing hypothalamic oxidative damage [180]. In addition, activation of Nrf2 is required in human β-cell proliferation in diabetic condition [181].

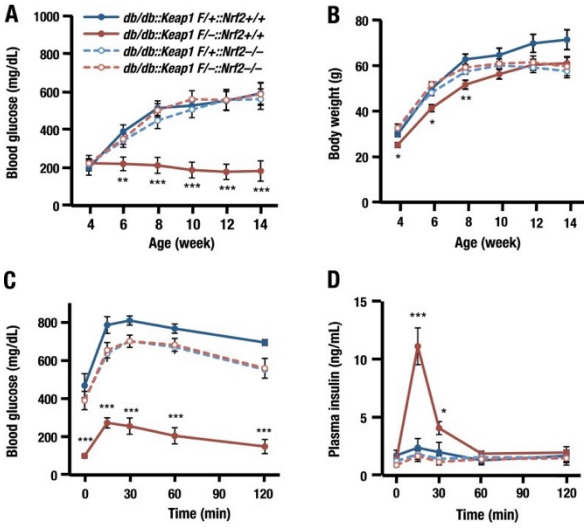


Figure 16. – Overexpression of Nrf2 prevents diabetes in *db/db* mice. (A) Blood glucose levels, (B) body weight (C) Oral glucose tolerance test (OGTT), (D) plasma insulin [179]

However, studies also yielded paradoxical but inspiring data. For example, despite improved glucose metabolism with Nrf2 induction in different organs such as pancreas, overall mutation of Nrf2 significantly improved glucose metabolism in both db/db and HFD-induced diabetic mice [182, 183]. Insulin sensitivity, insulin secretion and insulin release-related genes were all improved in Nrf2 knockout mice. Moreover, activation of Nrf2 by Keap 1 knockdown increased the markers of metabolic syndromes such as diet-induced obesity, hepatic steatosis,

and glucose intolerance in HFD induced diabetes [184]. Clearly, the role of Nrf2 in diabetes remains controversial and needs further studies to determine.

1.3.5.3 Role of Nrf2 in diabetic complications

The role of Nrf2 in diabetic complications has been investigated. For example, in mice with HFD-induced obesity, Nrf2 knockout developed a less insulin-resistant phenotype via participating in the regulation of FGF21 in liver and white adipose tissue [183]. Xue *et al*. group reported that incubation with Sulforaphane (an Nrf2 activator) with human microvascular endothelial cells prevented hyperglycemia-induced activation of the hexosamine and PKC pathways [185]. Multiple evidence have shown that in addition to adipose tissue and microvascular endothelial cells, activation of Nrf2 is beneficial for a variety of diabetic complications, including diabetic nephropathy, heart failure, cardiovascular disease and wound healing [178].

1.3.6 Role of Nrf2 in DN

As mentioned in 1.1.4, DN is one of the main complications of diabetes mellitus and oxidative stress contributes to the development of DN, Nrf2, an antioxidant factor, has been widely studied [137, 186]. In general, these studies can be classified by either Nrf2 deficiency or activation.

1.3.6.1 Deficiency of Nrf2 in DN

Nrf2 knockout mice has been successfully generated by Chan *et al*. in which part of exon 4 and all of exon 5 (the function regions of Nrf2) were replaced with bacterial gene LacZ [187]. An earlier study by Yoh, K. group have reported that in STZ-induced diabetes [188], WT-STZ mice developed high serum glucose, creatinine clearance, urinary protein and urinary nitrite/nitrate (NOx) while there was a trend of lower serum glucose in STZ-Nrf2KO mice (Figure 17A). Furthermore, Nrf2KO-STZ mice did not develop renal hyper-filtration and early renal injury (at 2-6 weeks, Figure 17B) but rather had significantly lower urinary protein level compared to WT-STZ mice (Figure 17C). However, Nrf2KO-STZ mice developed significant NO overproduction and oxygen radical formation compared to STZ-WT mice (Figure 16D).

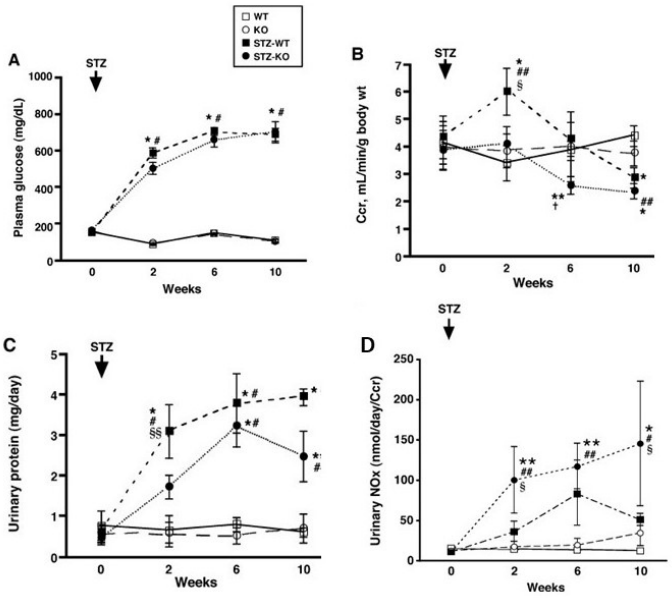


Figure 17. – Serum glucose (A), creatine clearance (B), urinary protein level (C), and urinary NOx production in WT, Nrf2KO, STZ-WT and STZ-KO mice [188].

Another study by Jiang, T. group has reported that Nrf2KO-STZ mice had higher ROS production, suffered from greater oxidative DNA damage and renal injury compared to WT-STZ mice. The underlying mechanisms were at least partially through inhibition of TGF-β1 and reduction of extracellular matrix production. This study also reported a higher ROS level, elevated Nrf2 expression and its downstream genes in human renal mesangial cells with diabetes [189].

Of note, one comprehensive microarray identified 2561 transcripts and 240 proteins in Nrf2 knockout mice that differed from WT counterparts [190]. In addition to genes related to detoxification and redox balance, Nrf2 also regulates a variety of genes that are involved in multiple physiological functions. For example, the transporter SNAT3 (slc38a3), a sodium-coupled neutral amino acid transporter, is responsible for the glutamine transportation and plays a significant role in the kidneys during metabolic acidosis [191]. The level of SNAT3 expression was dramatically decreased in Nrf2 knockout mice [192]. Our previous work has found that overexpression of catalase in the RPTCs significantly normalized systolic BP, attenuated renal injury, and inhibited RPTC Nrf2 and Agt expression. In both iRPTC (immortalized RPTCs) and mice,

overexpression of Nrf2 further stimulated Agt expression via binding to the Agt promoter. This study highlighted a novel link between Nrf2 and Agt, indicating that Nrf2 might participate in the regulation of iRAS [144]. Our further study has shown that in Akita mice, a type 1 diabetic model, knockout of Nrf2 significantly attenuated systolic BP, kidney hypertrophy and renal injury via lowering the expression of Agt, Ace but elevating the Ace2 and Ang 1-7 level [193]. Moreover, Nrf2/Agt pathway has been demonstrated to be affected by the insulin action in RPTCs. In this study, renal Nrf2 and Agt expression was down regulated in hyperinsulinemic-euglycemic mice, whereas heterogeneous nuclear ribonucleoprotein F and K expression was upregulated. This study documented that beneficial actions of insulin in DN appear to be mediated partially by suppressing Nrf2/Agt pathway and stimulating hnRNP F/K [194].

1.3.6.2 The genetic activation of Nrf2 in DN

As an antioxidant factor, activation of Nrf2 has been studied both genetically and pharmacologically. For instance, fully activation of Nrf2 by Keap 1-null mice died post-natally, probably from malnutrition resulting from hyperkeratosis in the esophagus and forestomach. Double knockout of Keap 1 and Nrf2 rescued this lethality [195]. Deletion of oesophageal Nrf2 in Keap 1-null mice allowed survival until adulthood, but these animals developed polyuria with low osmolality and bilateral hydronephrosis [196]. Furthermore, knockout of Keap 1 in renal tubular epithelial cells resulted in specific overexpression of Nrf2 and marked increase of renal pelvic expansion with hydronephrosis in both males and females at 3 months. Till 6 months, mice showed progressive hydronephrosis with significantly higher red blood cell count and hemoglobin, hematocrit, mean cell volume and mean cell hemoglobin concentration [197]. These data suggested a complicate role of Nrf2 in the kidneys, rather than an antioxidant factor.

1.3.6.3 The pharmacological activation of Nrf2 in DN

A number of Nrf2 activators such as sulforaphane and oltipraz have been applied to DN study [198]. In both type 1 and type 2 diabetic mice, sulforaphane can suppress renal inflammation, oxidative stress, and dysfunction via activation of Nrf2 [199, 200]. Dietary supplementation with sulforaphane down-regulated TGF-β1 expression and subsequent fibrosis in WT-STZ mice [201]. Our group has reported that oltipraz stimulated Nrf2 expression in

immortalized RPTCs and mouse kidney, which further led to enhanced expression of renal Agt level and caused kidney damage [144].

It needs to mention that as Nrf2 activators, Sulforaphane and Oltipraz appear to have opposite effects. This is probably due to their different structures (Figure 18). As introduced in 1.3.3, Oltipraz is a synthetic compound that belongs to the dithiolethione family. It can stimulate Nrf2 and its downstream genes including NQO1 and GSTs via inducing intermolecular disulfide bonds at Cys273 and Cys288 of two Keap 1 molecules. Sulforaphane is a natural compound that contains the isothiocyanate group. One study has shown that mutation of C151 to a serine in Keap 1 was able to repress Nrf2 on sulforaphane treatment, indicating that C151 might be one of the cysteines that responsive to sulforaphane [167].

a). Structure of Oltipraz b). Structure of Sulforaphane

Figure 18. – Structures of Oltipraz (a) and Sulforaphane (b).

One of the most well-studied Nrf2 activators is bardoxolone methyl (BM, CDDO-Me), a semi-synthetic triterpenoid. The BM was initially tested for cancer treatment in which yielded a raise of estimated GRF (eGFR) and Nrf2 downstream genes [202]. Although BM was reported toxic for rodents, a few analogues of BM have been studied on kidney diseases [203]. For example, in STZ-induced diabetic apolipoprotein $E^{-/-}$ mice, treatment with low dose of dh404 (3 and 10 mg/kg, an analog of BM), significantly attenuated urinary ACR, glomerular injury and improved renal tubular injury with upregulation of Nrf2-responsive genes; however, higher doses of dh404 (20 mg/kg) were found to be associated with increased expression of proinflammatory mediators MCP-1 and NF-κB. Further analysis found that oxidative stress such as 8-OhdG and urinary 8-isoprostane were significantly attenuated in all concentrations and particularly at the highest dose of 20 mg/kg of BD, indicating that a lack of protection by the highest dose of dh404 was no

correlation to oxidative stress [204]. Furthermore, administration of RTA dh404 (a synthetic triterpenoid) restored mean arterial pressure, decreased glomerulosclerosis and interstitial fibrosis via increasing Nrf2 expression and its target genes in 5/6 nephrectomy induced CKD rats [205]. Another study has shown that treatment of analogs of BM (RTA 405) in CKD rats worsened proteinuria, glomerulosclerosis, tubular damage followed by liver injury. It also caused severe changes in food intake and diuresis, accompanied by body weight loss and blood pressure elevation. These studies indicated that use of BM analogs in diabetic nephropathy are controversial and still needs further study to investigate [206].

1.3.7 Clinical trial of Nrf2

Despite controversial data of BM on kidney diseases, BM was further tested in clinical trials. A primary phase 2a clinical trial has found that 8-week treatment with BM on type 2 diabetes patients with moderate to severe CKD (baseline serum creatinine level ranged from 1.3 to 3.0 mg/dl), increased eGFR and creatinine clearance [207]. This clinical trial was followed by the BEAM, another clinical trial with a larger sample size. 227 patients with diabetes mellitus and CKD were administrated with either placebo or BM for 52 weeks. Data have shown that BM persistently ameliorated eGFR reduction during the treatment period without server adverse events [208].

However, a comprehensive clinical trial with BM (phase 3), involving 2185 patients with type 2 diabetes mellitus and stage 4 CKD was early terminated due to considerable adverse events of high mortality rates[209]. Compared to placebo group, patients in BM group had increased ACR and blood pressure, more cases of heart failure and death. Multiple reasons have been suggested to be responsible for this failed trial, for example, inappropriate experimental arrangements, largely depending on animal and cell data, ignoring negative reports of Nrf2 and the side effects of BM. More detailed reasons were also discussed, such as the augmentation of sodium retention and dysregulation of iRAS with BM treatment. To date, BM is currently being tested in several clinical trials (Table 2) [210, 211]. Thus, it is urgent and crucial to understand the role of Nrf2 in DN.

Clinical trial	Condition	Number of cases	Dosage, mg/day	Follow-up	eGFR increase, mL/min/1.73 m^2
Phase 1 2006–2008	Advanced solid tumor or lymphoid malignancy (Ccr ≥60 or Cr <2.0)# (eGFR <60)#	 36 10	 5–1,300† 5–1,300†	 21 days 21 days	 26.4% 35.6%
Phase 2 2008–2009	CKD with T2DM (1.3≤ Cr ≤3.0 for women, 1.5≤ Cr ≤3.0 for men)#	18	25–75†	8 weeks	7.2±5.3§
Phase 2 (BEAM) 2009–2010	CKD with T2DM (20≤ eGFR ≤45)#	57 57 56	25† 25–75† 25–150†	52 weeks 52 weeks 52 weeks	5.8±1.8◊, ¶ 10.5±1.8◊, ¶ 9.3±1.9◊, ¶
Phase 3 (BEACON) 2011–2012	CKD with T2DM (15≤ eGFR <30)#	1,088	20‡	9 months	5.5±0.2◊
Phase 2 (TSUBAKI) 2015–2017	CKD with T2DM (CKD stages 3–4)	54*	Dose titration‡	16 weeks	In progress
Phase 2/3 (CARDINAL) 2017–2020	Alport syndrome (30≤ eGFR ≤90)#	120*	5–30‡	12 or 48 weeks	In progress

Table 4. – Summary of clinical trials of CDDO-methyl ester in kidney diseases [211].

1.3.8 Nrf2 and Cancer

Besides the role of Nrf2 in oxidative stress and diabetes, Nrf2 has been also found that highly expresses in different cancers. However, the role of Nrf2 in cancer is still contradictory.

For the good side of Nrf2, it has been shown that Nrf2 protects against chemical carcinogen-induced tumor formation in the stomach, bladder, and skin [212]. For instance, when exposed to different chemicals, mice without Nrf2 were more susceptible to develop gastric neoplasia [213], bladder and skin tumors [214, 215], mainly due to Nrf2's role in reducing ROS and DNA damage. Treatment of sulforaphane, an Nrf2 activator, promoted cancer cell apoptosis via p53 mechanisms while induced phase II detoxification enzymes [216]. These studies suggested that Nrf2 plays an essential role in tumorigenesis inhibition and may be a potential therapeutic target.

However, the 'dark side' of Nrf2 in cancer has been also reported. It was suggested that constitutive expression of Nrf2 in lung, breast, head and neck, ovarian, and endometrial carcinomas partially promoted cancer cells proliferation and chemoresistance. Specially, activation of Nrf2 responded to drug resistance, resulting in both intrinsic and acquired chemoresistance [217]. The cancer cells respond to three chemotherapeutic drugs, namely cisplatin, doxorubicin, and etoposide, whereas increase of Nrf2 by tBHQ (an Nrf2 activator)

caused resistance of cancer cells to these drugs [218]. Hence, a number of Nrf2 inhibitors were currently suggested as potential targets for cancer treatment [217].

Taken together, the role of Nrf2 activation in cancer is paradoxical and clearly requires further studies to determinate.

1.4 Sodium-glucose co-transporter-2 (SGLT2)

1.4.1 Understanding the kidneys' role in blood glucose regulation

The kidney has often been overlooked as a significant player in glucose metabolism until the development of new types of glucose-lowering drugs, namely sodium–glucose co-transporter 2 inhibitors (SGLT2i). The kidneys participate in glucose control via three pathways [219]. First, it releases glucose into the circulation via gluconeogenesis. Second, it takes up the glucose from circulation to meet its energy needs, namely, glucose utilizations. Last, the kidneys reabsorb most of the glucose filtered by the glomeruli in renal tubules [220].

1.4.1.1 Gluconeogenesis and glucose utilizations in the kidneys

Gluconeogenesis is the formation of glucose-6-phosphate from a variety of precursors including lactate, glycerol, and amino acids, and its subsequent hydrolysis by glucose-6-phosphatase for generation of glucose [221]. The kidneys have been considered two different parts. Cells in the renal medulla can only use glucose for their needs. They are able to phosphorylate the glucose and accumulate glycogen but unable to release glucose into the bloodstream due to lack of the glucose-6-phosphatase. Cells in the renal cortex, however, contain gluconeogenic enzymes which allow cells to produce and release glucose into the blood, though these cells can barely synthesize glycogen due to very limited phosphorylating capacity [219].

In post absorptive period, human kidneys and liver provide approximately the same amounts of glucose through gluconeogenesis [221]. In fasting period, up to 20–25% glucose is derived from the kidneys while the remaining 75–80% is from liver [222]. When fasting continues, along with the depletion of glycogen stores, gluconeogenesis produces all the glucose for energy

needs with a subsequent increase of renal gluconeogenesis [221]. Specially, it has been reported that in both type 1 and type 2 diabetes, renal gluconeogenesis was enhanced about the same extent as hepatic glucose release [223, 224]. These findings established the importance of renal gluconeogenesis in glucose metabolism.

1.4.1.2 Glucose reabsorption

On a daily basis, the kidneys produce 15–55 g of glucose by gluconeogenesis, metabolize 25–35 g of glucose but filter up to 180 grams of glucose via glomerulus and reabsorb them in RPTCs [220].

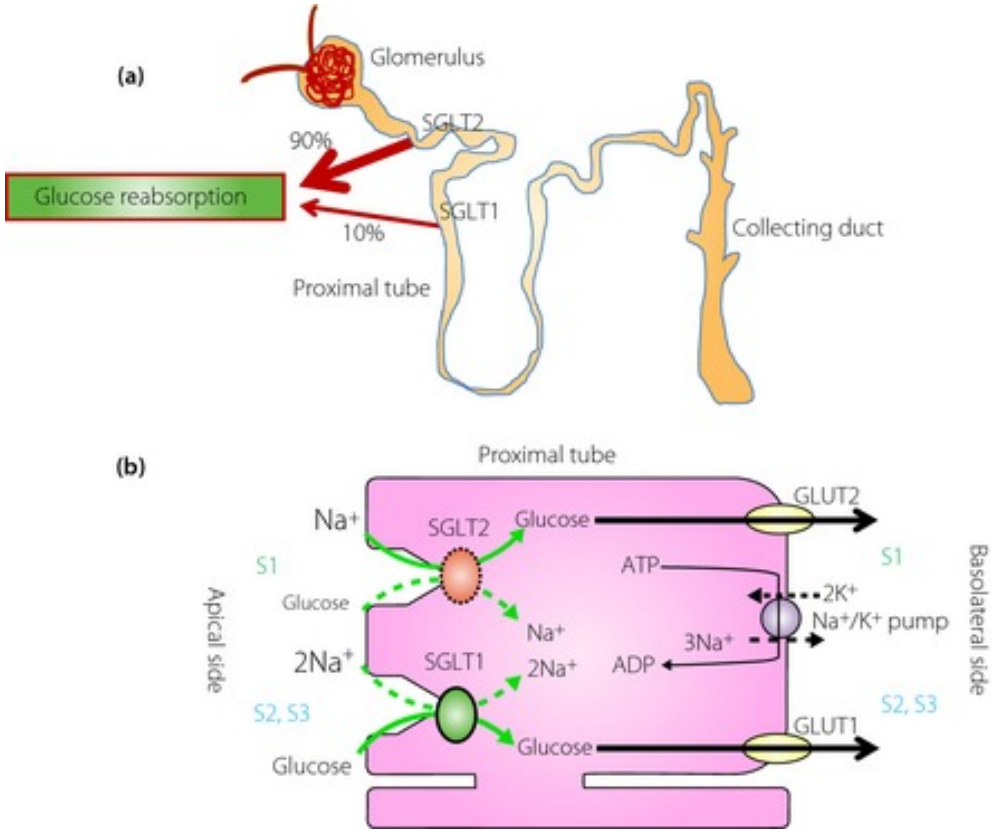


Figure 19. – Renal glucose handling. (a) Glucose reabsorption. (b) Glucose reabsorption via SGLT2 and SGLT1 in the proximal renal tubular cell [225].

As shown in Figure 19, glucose in plasma is freely filtered by the glomerulus and almost all of the filtered glucose is reabsorbed by RPTCs via glucose transporters SGLT2 and SGLT1. In normal condition, the amount of glucose reabsorption increases linearly with amount of filtered glucose by the glomerulus, till it reaches maximal absorptive capacity, namely the renal threshold (Tm) (Figure 20). In human, the Tm equates a filtration rate at 250–350 mg/min per 1.73 m^2 [21], which occurs at plasma glucose concentrations of 11.0 mmol/l in healthy adults. Above this plasma glucose concentration, the percentage of glucose reabsorption decreases and excessive glucose is excreted into urine, namely, glucosuria [221].

Of note, it has been well recognized that glucosuria in diabetic condition does not occur at plasma glucose levels that would normally produce glucosuria in non-diabetic condition. This is the result of enhanced expression of SGLT2 transporters and augmented glucose reabsorption capability from glomerular filtrate in people with diabetes [221, 226].

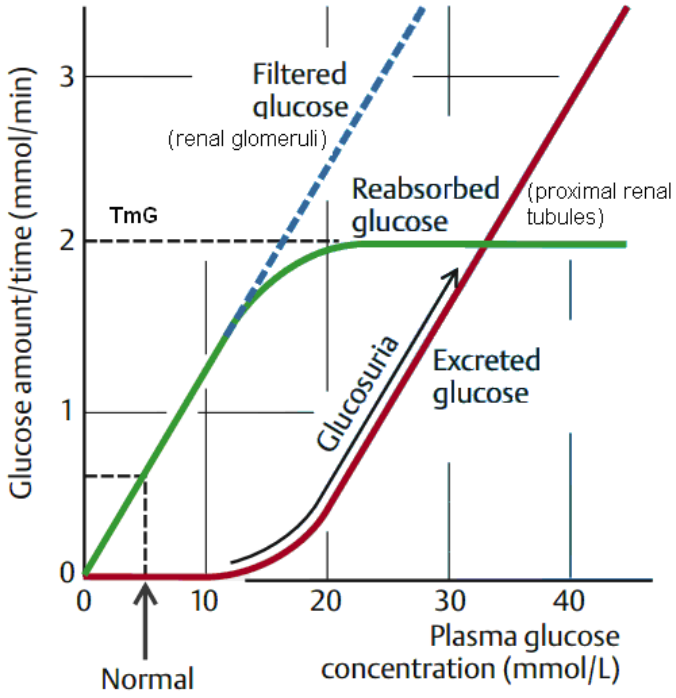


Figure 20. – Threshold of renal reabsorption of glucose [219]

1.4.2 Glucose transporters

During the process of reabsorption, glucose is first transported from proximal tubule lumen to proximal tubule cells by crossing the apical brush border and then the basolateral side (Figure 19). Two transporter families, the sodium-glucose co-transporters (SGLTs) in the apical brush border and the facilitative glucose transporters (GLUTs) family in the basolateral side, participate in this reabsorption.

1.4.2.1 SGLTs

The SGLTs family is composed by six members. Among them, SGLT2 and SGLT1 are the best-characterized members, while SGLT3-6 have not yet been fully understood. As shown in Figure 19, SGLT2 lies in S1 and S2 segments of RPTCs, which is a low-affinity but high-capacity transporter with a coupling ratio of Na^+ at 1:1. SGLT2 has been established as one of the main glucose transporters as it is responsible for about 90% of glucose reabsorption. The remaining ~10% of glucose reabsorption is transported by SGLT1, another transporter that mainly locates in the S3 of RPTCs. In contrast, SGLT1 is a high-affinity and low-capacity transporter with a coupling ratio of Na^+ at 2:1 [225]. Although some studies have suggested that only ~3% of glucose is reabsorbed by SGLT1 under normo-glycemic conditions, capacity of glucose reabsorption of SGLT1 can be dramatically enhanced as a compensatory effect when SGLT2 was inhibited.

The significance of SGLT2 and SGLT1 in glucose reabsorption has been well established. For instance, glucose reabsorption was absent during pharmacologic SGLT2 inhibition in SGLT1 KO mice and in SGLT1/SGLT2 double knockout mice [227-229]. In diabetic condition, knockout of SGLT2 significantly attenuated hyperglycemia and glomerular hyperfiltration [230]. In human, individuals with SGLT1 mutations exhibited malabsorption, severe osmotic diarrhea and dehydration, while people who lack SGLT2 genetically had familial renal glucosuria [231, 232]. Furthermore, a number of clinical trials have proved SGLT2i as one of the most novel and promising approach in the treatment of diabetes [233].

1.4.2.2 GLUTs

The GLUT family is a group of membrane proteins that facilitates glucose transportation across the plasma membrane [234]. The most well-studied GLUTs are GLUT1–5 [235]. Among them, the GLUT3 is mainly responsible for glucose uptake in the brain, whereas the GLUT4 functions dominantly in muscle and fat. The GLUT2, together with the GLUT1, is considered important in the glucose handling of kidney. GLUT2 is low-affinity transporter that expresses in different cell types including hepatocytes, intestines and pancreatic β-cells as well as early RPTCs. In contrast, GLUT1 is a high-affinity transporter that mainly expresses in erythrocytes and late RPTCs [234]. Studies have shown that loss of GLUT2 resulted in massive glucosuria in both mice and humans [236]. In STZ-induced diabetic rats, GLUT2 but not GLUT1 was increased to adapt for hyperglycemia condition [237]. However, detailed mechanisms of GLUT1 and GLUT2 in glucose transportation still need further studies to clarify [238].

1.4.3 The regulation of SGLT2

Although SGLT2 plays a key role in glucose reabsorption, the molecular regulation of SGLT2 remains poorly understood. A few pathways including sodium level, hyperglycemia or insulin have been proposed that involve in the modulation of SGLT2.

1.4.3.1 Hyperglycemia and insulin

S. Vestri *et al.* hypothesized that different rate of Na^+ filtration may regulate glucose transporters. Their data, however, showed that the intracellular glucose concentration rather than sodium level was the main player [239]. Indeed, hyperglycemia induces both SGLT1 and SGLT2 mRNAs in diabetic condition [240]. In Vallon's study, administration of SGLT2 inhibitor (empagliflozin) in type 1 diabetic Akita mice upregulated renal SGLT2 expression [240]. Ghezzi's group found that in HEK 293T cells, both expression of hSGLT2 and glucose transportation were upregulated by high glucose-induced activation of PKA and PKC. However, this study also reported that phosphorylation of SGLT2 on Ser624 by insulin is necessary for activation of SGLT2 [241]. This finding is supported by Nakamura, *et al.* group, which addressed that it was not high glucose, but insulin participated in the regulation of SGLT2. In their study, insulin stimulated SGLT2 in a dose-dependent manner and this effect could be blocked by an antioxidant factor (N-acetylcysteine)

[242]. Although insulin/SGLT2 pathway supports the augment of SGLT2 expression in type 2 diabetic db/db mice, it cannot explain the upregulated SGLT2 level in hypo-insulinemic type 1 diabetic Akita mice [230, 240].

In addition to hyperglycemia and insulin, the hepatocyte nuclear factor (HNF)-1α, a transcription factor, has been reported to participate in the regulation of SGLT2. In this study, HNF-1 alpha expression and activity was positively correlated with SGLT2 mRNA expression via binding to SGLT2 promoter in diabetic rats [243].

1.4.3.2 SGLT2 and iRAS

Activation of iRAS plays a key role in the progression of DN [244]. Our group has demonstrated that overexpression of Agt in RPTCs significantly increased hypertension, fibrosis and kidney injury [85]. Interestingly, Woods' group has reported that administration of canagliflozin on HFD-induced diabetic mice significantly ameliorated hyperglycemia, hypertension and augmented renal Agt level [245]. However, one of our studies recently found that in type 1 diabetic Akita mice, insulin, rather than canagliflozin, lowered iRAS activity [246]. Similarly, incubation of canagliflozin with HK2 cells or treatment of WT mice with canagliflozin had no effect on Agt expression [247]. Hence, whether SGLT2 reacts with Agt or iRAS remains not clear [248, 249].

1.4.3.3 SGLT2 and oxidative stress

Oxidative stress is a well-recognized biological process leading to the progression of DN. Recent studies have identified that SGLT2 inhibitors, beyond glucose-lowering effects, possess potent antioxidant effect. Catalase is one of the key enzymes that protects cell from oxidative stress by catalyzing hydrogen peroxide to water and oxygen. Overexpression of catalase in diabetic Akita mice normalized systolic blood pressure and attenuated renal injury [144]. Administration of phlorizin (a SGLT2i) in STZ-induced diabetic mice decreased catalase, glutathione peroxidase, superoxide dismutase activities and oxidative stress [250]. Long-term treatment with dapagliflozin ameliorated hyperglycemia, β-cell damage, albuminuria, glomerular mesangial expansion and interstitial fibrosis as well as oxidative stress markers in db/db mice, suggesting that the effect of dapagliflozin on improving hyperglycemia involves inhibition of

oxidative stress [251]. However, it is unknown whether the effect SGLT2 inhibition on oxidative stress is direct or glucose lowering-effect dependent. For example, the ameliorated oxidative stress may result from less AGE production due to decreased hyperglycemia by SGLT2i administration [252].

Despite effect of SGLT2i treatment on oxidative stress, whether oxidative stress participates in the activation of SGLT2 in diabetes has never been studied. For example, Nrf2, a transcription factor that abundantly expresses in RPTCs, is activated by oxidative stress in hyperglycemia and subsequently initiates transcription of downstream genes. Our preliminary data have shown that Nrf2 also participates in glucose regulation and its binding sequences present in SGLT2 promoter. Apparently, understanding their relation will provide a novel perspective of activation of SGLT2 in diabetic condition.

1.4.4 Effects of SGLT2 inhibition

Phlorizin, a pilot of SGLT2i, was early confirmed of its effect on lowering blood glucose [253]. Structure of phlorizin was modified and selected to reduce the side effect. Finally, dapagliflozin, shortly followed by canagliflozin and empagliflozin, has been approved as SGLT2i agent on T2DM patients in United States and European Union in 2012. Other three agents of SGLT2i, ipragliflozin, tofogliflozin, and luseogliflozin were approved in Japan [254]. Although there are differences among SGLT2i, most of them share similar or common effects, not limited to glycemic control, GFR reduction, weight loss and amelioration of hypertension, but also protecting other organs such as heart and pancreases.

1.4.4.1 The effect of SLGT2i in glycemic control

It has been well established that SGLT2i can reduce hyperglycemia and glycated hemoglobin (HbA1c) in a dose-dependent manner in diabetic patients [255-257]. For example, in one randomized, double-blind study with 1,450 patients, treatment with canagliflozin 100 and 300 mg daily decreased HbA1c −0.65% and −0.74%, respectively [257]. Particularly, combined with other anti-hyperglycemic medications, SGLT2 inhibitors yielded additional improvement in glucose control [258]. Unlike insulin, SGLT2i monotherapy was not associated with an increased hypoglycemic risk [259]. The underlying mechanisms of SGLT2i's effect on glucose lowering have

been investigated in patients with T2DM and healthy subjects. Except reducing glucose reabsorption capacity, SGLT2i can also decrease Tm. In human, a normal threshold for reabsorption of glucose corresponds to a serum glucose concentration of 180 mg/dL. In patients with diabetes, the threshold can increase up to 220 to 240 mg/dL in T2DM, whereas selective inhibition of SGLT2 can reduce it to as low as 40 to 120 mg/dL [37]. Even in healthy subjects, SGLT2 inhibitor (Empagliflozin) induces glucosuria in a dose-dependent manner [260].

1.4.4.2 The effect of SLGT2i on GFR

In diabetic condition, increased amount of glucose filtered by the glomerulus leads to enhanced tubular glucose load and reabsorption, resulting in decrease of Na^+ and Cl^- level in tubular fluid. This is sensed by macula densa, which further increases GFR. This response is called tubule-glomerular feedback (TGF). Treatment of SGLT2i leads to excessive Na^+ level in tubular fluid with a decrease of GFR. The decreased GFR further reduces filtered glucose and decrease anti-diabetic efficacy of SGLT2i. Hence, the glucose-lowering effects of SGLT2 inhibition relies on both serum glucose concentration and GFR, two parameters that determine glucose amount by SGLT2 inhibition. Therefore, current recommendations suggest that dapagliflozin and canagliflozin should be used only in patients with an eGFR >60 ml/min/1.73 m^2 and ≥45 ml/min/1.73 m^2, respectively [254, 261]. However, one study has recently reported that treatment of SGLT2i in type 2 diabetic patients with stage 3 chronic kidney disease (eGFR between 30–60 ml/min) still reduced Hb A_1C [259]. The most recent clinical trial, DAPA-CKD, enrolled 4304 CKD patients with an eGFR of 25-75 mL/min/1.73 m^2 was stopped early for overwhelming efficacy, suggesting a wider range of SGLT2i application in patients [262].

1.4.4.3 The effect of SLGT2i on body weight

Weight loss is another beneficial effect of SGLT2i treatment. In various studies including randomized controlled trials, patients who received SGLT2 inhibitors lost weight approximately 1 to 3 kg in a dose-dependent manner [263] [36]. Clinical data showed that body weight reduction by SGLT2i is maintained over 2 years [264]. Many factors contribute to the effect of SGLT2i on body weight reduction. For example, urinary glucose excretion directly causes body weight loss through energy loss. Data have shown that inhibition of SGLT2 results in elimination of about 60–

100g of glucose per day. However, the body weight loss via glycosuria is substantially less than expected due to self-regulation of glucose homeostasis [265]. Overall, it has been now considered that approximately 50% to 75% of total weight loss was from body fat loss, while 15% to 35% was due to extracellular water reduction caused by glucosuria [263].

1.4.4.4 The effect of SLGT2i on hypertension

DN is often accompanied with hypertension, and management of hypertension is critical in the therapy of DN. All FDA-approved agents of SGLT2i, namely canagliflozin, empagliflozin, dapagliflozin and ertugliflozin, can reduce both systolic and diastolic blood pressure [266]. It has been suggested that at least six molecular pathways were involved in blood pressure reductions by SGLT2i [267]. Briefly, SGLT2i decrease hypertension: i) via increasing the natriuretic/diuretic effects and subsequently increase blood viscosity [268]; ii) via modulating autonomous nervous system activity [269]; iii) partially via body weight reduction [270]; iv) by directly affecting NO synthase activity [271]; v) by directly improving renal function [272]; vi) via down-regulation of the oxidative stress. In addition, one study has provided that inhibition of SGLT2 may directly downregulate NHE_3 and lead to blood pressure reduction [273]. In summary, although not yet approved as antihypertensive agents, the role of SGLT2i on lowering blood pressure has been well-established.

1.4.4.5 Other renal effects of SGLT2 inhibition

A number of studies have proved that SGLT2i provide multiple beneficial functions in the kidneys. For example, treatment with empagliflozin in patients with type 2 diabetes reduced the macro-albuminuria [274]. Similar results have been reported in trials with dapagliflozin in patients with diabetes and hypertension [275]. Another study has reported that decrease of urinary ACR may result from decreased intra-glomerular pressure and reduced tubular cell injury [276, 277]. Furthermore, studies have shown that SGLT2i can decrease renal inflammation and fibrosis [276]. Empagliflozin treatment showed significant reductions in glomerular hypertrophy and mesangial matrix expansion as well as markers of inflammation in ob/ob mice [278]. Another study has reported that 12-week treatment with SGLT2i in db/db mice dose-dependently decreased mesangial expansion, interstitial fibrosis and inflammation as well as oxidative stress markers

including MCP-1, intracellular adhesion molecule-1, and TGF-β1 [251]. From stored plasma samples in a 2-year clinical trial of DN, treatment of canagliflozin decreased TNFR1, IL-6, MMP7 and FN1 level [279].

1.4.4.6 The effect of SGLT2i in the heart

Approximately 50% of diabetic patients suffer from heart failure [280]. Currently, the first-line agent of hyperglycemia control in T2DM patients is metformin. According to recommendations by the American Diabetes Association and European Association, the second choice of drug on top of metformin is SGLT2i, glucagon-like peptide-1 receptor agonists, dipeptidyl peptidase 4 inhibitors, sulfonylureas and thiazolidinediones, as well as basal insulin [281]. Treatment with SLGT2i has yielded beneficial effects on diabetic heart failure. In EMPA-REG OUTCOME trial, 7020 participants with type 2 diabetes with high cardiovascular risk and a baseline eGFR≥30 ml/min per 1.73 m^2 were randomly assigned to empagliflozin or placebo and followed up for 3.1 years. Results have shown that treatment with empagliflozin slowed progression of albuminuria, reduced eGFR and risk of renal outcome by 39% (incident of worsening nephropathy or cardiovascular death). Moreover, there was a 14% reduction of primary outcome (a composite of cardiovascular death, nonfatal myocardial infarction or nonfatal stroke). In addition, hospitalization for heart failure and all-cause mortality were both greatly reduced [274]. The most recent clinical trial (DAPA-HF), targeting at effect of SGLT2i on heart failure, was published in 2019 [64]. 4744 patients with heart failure and reduced ejection fraction were randomly assigned to receive either dapagliflozin or placebo on top of other recommended therapy. Over a median of 18.2 months, worsening heart failure and death from cardiovascular causes were significantly lower in the dapagliflozin group without common serious side effects. Particularly, there was a cardioprotective effect regardless of presence or absence of diabetes. These findings established benefits of SGLT2 inhibition on cardiovascular diseases. The underlying mechanisms of SGLT2i's protection of heart failure is not yet totally understood. Proposed pathway includes that SGLT2i offers an attractive fuel (β-hydroxybutyrate) for oxidation, inhibit the NHE3 and reduces blood pressure [282, 283]. Additionally, diuretic effects of SGLT2i have also been proposed that involves in the benefit on SGLT2i on heart diseases. However, it was

questioned by DAPA-HF trial, since intensification of diuretics therapy did not reduce cardiovascular death [284].

1.4.4.7 The effects of SGLT2i on other organs

Type 2 diabetes is associated with insulin resistance in peripheral tissues and pancreatic beta cell failure. Several studies with canagliflozin and empagliflozin have demonstrated that SGLT2i improve insulin response in patients with type 2 diabetes [285]. For instance, treatment of T2DM patients with empagliflozin increased β-cell glucose sensitivity and improved pancreatic beta cell function, probably via amelioration of glucose toxicity [286]. Additionally, SGLT2i has also been reported protective for adipose tissue, liver, endothelial function and circulating progenitor cells in diabetic condition [287, 288].

1.4.5 The side effect of SGLT2i

Although well tolerated in general, SGLT2i still possesses a few well-recognized adverse effects [289]. One most common side effect of SGLT2i is increased risk of balanitis and vulvovaginitis, which were caused by consistent excretion of glucose in urine. Urinary tract infections also appear in patients on SGLT2i. Another common side effect of SGLT2i is the volume depletion induced by the osmotic diuresis, which could cause dehydration, postural dizziness and orthostatic hypotension. Moreover, use of the SGLT2i may cause acute kidney injury and diabetic ketoacidosis.

Taken together, the kidneys participate in the regulation of glucose homeostasis. SGLT2i, the most recent glucose-lowering agent, achieved multiple reno-protective and cardiovascular effects. Despite these well-established beneficial effects, mechanisms of SGLT2i's action and upstream regulation of SGLT2 are still not fully understood. Investigation of the underlying mechanisms might provide us new therapeutic approaches in the therapy of DN.

1.5 Mice models of DN study

1.5.1. T1DM mice model

1.5.1.2 STZ-induced diabetes

T1DM is an autoimmune destruction of the pancreatic beta cells that characterized by lack of insulin. Streptozotocin (STZ)-induced diabetes is one of the most commonly used T1DM mice models. STZ has been discovered from a microbe *Streptomycetes achromogenes* and is selectively toxic to the beta cells. Injection of STZ can rapidly disrupt pancreas islet and induce hyperglycemia [290]. Two experimental protocols of STZ injection are being used. The first one is a single high-dose STZ intraperitoneal injection that results in pancreatic beta cells necrosis within 48-72h. It is one of the most efficient strategies to induce hyperglycemia. However, the single high-dose STZ model is often accompanied by high toxicity in liver, kidney and other organs. Studies have shown that single injection of high-dose STZ caused acute tubular necrosis and impaired renal function, limiting its application in the study of DN [291, 292]. Compared to high-dose STZ, low-dose STZ injection on 5 consecutive days are highly recommended due to its less toxicities and high efficiency [293].

1.5.1.2 Akita mice

Akita mice carry a single nucleotide mutation in *insulin 2* gene (ins2+/c96y), resulting in insulin and β cells depletion. Akita mice develop severe insulin-dependent diabetes (characterized by hyperglycaemia, hypo-insulinaemia, polyuria and polydipsia) at the age of 3 to 4 weeks [290, 294]. Akita mice are one of the unique mice models in study of DN and exist in a few strains. On a C57BL/6J background, Akita mice develop severe hyperglycemia, hypertension, hyperfiltration, kidney hypertrophy, fibrosis and modest albuminuria at week 3-4. But the hyperglycemia is worse in male mice than in female mice [294]. Studies have showed that male Akita mice at 20 weeks develop hyperglycemia, hypertension, enhanced GFR, albuminuria, kidney hypertrophy and fibrosis as well as ROS generation. Morphologically, the kidneys of Akita mice at 20 weeks show mesangial matrix expansion, basement membrane thickening, depletion of podocytes, tubular dilation and tubule cells detachment as well as fibrotic markers such as TGF-β1, collagen IV and

FN1 [107, 193]. These characteristics represent human diabetic kidney disease. Hence, Akita mice are a suitable mouse model for the study of DN [295].

1.5.2 Type 2 diabetic mice model

Type 2 diabetes is associated with insulin resistance and insufficiency. Currently, most of type 2 diabetic mice models have obesity, reflecting that obesity is closely linked to type 2 diabetes development in human [296]. Genetic modification or high fat diet (HFD) induction are the most commonly used methods to induce the obesity. Leptin is a hormone that cause hyperphagia and obesity. Disruption of leptin or leptin receptor generate widely-used obesity mice model, Lep-ob/ob or Lepr-db/db, respectively [290]. Db/db mice exhibit obesity and progress to hyperinsulinemia and hyperglycemia at week 3–4. Previous work has documented that db/db mice on a BKS background develop T2DM phenotypes including increased body weight, hyperglycemia and kidney injury at week 20. Moreover, activation of oxidative stress and iRAS in the kidneys have also been documented [141]. Other types of type 2 diabetic mice models including Lep-ob/ob and HFD are also well-described mice models in the study of DN [290].

1.5.3 Transgenic (Tg) mice with androgen-regulated protein (KAP) promoter

KAP is abundantly expressed in the RPTCs of male mice or testosterone-induced female mice [297]. The expression of KAP is largely controlled by testosterone. KAP gene is one of the most abundant and specific genes that express in mouse RPTCs and possesses a robust and efficient promoter [298]. The KAP promoter has been used for generation of transgenic mice. For instance, human angiotensinogen (hAgt) gene including exons II, III, IV and V was successfully fused with KAP promoter for the generation of hAgt-Tg mice [297]. The KAP2 construct is a modified form of the KAP-hAgt at exon II. With this promoter, our lab has successfully generated rAgt-Tg mice, rCat-Tg, rat hnRNP F-Tg mice and rNrf2-Tg (Figure 1, chapter 3), which specifically overexpress target genes in mouse RPTCs.

1.5.4 Mouse models used for current studies.

1.5.4.1 Generate of Akita Nrf2 KO

Fertile heterozygous Akita mice with spontaneous mutation of *insulin 2* (*Ins2*) gene (C57BL/6-*Ins2*^{Akita}/J) and homozygous Nrf2 knockout (KO) (B6.129X1-Nef2/2^{tm1Ywk}/J) mice were purchased from Jackson Laboratories. We generated Akita Nrf2 KO mice by crossbreeding female homozygous Nrf2 KO mice with male Akita mice. Akita Nrf2 KO mice are homozygous for Nrf2 KO but heterozygous for *Ins2* gene mutation. The detailed breeding map is shown as below.

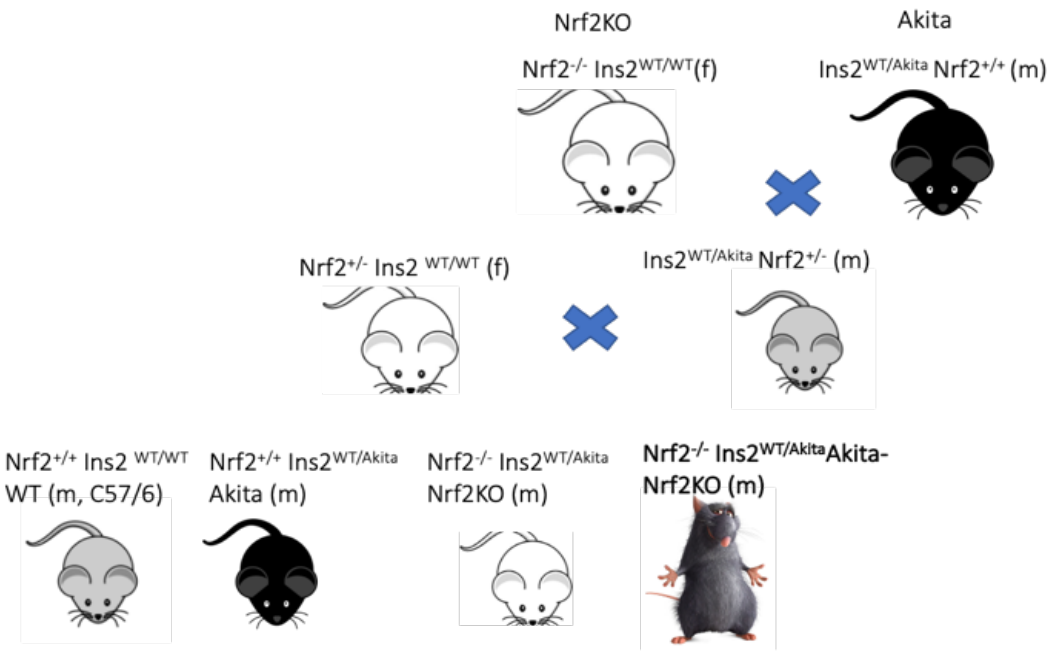


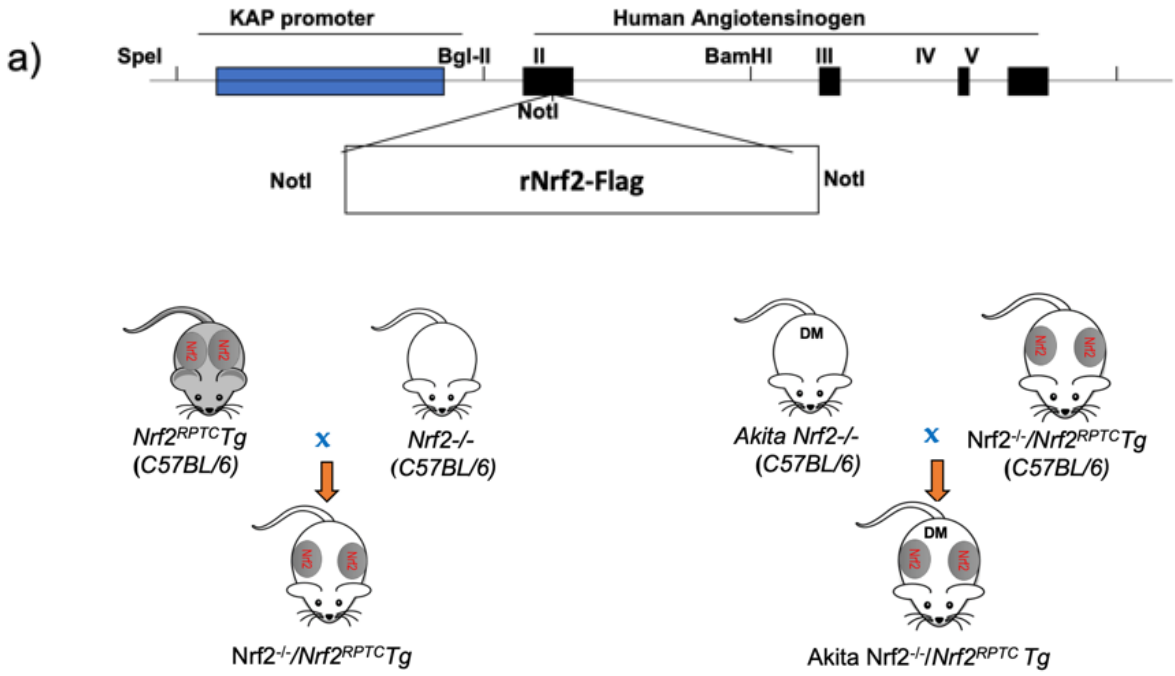
Figure 21. – Generation of Akita Nrf2 KO mice.

1.5.4.2 Generation of Nrf2^{RPTC}Tg mice and Akita Nrf2$^{-/-}$ /Nrf2^{RPTC}Tg mice.

Specific overexpression of rat Nrf2 in mice RPTCs were generated for this study. In brief, as shown in Figure 22a, rat Nrf2 cDNA was cloned from rat kidney total RNA by RT-PCR, fused with Flag tag at 3' end and inserted into a plasmid containing the kidney androgen regulated promoter (KAP2) at Not1 site. Isolated KAP2-Nrf2-Flag transgene was then microinjected into one-cell fertilization mouse embryos by a standard procedure (performed by Cyagen Biosciences

Inc (www.cyagen.com)). Positive founders were then crossed with WT C56BL/6 mice for F1 and further generation.

As shown in Figure 22b & c, Akita *Nrf2*$^{-/-}$/*Nrf2*^{RPTC} Tg mice were generated by crossbreeding female *Nrf2*^{RPTC} Tg mice (C57BL6) with male Akita *Nrf2*$^{-/-}$ mice (C57BL6).



b) *Nrf2*$^{-/-}$/*Nrf2*^{RPTC} Tg

C) Akita *Nrf2*$^{-/-}$/*Nrf2*^{RPTC} Tg

Figure 22. – Generation of *Nrf2*$^{-/-}$/*Nrf2*^{RPTC} Tg mice and Akita *Nrf2*$^{-/-}$/*Nrf2*^{RPTC} Tg mice

1.6 Objectives and hypothesis of this study

Nrf2 is a transcription factor that regulates expression of antioxidant genes through binding to the ARE. Nrf2 has been accepted as an antioxidant factor and abundantly expresses in RPTCs [299]. However, BEACON trial with bardoxolone methyl (Nrf2 activator) in patients with type 2 diabetes and chronic kidney disease (CKD) was early terminated due to increased albumin/creatinine ratio (ACR), blood pressure, heart failure and cardiovascular deaths in patients who received bardoxolone methyl [209]. Despite these unexpected clinical results, more clinical trials are still ongoing [211]. Thus, understanding the role of Nrf2 in DN is urgent and crucial.

Our lab recently reported that overexpression of catalase in RPTCs ameliorated systemic hypertension, RPTC apoptosis decreased oxidative stress and Nrf2-stimulation of *angiotensinogen* (*Agt)* gene expression in diabetic Akita catalase -Tg mice [144]. Nrf2-stimulation of intrarenal *Agt* gene expression contributes to the development of systemic hypertension and nephropathy in diabetes via binding to the Agt promoter. However, whether Nrf2 affects iRAS have ever been studied.

Nrf2 has been reported that related to glucose homeostasis in diabetes [178, 179, 182]. Our preliminary data found lower glycemia in both Akita Nrf2 KO and db/db Nrf2 KO compared to Akita and db/db, respectively. In the kidneys, 90% of glucose filtered by the glomerulus is reabsorbed by SGLT2 in RPTCs. Selective inhibition of SGLT2 has become the most novel and promising therapeutic option in the treatment of diabetes. We further found that Nrf2 putative binding site exists in the SGLT2 gene promoter and transfection of Nrf2 cDNA stimulates SGLT2 gene expression in HK2 cells. Whether the effect of Nrf2 on glucose homeostasis is involved SGLT2 has not been studied.

Taken together, we hypothesised that Nrf2 contributes to the progression of DN via up-regulation of iRAS and SGLT2 gene.

Aims

(1) To study the effect of $Nrf2^{-/-}$ on DN

(2) To investigate the role of Nrf2 with iRAS

(3) To study the effect of overexpression of Nrf2 in RPTCs on DN in Akita $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg mice

(4) To investigate molecular mechanism(s) of Nrf2 regulation of SGLT2 gene expression in human immortalized RPTCs (HK2) in vitro.

(5) To study the effect of $Nrf2^{-/-}$ on DN in male and female db/db mice

Chapter 2 – Article 1

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Nrf2 Deficiency Upregulates Intrarenal Angiotensin-converting Enzyme-2 and Angiotensin 1-7 Receptor Expression and Attenuates Hypertension and Nephropathy in Diabetic Mice

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Abstract

We investigated the role of nuclear factor erythroid 2-related factor 2 (Nrf2) *in r*enin-angiotensin system (RAS) gene expression in renal proximal tubule cells (RPTCs) *and* in the development of systemic hypertension and kidney injury in diabetic Akita mice. We used adult male Akita Nrf2 knockout (KO) mice and Akita mice treated with trigonelline (an Nrf2 inhibitor) or oltipraz (an Nrf2 activator). We also examined immortalized rat RPTCs (IRPTCs) stably transfected with control plasmids or plasmids containing rat angiotensinogen (Agt), angiotensin-converting enzyme (ACE), angiotensin-converting enzyme-2 (Ace2) or angiotensin 1-7 receptor (MasR) gene promoters. Genetic deletion of Nrf2 or pharmacological inhibition of Nrf2 attenuated hypertension, renal injury, and tubulointerstitial fibrosis, and lowered the urinary albumin/creatinine ratio as well as upregulated RPTC Ace2 and MasR expression, increased urinary angiotensin 1-7 levels parallel with down-regulation of Agt, ACE and pro-fibrotic gene expression compared to non-treated Akita mice. In cultured IRPTCs, Nrf2 small interfering RNA transfection or trigonelline treatment prevented high glucose-stimulation of Nrf2 nuclear translocation, Agt and ACE transcription with augmentation of Ace2 and MasR transcription, which was reversed by oltipraz. These data identify a novel mechanism, Nrf2-mediated stimulation of intrarenal RAS gene expression, by which chronic hyperglycemia induces hypertension and renal injury in diabetes.

Introduction

Nuclear factor erythroid 2-related factor 2 (Nrf2) functions as a master regulator of redox balance in cellular cytoprotective responses (1). Under baseline conditions, Nrf2 is sequestered in the cytoplasm, stabilized by Kelch-like ECH-associated protein 1 (Keap 1) and rapidly degraded by proteasomes. In the presence of oxidative stress, Nrf2 is released from Keap 1, translocates to the nucleus and forms heterodimers with small musculoaponeurotic fibrosarcoma proteins (sMafs), which bind to the anti-oxidant response element in the promoters of various genes, including anti-oxidant and detoxifying genes, and enhances their expression (1-3). Although Nrf2 is abundantly expressed in non-diabetic and diabetic kidneys (4-6), its physiological role in the kidneys is undefined.

Studies in rodents with the Nrf2 activators bardoxolone methyl (BM) analogs RTA 405 and dh404 have yielded conflicting results. BM analogs were reported to exert potent anti-diabetic effects in mice with diet-induced diabetes and in rodent models of T2D and obesity (7,8). Others found that BM analogs increase albuminuria and blood pressure along with weight loss in Zucker diabetic fatty rats (9) and at high doses, worsen diabetes-associated atherosclerosis and kidney disease in diabetic apoE$^{-/-}$ mice (10). A phase 2 clinical trial with BM in human T2D with stage 3b or 4 chronic kidney disease reported reductions of serum creatinine levels and slight increases of estimated GFR (11), suggesting a renoprotective action. However, phase 3 clinical trials with BM involving T2D patients with stage 4 (advanced) diabetic kidney disease were discontinued after 9 months of follow-up because of increased mortality and heart failure rates, as well as development of hypertension and albuminuria without favorable effects on GFR (12). Thus, whether Nrf2 activation is beneficial in diabetic patients with kidney disease remains to be investigated. Currently, three Phase 2/3 clinical trials are underway to test the safety and efficacy of BM.

We reported previously that catalase (Cat) overexpression, specifically in renal proximal tubule cells (RPTCs), curbs systemic hypertension and RPTC apoptosis (13-15), and prevents oxidative stress and Nrf2-stimulation of *angiotensinogen* (*Agt)* gene transcription in diabetic Akita *Cat*-Tg (transgenic) mice (5) indicating that Nrf2-stimulation of intrarenal *Agt* gene expression

contributes to the development of systemic hypertension and nephropathy in diabetes. However, little information is available as to whether Nrf2 affects the expression of other renin-angiotensin system (RAS*)* components, including angiotensin-converting enzyme (ACE), angiotensin-converting enzyme-2 (Ace2) and angiotensin 1-7 receptor (MasR) in diabetic RPTCs, which may be crucial in the development of hypertension and nephropathy in diabetes.

In the present study, we investigated the relationship between Nrf2 and intrarenal RAS gene expression, systemic hypertension and renal injury in Akita mice, a murine model of type 1 diabetes mellitus and in RPTCs cultured in high-glucose (HG) milieu.

Materials and methods

Chemicals and constructs

D-glucose, D-mannitol, the alkaloid trigonelline (*$C_7H_7NO_2$, an Nrf2 inhibitor)* and oltipraz (an Nrf2 activator) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Normal glucose (NG, 5 mmol/l D-glucose)-Dulbecco's Modified Eagle's Medium (DMEM, Catalogue No. 12320), penicillin/streptomycin and fetal bovine serum (FBS) were procured from Invitrogen, Inc. (Burlington, ON, Canada). The antibodies used in the present study are listed in **Table 1**. pGL4.20 vector containing luciferase reporter was obtained from Promega (Sunnyvale, CA, USA). pGL4.20 containing rat *Agt* gene promoter (N-1495/N+18) and rat *Ace2* gene promoter (N-1091/+83) has been described previously (16,17). The rat *ACE* gene promoter (N-1675/+95) and the rat *MasR* gene promoter (N-1811/+100) were cloned from rat genomic DNA with specific primers (**Table 2**) and then inserted into pGL4.20 plasmid at Bgl II/Xho I restriction sites. Scrambled (Scr) Silencer Negative Control #1 and *Nrf2* siRNAs were obtained from Ambion, Inc. (Austin, TX, USA). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). Restriction and modifying enzymes were procured from commercial sources.

Generation of Akita Nrf2 KO mice

Fertile heterozygous Akita mice with spontaneous mutation of *insulin 2* (*Ins2*) gene (C57BL/6-*Ins2*^{Akita}/J) and homozygous Nrf2$^{-/-}$ knockout (KO) (B6.129X1-Nef2/2^{tm1Ywk}/J) mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA (http://jaxmice.jax.org). We

generated Akita *Nrf2* KO mice by cross-breeding female homozygous *Nrf2* KO mice with male heterozygous Akita mice (N.B.: Homozygous Nrf2 KO mice are viable and fertile (4) whereas homozygous Akita mice are infertile). Akita *Nrf2* KO mice are homozygous for *Nrf2* KO but heterozygous for *Ins2* gene mutation.

Pathophysiology

Male adult (12-week-old) non-Akita wild type (WT), *Nrf2* KO, Akita and Akita *Nrf2* KO mice (10 per group) were studied. All animals received standard mouse chow and water *ad libitum*. Animal care and experimental procedures were approved by the CRCHUM Animal Care Committee.

Systolic blood pressure (SBP) was tracked with a BP-2000 tail-cuff pressure monitor (Visitech Systems, Apex, NC, USA) every morning, at least 2-3 times per week, for 8 weeks (5,6,14-22). Each animal was habituated to the procedure for at least 15-20 min per day for 5 days before the first SBP measurement. SBP values are presented as means ± SEM of 2 to 3 determinations per week per mouse per group.

Glomerular filtration rate (GFR) was estimated with fluorescein isothiocyanate inulin, as recommended by the Animal Models of Diabetic Complications Consortium (http://www.diacomp.org/) with slight modifications (5,6,16,17).

Blood glucose (BG) levels, after 4-5 h of fasting, were measured with the Accu-Check Performa System (Roche Diagnostics, Laval, QC, Canada). The mice were housed individually in metabolic cages for 8 h during the daytime prior to euthanasia at the age of 20 weeks. Body weight (BW) was recorded. Urine samples were collected and assayed for albumin and creatinine by albumin enzyme-linked immunosorbent assay (ELISA, Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA) (5,6,16,17).

After the animals were euthanized, the kidneys were removed, decapsulated and weighed. Left kidneys were processed for histology and immunostaining. Right kidneys were harvested for isolation of RPTs by Percoll gradient (5,6,16,17). Aliquots of freshly isolated RPTs from individual mice were immediately processed for total RNA and protein analysis.

In separate experiments, adult male Akita mice (age 11 weeks) were divided into 3 groups (9 mice per group) and treated with 0.9% NaCl, *i.p.* or trigonelline (0.02 $mg.kg^{-1}.day^{-1}$, *i.p.* in 0.9% NaCl) from week 12 and then with or without oltipraz (150 $mg.kg^{-1}day^{-1}$, by gavage in corn oil) starting at week 14 every other day until week 17, according to published protocols including ours (5,23,24).

Histology

4-5 sections per kidney and 3 mouse kidneys per group were immunostained using the standard avidin-biotin-peroxidase complex method (ABC Staining, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (5,6,16,17). Tissue sections were counterstained with hematoxylin and analyzed by light microscopy by 2 investigators blinded to the treatments.

Oxidative stress in RPTs was assessed by dihydroethidium (DHE, Sigma) and 5-(6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Life Technologies, Burlington, ON, Canada) staining of frozen kidney sections and confirmed by standard assays of reactive oxygen species (ROS) generation (5,6,15,16,25) in isolated RPTs. Tubular luminal area, mean glomerular and RPTC volumes were assessed, as described elsewhere (5,6,16,17,25).

Western blotting (WB)

WB was performed as described previously (5,6,16,17,25). The relative densities of Nrf2, Keap 1, NAD(P)H quinone oxidoreductase 1 (NQO-1), Agt, ACE, Ace2, MasR and β-actin bands were quantified by densitometry, with ImageQuant software (version 5.1, Molecular Dynamics, Sunnyvale, CA, USA).

Real time-quantitative polymerase chain reaction (RT-qPCR)

RPT *Nrf2, Keap 1, NQO-1, Agt, ACE, Ace2, MasR* and *β-actin* mRNA levels were quantified by RT-qPCR with forward and reverse primers (Table 2) (5,6,16,17,25).

Urinary angiotensin II (Ang II) and Ang 1-7 measurement

Urinary Ang II and Ang 1-7 levels were quantified by ELISA (Immuno-Biological Laboratories, Inc., Minneapolis, MN, USA) and normalized by urinary creatinine levels, as described previously (6,15-17,20,22,25).

Cell culture

Rat IRPTCs (passages 13 through 18) (26) were studied. The plasmids pGL4.20-*Agt (N-1495/+18)*, pGL4.20-*ACE (N-1675/+95), pGL4.20-Ace2 (N-1091/+83)* and *pGL4.20-MasR (N-1811/+100)* were stably transfected into IRPTCs (16,17).

To study the effects of HG, trigonelline and oltipraz, IRPTCs at 75-85% confluency and stable transformants were synchronized overnight in serum-free DMEM containing NG (5 mmol/l D-glucose), then incubated in 5 mmol/l D-glucose plus 20 mmol/l D-mannitol or 25 mM D-glucose DMEM containing 1% depleted FBS (charcoal stripped FBS) for 24 h in the presence or absence of trigonelline ± oltipraz (5,6). *Agt, ACE, Ace2* and *MasR* mRNA levels were quantified by RT-qPCR, and corresponding *Agt, ACE, Ace2* and *MasR* promoter activities were measured by luciferase activity assay (5,6,16,17). IRPTCs stably transfected with the plasmid pGL4.20 served as controls.

In additional experiments, stable transformants were transiently transfected with *Nrf2* or scrambled (Scr) siRNA (5,6), and the effects of HG on *Agt, ACE, Ace2* and *MasR* gene promoter activities were analyzed after 24 h of incubation.

Statistical analysis

The data are expressed as means ± SEM. Statistical comparisons were made by Student's t-test or 1-way analysis of variance and the Bonferroni test as appropriate. $p<0.05$ values were considered to be statistically significant.

Results

Nrf2 expression in Akita and Akita Nrf2-KO mouse kidneys

We confirmed the presence of mutated *Ins2* gene in RPTs isolated from Akita and Akita *Nrf2* KO mice but not in WT and *Nrf2* KO mice (**Fig. 1A** (panel i)). The *Nrf2* gene was detected in RPTs of WT and Akita mice but not in *Nrf2* KO and Akita *Nrf2* KO animals (**Fig. 1A** (panel ii)).

Average SBP was ~20 mm Hg higher in Akita mice at age 12 weeks than in WT mice ($p < 0.005$) and remained significantly elevated for the study's duration (**Fig. 1B**; **Table 3**). Genetic deletion of *Nrf2* significantly decreased SBP as compared to Akita mice. Nrf2 immunostaining was more pronounced in nuclei of RPTCs from Akita mice than in WT mice and was barely detectable in *Nrf2* KO and Akita *Nrf2* KO mice (**Fig. 1C**). NQO-1 expression was also higher in RPTCs from Akita mice than in WT mice and was markedly reduced in *Nrf2* KO and Akita *Nrf2* KO mice (**Fig. 1D**). Note: NQO-1 is a member of phase II detoxifying enzymes (27) and Nrf2 binds to the antioxidant response element (ARE) in the promoter of NQO-1 gene and stimulates NQO-1 gene transcription (28). Thus, NQO-1 has been implied as a downstream mediator of the Nrf2 pathway (29). In contrast, no differences in Keap 1 immunostaining were detected among the groups studied (**Fig. 1E**). Nrf2, NQO-1 and Keap 1 protein expression in RPTs assessed by WB (**Fig. 1F, 1G** and **1H**, respectively) and their respective mRNA expression assayed with real time (RT)-qPCR (**Fig. 1I**, **1J** and **1K**, respectively) were consistent with these changes.

Pathophysiological measurements in mice

Table 3 reports the results of physiological measurements in WT, *Nrf2* KO, Akita and Akita *Nrf2* KO mice at the age of 20 weeks. As anticipated, blood glucose levels were significantly higher in Akita and Akita *Nrf2* KO mice than in WT or *Nrf2* KO mice. BG levels were similar in Akita *Nrf2* KO and Akita mice. Due to the limitation of the range of detection of the Accu-Check Performa glucose meter (up to 33.3 mmol/L), we cannot exclude the possibility that undetected differences in blood glucose might have existed between the diabetic groups, but these were not sufficient to affect body weight, whereas Akita *Nrf2* KO mice exhibited significantly lower SBP than Akita mice. While *Nrf2* KO had no detectable effects on BW, it decreased, though never completely normalized, kidney weight (KW)/BW and KW/tibial length (TL) ratios, urinary albumin-creatinine ratio (ACR), glomerular tuft volume, tubule lumen area, proximal tubular cell volume and urinary Ang II levels in Akita *Nrf2* KO compared to Akita mice. Urinary Ang 1-7 levels were normalized in Akita *Nrf2* KO compared to Akita, whereas *Nrf2*-deficiency did not affect GFR/BW.

Histology

Confirming earlier observations (5,6,15,16,25), the kidneys of Akita mice exhibited

structural damage. The histological changes included proximal tubule cell atrophy, tubular luminal dilatation with accumulation of cell debris, and increased extracellular matrix proteins in glomeruli and tubules (**Fig. 2A** and **Table 3**). *Nrf2* KO markedly reversed, albeit never completely resolved, these abnormalities.

We detected significantly stronger staining for DHE (**Fig. 2B**) and DCFDA (**Supplementary Fig. 1A**) in RPTs from Akita mice than in WT and *Nrf2* KO mice, but the staining did not differ from that in Akita *Nrf2* KO mice. These findings were confirmed by semi-quantification of DHE (**Fig. 2E**) and assessment of ROS levels in RPTs by the lucigenin assay (**Fig. 2F**).

Immunostaining for Cat (**Fig. 2C**) and NADPH oxidase 4 (Nox4) (**Fig. 2D**) were lower and higher, respectively, in RPTCs from Akita mice than in WT or *Nrf2* KO mice. Nrf2 deficiency did not affect Cat and Nox4 expression compared to Akita mice. These findings were confirmed by quantification of Cat and NADPH oxidase activity in RPTs (**Fig. 2G** and **2H**, respectively) and by RT-qPCR of mRNAs (**Fig. 2I** and **2J**, respectively) from isolated RPTs. No changes of Nox1 and Nox2 mRNA levels were detected in the different groups (**Supplementary Fig. 2A** and **2B**, respectively).

Effects of Nrf2 deletion on Agt, ACE, Ace2 and MasR expression in Akita mice

Immunostaining revealed higher Agt (**Fig. 3A**) and ACE (**Fig. 3B**) expression in RPTCs from Akita mice compared to WT or *Nrf2* KO mice. In contrast, Ace2 (**Fig. 3C**) and MasR (**Fig. 3D**) expression were lower in RPTCs from Akita mice compared to WT or *Nrf2* KO mice. Akita *Nrf2*-deficient mice exhibited enhanced Ace2 and MasR expression (**Fig. 3C** and **3D**, respectively). Moreover, Ace2 and MasR expression was higher in RPTCs from *Nrf2* KO than in WT mice (**Fig. 3C** and **3D**, respectively). These findings were confirmed by WB for Agt, ACE, Ace2 and MasR (**Fig. 3E**-**3H**, respectively) and by RT-qPCR of their respective mRNAs (**Fig. 3I-3L**) from isolated RPTs.

Effects of Nrf2 deficiency on tubulointerstitial fibrosis in Akita mice

Masson's trichrome staining revealed higher collagenous matrix protein expression **(Supplementary Fig. 1B**) in the tubulointerstitium of Akita mice compared to WT or *Nrf2* KO mice with more pronounced attenuation in Akita *Nrf2* KO mice. Increased immunostaining of TGF-β1, FN1 and Col 1 (**Supplementary Fig. 1C, 1D** and **1E,** respectively) was also detected in the

tubulointerstitium of Akita mice compared to WT or *Nrf2* KO mice. Genetic deletion of *Nrf2* in Akita mice markedly attenuated TGF-β1, FN1 and Col 1 expression (**Supplementary Fig. 1C**, **1D** and **1E**, respectively). Semi-quantification of Masson staining (**Supplementary Fig. 1F**) and RT-qPCR of *TGF-β1, FN1* and *Col I* mRNA from isolated RPTs (**Supplementary Fig. 1G, 1H** and **1I,** respectively) confirmed these findings.

Effects of Nrf2 siRNA on Agt, ACE, Ace2 and MasR gene expression in isolated IRPTCs

Consistent with our previous observations (5,6), HG enhanced Nrf2 expression in both cytoplasmic (**Fig. 4A**) and nuclear fractions (**Fig. 4B**) of IRPTCs compared to NG. *Nrf2* siRNA transfection reduced cytoplasmic Nrf2 in IRPTCs cultured in NG and HG. It had no effect on nuclear Nrf2 in NG but lowered Nrf2 expression in HG in IRPTCs. Transient *Nrf2* siRNA transfection also significantly inhibited *Nrf2* but not *NQO-1* mRNA expression in IRPTCs in NG and prevented HG-stimulation of *Nrf2* and *NQO-1* mRNA expression compared to Scr siRNA (**Fig. 4C** and **4D,** respectively). Furthermore, *Nrf2* siRNA transfection prevented increases of *Agt* and *ACE* and decreases of *Ace2* and *MasR* in response to HG at the mRNA level (**Fig. 4E, 4F, 4G** and **4H,** respectively), as well as their respective promoter activities (**Fig. 5A, 5B, 5C** and **5D,** respectively).

To confirm the impact of Nrf2 on *Agt, ACE, Ace2* and *MasR* expression in IRPTCs, we studied the effects of the Nrf2 inhibitor alkaloid trigonelline $\pm$ the Nrf2 activator oltipraz. Trigonelline treatment prevented HG stimulation of *Agt* and *ACE* and suppression of *Ace2* and *MasR* mRNA expression in IRPTCs, and these actions were abrogated by oltipraz (**Fig. 5E, 5F, 5G** and **5H**, respectively).

Effect of trigonelline and oltipraz on expression of intrarenal RAS in Akita mice

Trigonelline administration did not affect BG (**Fig. 6A**) whereas it significantly lowered SBP in Akita mice (**Fig. 6B** and **Table 4**). This was reversed by oltipraz treatment. Trigonelline significantly decreased the KW/BW and KW/TL ratios but not urinary ACR and Ang II/creatinine levels (**Table 4**). These actions were also reversed by oltipraz. Interestingly, trigonelline treatment completely normalized urinary Ang 1-7/creatinine levels in Akita mice compared to non-Akita mice, without affecting GFR/BW.

To test the antioxidant capacity of trigonelline, we examined the effect of trigonelline on oxidative stress in kidneys of Akita mice. Treatment of Akita mice with trigonelline markedly attenuated DHE staining (**Fig. 6C,** semi-quantification of DHE staining is shown on **Fig. 7A**) and reduced ROS levels in RPTs assessed by the lucigenin assay (**Fig. 7B**). Furthermore, trigonelline prevented HG-induced ROS generation in IRPTCs in a concentration-dependent manner (**Supplementary Figure 3A**). Thus, trigonelline possesses an intrinsic anti-oxidant property to lower ROS generation in diabetic RPTCs.

Trigonelline also markedly attenuated renal damage, including glomerulosclerosis, tubule lumen dilatation and accumulation of cell debris as assessed with PAS staining (**Fig. 6D**) and these changes were reversed by oltipraz. Consistently, RPTCs of Akita mice exhibited higher immunostaining for Nrf2 (**Fig. 6E**), Agt (**Fig. 6F**) and ACE (**Fig. 6G**) than RPTCs of WT mice. In contrast, lower immunostaining of Ace2 (**Fig. 6H**) and MasR (**Fig. 6I**) was observed in Akita mice. Trigonelline reduced Nrf2, Agt and ACE expression (**Fig. 6E, 6F** and **6G,** respectively) and increased Ace2 and MasR expression (**Fig. 6H** and **6I**, respectively) to levels comparable to those detected in WT mice, and these effects were reversed by oltipraz. Trigonelline did not affect Keap 1 expression in Akita mice (**Fig. 6J**). WB of Nrf2, Agt, Keap 1 (**Fig. 7C** (panel i)**, 7D, 7E** and **7F**), respectively), ACE, Ace2 and MasR (**Fig. 7C** (panel ii)**, 7G, 7H** and **7I)**, respectively) and RT-qPCR of *Nrf2*, *Agt, Keap 1, ACE, Ace2* and *MasR* mRNA expression (Supplementary **Fig. 2C** to **2H**, respectively) confirmed these findings. Furthermore, trigonelline treatment prevented Nrf2 nuclear translocation and decreased cytosolic Nrf2 expression in RPTs of Akita mice (**Fig. 7J-7L**) and in IRPTCs cultured in HG (**Fig. 7M-7O**). These effects were reversed by oltipraz.

Discussion

Our results document that selective genetic deletion of *Nrf2* or pharmacological blockade of Nrf2 with trigonelline in Akita mice effectively upregulates RPTC *Ace2/MasR,* and suppresses *Agt/ACE* expression, resulting in attenuation of systemic hypertension and kidney injury. Consistently, in cultured IRPTCs, *Nrf2* siRNA transfection or trigonelline treatment prevents HG-induced upregulation of *Agt/ACE* and downregulation of *Ace2/MasR* gene expression. The effects

of trigonelline were reversed by oltipraz both *in vitro* and *in vivo*. These data identify a novel mechanism underlying Nrf2 activation by oxidative stress (secondary to hyperglycemia) that stimulates intrarenal RAS gene expression and activation, leading to the development of hypertension and nephropathy in diabetes.

Akita mice, an autosomal dominant model of spontaneous type 1 diabetes (T1D) with a mutated *Ins2* gene, have decreased numbers of pancreatic islet β-cells and develop hyperglycemia as early as 3-4 weeks of age (30). By age 13 to 30 weeks, male Akita mice manifest impaired renal function and increased oxidative stress markers in their RPTs (31,32). Akita mice represent a useful model to study the early to moderately advanced renal morphological changes in T1D patients.

In the present study, we found that Nrf2 and NQO-1 expression is increased in RPTs of 20-week-old Akita mice compared to WT mice. This was associated with marked increases in ROS generation, NADPH oxidase activity and *Nox4* mRNA expression, whereas Cat activity and Cat mRNA expression was lower in RPTCs of Akita mice than in WT mice. These changes were similar to those detected in Akita Nrf2 KO mice, indicating that hyperglycemia-induced oxidative stress and higher Nox4 activity with suppressed Cat activity may contribute to renal injury in Akita mice.

We do not presently understand why increased Nrf2 expression and activity (reflected by heightened NQO-1 expression) cannot attenuate oxidative stress (as evidenced by augmented DHE and DCFDA staining and ROS generation) in Akita mice. Our data, including our previous reports (5,6) and studies in diabetic rats by other groups (33,34), document reduced Cat activity in diabetic mice. Thus, one possibility is that hyperglycemia would result in decreased sirtuin-1 and Foxo3α expression and consequently lower Cat expression, thereby enhancing ROS generation in diabetic kidneys (35,36). Another possible explanation is that hyperglycemia enhances NF-κB activation to compete for Nrf2 stimulation of transcription of antioxidant genes including *Cat*. This latter possibility is supported by the findings that the phosphorylated NF-κB p65 subunit and Nrf2 both bind to the same domain of CREB-binding protein (CBP), a coactivator of Nrf2 (37). The phospho-NF-κB p65 subunit could then attenuate the transcription of antioxidant response element (ARE)-dependent genes by depriving CBP from Nrf2 through a

competitive mechanism (37).

The mechanisms leading to SBP elevation in Akita mice are incompletely understood. The possibility that downregulation of *Ace2* and *MasR* gene expression and upregulation of *Agt* and *ACE* gene expression, yielding higher Ang II/Ang 1-7 ratios that facilitate the development of hypertension has received considerable attention (38-40). Indeed, our data disclose significantly higher RPTC Agt and ACE expression and urinary Ang II levels with lower RPTC Ace2 and MasR expression and urinary Ang 1-7 levels in Akita than in WT mice. These observations are consistent with our earlier findings of elevated ACE and depressed Ace2 expression in the kidneys of Akita *Agt*-Tg mice (20), suggesting that upregulation of Ace2/MasR expression to increase Ang 1-7 level is important to down-regulate Agt/ACE expression and prevent systemic hypertension in Akita mice (25). These findings are consistent with clinical reports of heightened intrarenal RAS and urinary Ang II expression with lower Ace2 expression in hypertensive diabetic patients (41-45).

The exact mechanism(s) by which Nrf2 deficiency leads to downregulation of renal Agt/ACE and upregulation of Ace2/MasR gene expression in diabetes remain(s) unclear. One possibility is that hyperglycemia/ROS augment Nrf2 activation by promoting its dissociation from Keap 1 and translocation into the nucleus. Nrf2 will then bind to Nrf2-binding sites in the Agt/ACE gene promoter regions and promote Agt/ACE gene expression. Previously, we showed that Nrf2 binds to Nrf2-binding sites in the Agt gene promoter (5). Thus, Nrf2 deletion in Akita mice should diminish Agt gene expression. Consistently, Nrf2 siRNA transfection diminished HG-stimulation of Agt/ACE gene promoter activity and upregulation of Ace2/MasR gene promoter in IRPTCs. These observations could be explained by the presence of Nrf2-REs (response elements) in Agt gene promoter (5) and of potential putative Nrf2-REs in ACE, Ace2 and MasR gene promoters. Indeed, studies are ongoing in our laboratory to identify these putative Nrf2-REs.

How might oxidative stress lead to interstitial fibrosis in Akita mice? One possibility is that augmented Agt/ACE expression with Ang II elevation via ROS generation stimulates TGF-β1 and subsequently enhances the expression of extracellular matrix proteins and profibrotic genes in RPTCs, resulting in interstitial fibrosis (46). Indeed, neutralizing TGF-β1 with antibody was reported to alleviate fibrosis and tubule cell apoptosis in animal models of diabetes (47). We also

detected higher TGF-β1, FN1 and Col I protein and mRNA expression in RPTs of Akita mice than in WT controls. These increases were mitigated in Akita Nrf2 KO mice, linking Nrf2 with intrarenal RAS activation and upregulation of TGF-β1 expression in RPTs and consequently to interstitial fibrosis in Akita mice. Thus, our findings support the notion that Nrf2 activation aggravates tubulointerstitial fibrosis with nephropathy progression in diabetes.

At present, our data do not allow delineating whether kidney damage in Akita mice is due to hyperglycemia or hypertension per se. We have previously reported that dual RAS blockade normalizes Ace2 expression and prevents hypertension and tubular apoptosis in Akita angiotensinogen-transgenic mice without affecting hyperglycemia (20), suggesting the hypertension induced by intrarenal RAS activation plays a predominant role in DN progression. Furthermore, our recent observation that insulin treatment normalizes hyperglycemia and hypertension, inhibits Nrf2 gene expression and prevents Nrf2-stimulation of *Agt* gene expression in RPTs of Akita mice (6), would indicate that hyperglycemia can evoke intrarenal RAS genes expression and subsequently hypertension development in diabetes. Thus, these studies point to the importance of hyperglycemia–induced intrarenal RAS activation via Nrf2 expression in the development of hypertension in Akita mice. Clearly, more studies are needed to define the contribution of hypertension and hyperglycemia to kidney damage in diabetes.

The exact mode of the mechanism by which trigonelline decreases Nrf2 protein levels in RPTs of Akita mice is not known. However, we have found that trigonelline decreases oxidative stress (DHE staining) in kidneys of Akita mice and inhibits Nrf2 promoter activity, Nrf2 mRNA expression and Nrf2 nuclear translocation in IRPTCs in HG. These data would indicate that trigonelline inhibits Nrf2 protein expression via inhibition of Nrf2 gene transcription by lowering oxidative stress in diabetic RPTCs. These data are consistent with our previous report that overexpression of catalase in RPTCs inhibits Nrf2 expression in Akita mice (5). Furthermore, lowering Nrf2 nuclear translocation by trigonelline may prevent positive auto-feedback of Nrf2 on *Nrf2* gene transcription (48). Clearly, more studies are needed to elucidate the underlying mechanism of trigonelline action on Nrf2 expression.

In summary, our findings document that selective *Nrf2* KO upregulates renal *Ace2/MasR* gene expression with downregulation of *Agt/ACE* gene expression and prevents systemic hypertension and renal injury in diabetes. Our results imply an important role of oxidative stress induced *Nrf2* expression and activation in the development of hypertension and renal injury in diabetes by altering local intrarenal RAS expression.

Abbreviations

ACE, angiotensin-converting enzyme; Ace2, angiotensin-converting enzyme-2; ACR, albumin-creatinine ratio; Agt, angiotensinogen; Ang 1-7, angiotensin 1-7; Ang II, angiotensin II; ARE, antioxidant response element; BM, bardoxolone methyl; BW, body weight; Cat, catalase; Col I, collagen I; DCFDA, 5- (6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FN1, fibronectin-1; GFR, glomerular filtration rate; HG, high glucose; Ins2, insulin 2 gene; IRPTCs, immortalized renal proximal tubular cells; Keap 1, Kelch-like ECH-associated protein 1; KO, knockout; MasR, angiotensin 1-7 receptor; Nox4, NADPH oxidase 4; NQO-1, NAD(P)H quinone oxidoreductase 1; NG, normal glucose; Nrf2, nuclear factor erythroid 2-related factor 2; PAS, periodic acid Schiff; RAS, renin-angiotensin system; REs, response elements; ROS, reactive oxygen species; RPTs, renal proximal tubules; RPTCs, renal proximal tubule cells; RT-qPCR, real time-quantitative polymerase chain reaction; SBP, systolic blood pressure; Scr, scrambled; siRNAs, small interfering RNAs; SEM, standard error of the means; T1D, T2D, type 1 and 2 diabetes; Tg, transgenic; TL, tibial length; WB, Western blotting; WT, wild type

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Disclosure

None.

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Figure legends

Figure 1. Generation of Akita *Nrf2* KO mice. (A) Genotyping of (panel i) mutated and normal *Ins2* gene and (panel ii) of *Nrf2-LacZ* (*Nrf2* KO) and normal *Nrf2* gene in wild type (WT), *Nrf2* KO, Akita and Akita *Nrf2* KO mice by specific PCR analysis. (B) Longitudinal changes in mean SBP in male WT (●), *Nrf2* KO (■), Akita (▲) and Akita *Nrf2* KO (▼) mice. Values are means ± SEM, n = 10 for each group. **p<0.01 and ***p<0.005 Akita compared to WT mice; $^{+}$p<0.05 and $^{++}$p<0.01 Akita compared to Akita *Nrf2* KO mice. Immunohistochemical staining for Nrf2 (C), NQO-1 (D), and Keap 1 (E) expression in kidney sections (magnification x600), WB analysis of Nrf2 (F), NQO-1 (G) and Keap 1 (H) protein expression and RT-qPCR analysis of *Nrf2* (I), *NQO-1* (J) and *Keap 1* (K) mRNA levels in RPTs of WT, *Nrf2* KO, Akita and Akita *Nrf2* KO mice. Values are means $\pm$ SEM, n=6. *p<0.05; ***p<0.005; ns, not significant. WT (empty bars), *Nrf2* KO mice (light grey bars), Akita (solid black bars) and Akita *Nrf2* KO mice (dark grey bars).

Figure 2. Characterization of Akita *Nrf2* KO mice. (A) Periodic acid Schiff (PAS) staining (magnification x600), (B) DHE (red) and DAPI staining (blue) (magnification x200), (C) Cat and (D) Nox4 immunostaining in kidney sections (magnification x600) from male WT, *Nrf2* KO, Akita and Akita *Nrf2* KO mouse kidneys at age 20 weeks. Semi-quantification of DHE fluorescence (E), ROS production (F), Cat activity (G), NADPH oxidase activity (H), *Cat* mRNA (I), and *Nox4* mRNA (J) expression in RPTs of WT controls, *Nrf2* KO, Akita and Akita *Nrf2* KO mice. Values are expressed as means ± SEM, n=8 per group. *p<0.05; **p<0.01; ***p<0.005; ns, not significant. WT (empty bars), *Nrf2* KO mice (light grey bars), Akita (solid black bars) and Akita *Nrf2* KO mice (dark grey bars).

Figure 3. Agt, ACE, Ace2 and MasR expression in mouse kidneys at week 20. Immunohistochemical staining of Agt (A), ACE (B), Ace2 (C) and MasR (D) in mouse kidneys. Magnification X600. WB of Agt (E), ACE (F), Ace2 (G) and MasR (H) expression and RT-qPCR analysis of *Agt* (I), *ACE* (J), *Ace2* (K) and *MasR* (L) mRNA levels in RPTs of WT, *Nrf2* KO, Akita and Akita *Nrf2* KO mice. Values are expressed as means ± SEM (n=8). *p<0.05; **p<0.01; ***p<0.005; ns, not significant. WT (empty bars), *Nrf2* KO mice (light grey bars), Akita (solid black bars) and Akita *Nrf2* KO mice (dark grey bars).

Figure 4. **Effect of *Nrf2* siRNA on *Nrf2*, *NQO-1, Agt*, *ACE, Ace2* and *MasR* gene expression in immortalized rat RPTCs (IRPTCs) in NG and HG medium.** (A) Effect of *Nrf2* siRNA or scrambled (Scr) siRNA on Nrf2 protein expression in cytoplasmic fraction and (B) nuclear fraction of IRPTCs incubated in NG (normal glucose) and HG (high glucose) medium and quantified by WB. NE-PER Nuclear and Cytoplasmic Extraction Reagents were used for the isolation of cytoplasmic and nuclear fractions from IRPTCs according to the manufacturer's protocol (Catalogue No. 78833, ThermoScientific, Pierce Biotechnology, Rockford, IL, USA). Effect of *Nrf2* siRNA or Scr siRNA on *Nrf2* (C), *NQO-1* (D), *Agt* (E), *ACE* (F), *Ace2* (G) and *MasR* (H) mRNA expression in IRPTCs incubated in NG and HG medium and quantified by RT-qPCR. Cells were harvested after 24 h of incubation. mRNA levels in cells incubated in NG medium with Scr RNA are expressed as arbitrary unit 1. The results are reported as percentages of control values (means ± SEM, n=3 for three separate experiments done in triplicates. *p<0.05; **p<0.01; ***p≤0.005; ns, not significant. NG + Scr siRNA (empty bars), NG *+ Nrf2* siRNA (light grey bars), HG + Scr siRNA (solid black bars) and HG + *Nrf2* siRNA (dark grey bars).

Figure 5. Effect of *Nrf2* siRNA and trigonelline on *Agt, ACE, Ace2* and *MasR* promoter activity and mRNA in IRPTCs in HG medium. Dose-dependent effect of *Nrf2* siRNA and scrambled (Scr) siRNA on *Agt* (A), *ACE* (B), *Ace2* (C) and *MasR* (D) promoter activity in stably transfected IRPTCs incubated in HG medium and quantified by luciferase activity assay. Effect of trigonelline (Trig) ± oltipraz (Olz) on *Agt* (E), *ACE* (F), *Ace2* (G) and *MasR* (H) mRNA expression in IRPTCs incubated in HG medium and quantified by RT-qPCR. Cells were harvested after 24 h of incubation. Promoter activity and mRNA levels in cells incubated in NG medium are expressed as 100% control or arbitrary unit 1, respectively. The results are reported as percentages or fold of change of control values (means ± SEM, n=3 for three separate experiments done in triplicates. *p<0.05; **p<0.01; ***p<0.005; ns, not significant. NG + Scr siRNA (empty bars), NG *+ Nrf2* siRNA (light grey bars), HG + Scr siRNA (solid black bars) and HG + *Nrf2* siRNA (dark grey bars).

Figure 6. Effect of trigonelline on Nrf2, Agt, ACE, Ace2 and MasR expression in Akita mice *in vivo*. Longitudinal changes in mean BG (A) and SBP (B) in male WT (●), Akita (■), Akita + trigonelline (Trig) (▲) and Akita + Trig + Oltipraz (Olz) (▼) mice. Values are means ± SEM, n = 9 for each group. ***p<0.005 Akita compared to WT mice; $^{+}$p<0.05 Akita compared to Akita + Trig

mice; #p<0.05 Akita + Trig compared to Akita + Trig + Olz mice. (C) DHE (red) and DAPI staining (blue) (magnification x200), (D) PAS staining and immunohistochemical staining for Nrf2 (E), Agt (F), ACE (G), Ace2 (H), MasR (I) and Keap 1 (J) in the kidneys of WT and Akita ± trigonelline and oltipraz. Magnification X600.

Figure 7. Effect of trigonelline on oxidative stress and RPT *Nrf2*, *Agt, ACE, Ace2* and *MasR* expression in mice *in vivo*. (A) Semi-quantification of DHE fluorescence, (B) ROS production, (C) WB of (panel i) Nrf2, Agt and Keap 1 expression and (panel ii) ACE, Ace2 and MasR expression in RPTs of WT and Akita mice ± trigonelline and oltipraz. WB quantification of Nrf2 (D), Agt (E), Keap 1 (F), ACE (G), Ace2 (H) and MasR (I) expression in RPTs of WT and Akita mice ± trigonelline and oltipraz. (J) WB of Nrf2 protein expression and quantitation in nuclear fraction (K) and cytosolic fraction (L) in RPTs of WT and Akita mice ± trigonelline and oltipraz, respectively. (M) WB of Nrf2 protein expression and quantitation in nuclear fraction (N) and cytosolic fraction (O) of IRPTCs incubated in NG, HG and HG ± trigonelline and oltipraz, respectively. Values are expressed as means ± SEM (n=6 per group). *p<0.05; **p<0.01; ***p<0.005; ns, not significant. WT (empty bars), Akita mice (light grey bars), Akita mice treated with trigonelline (Trig, solid black bars) and Akita mice treated with Trig and oltipraz (Olz, dark grey bars).

Table 1. Antibodies

Name of Antibody	Source of Antibody	Species Raised in Mono/Polyclonal	RRID	Dilution for WB and/or IHC
Nrf2	Abcam	Rabbit; polyclonal	AB_881705	WB (1:2000); IHC (1:400)
NQO-1	Santa-Cruz Biotechnology	Goat; polyclonal	AB_2154339	WB (1:2000)
NQO-1	Abcam	Mouse; monoclonal	AB_881738	IHC (1:400)
Keap 1	Abcam	Rabbit; polyclonal	AB_1141055	WB (1:2000); IHC (1:400)
Agt	Generated in our lab (1)	Rabbit; polyclonal	AB_2631321	WB (1:2000); IHC (1:200)
ACE	Santa Cruz Biotechnology	Goat; polyclonal	AB_2273625	WB (1:2000); IHC (1:250)
Ace2	R&D Systems	Goat; polyclonal	AB_355722	WB (1:2000); IHC (1:250)
MasR	Novus Biologicals	Rabbit; polyclonal	AB_11039164	WB (1:2000); IHC (1:200)
β-Actin	Sigma-Aldrich	Mouse; Monoclonal	AB_476744	WB (1:20000)

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Table 2. Primer sequences for genotyping and RT-qPCR

Gene (Species)	Sense and Anti-Sense Primers	Reference Sequences
Primers for Genotyping		
Ins2 (mouse)	S: TGCTGATGCCCTGGCCTGCT AS: TGGTCCCACATATGCACATG	NT_039437.7
Nrf2 (mouse genotyping)	Common-S: GCCTGAGAGCTGTAGGCCC WT-AS: GGAATGGAAAATAGCTCCTGCC Mutant-AS: GACAGTATCGGCCTCAGGAA	
Primers for RT-qPCR		
Nrf2 (mouse/rat)	S: CGCCGCCTCACCTCTGCTGCCAGTAG AS: AGCTCATAATCCTTCTGTCG	NM_010902.3
Keap 1 (mouse/rat)	S: CATCCACCCTAAGGTCATGGA AS: GACAGGTTGAAGAACTCCTCC	NM_016679.4
NQO-1 (mouse/Rat)	S: TATCCTTCCGAGTCATCTCTAGCA AS: TCTGCAGCTTCCAGCTTCTTG	NM_008706.5
Nox4 (mouse)	S: TGGCCAACGAAGGGGTTAAA AS: GATGAGGCTGCAGTTGAGGT	NM_015760.4
Catalase (mouse)	S: CGACCAGATGAAGCAGTGGA AS: CCACTCTCTCAGGAATCCGC	NM_009804.2
Agt (mouse/rat)	S: CCACGCTCTCTGGATTTATC AS: ACAGACACCGAGATGCTGTT	NM_031144.3
ACE (mouse)	S: AGGAGTTTGCAGAGGTCTGG AS: GGAAGCAGACCTTGCCAGTG	NM_207624
ACE (rat)	S: GAGCCATCCTTCCCTTTTTC As: GGCTGCAGCTCCTGGTATAG	NM_012544.1
Ace2 (mouse)	S: AGGAGGAAGTTGATGGATACCTA AS: GGCTCAGTCAGCATAGAGTTT	NM_027286
Ace2 (rat)	S: ACAGTTCCTTTTGGGGAGGC AS: GTGACAGGAGGCTCGTAAGG	NM_001012006.1
MasR (mouse)	S: GCATTCGTCTGTGCCCTTCT	NM_008552.4

Table 3. Physiological and histological measurements

	WT	Nrf2 KO	Akita	Akita Nrf2 KO
Blood glucose (BG, mM) (n=10)	9.55±0.53	11.79±0.46	32.11±0.51***	32.32±0.68***
Systolic blood pressure (SBP, mmHg) (n=10)	111.3±0.71	115.9±2.39	132.4±2.34***	121.4±1.57^{**++}
Body weight (BW, g) (n=10)	33.08±0.45	33.64±0.81	23.92±0.58***	24.13±0.42***
Kidney weight (KW, mg) (n=10)	339.5±6.99	328.2±9.12	540.0±11.55***	454.2±10.76^{***+++}
KW/BW (mg/g) (n=10)	10.11±0.26	10.02±0.35	22.45±0.89***	17.73±0.70^{***+++}
Tibia length (TL, mm) (n=10)	23.52±0.65	23.3±0.17	21.4±0.27**	21.91±0.30*
KW/TL (mg/mm) (n=10)	13.46±0.62	13.69±0.29	25.91±0.84***	20.17±0.60^{***+++}
GFR/BW (ml/min/g) (n=10)	7.8±0.37	9.03±0.47	19.2±1.56***	18.64±1.29***
Glomerular tuft volume (*10^3µm^3) (n=6)	180.4±4.0	179.0±4.9	291.2±10.28***	237.9±7.43^{***+++}
Tubular luminal area (µm^2) (n=6)	52.59±2.27	51.5±1.86	108.59±3.13***	87.39±2.31^{***+++}
RPTC volume (*10^3µm^3) (n=6)	7.44±0.57	7.78±0.29	11.64±0.43***	9.99±0.34^{***+}
ACR (µg/mg) (n=10)	22.36±1.71	25.29±6.09	134.1±19.04***	56.98±15.88^{++}
Urinary AngII/Creatinine ratio (ng/mg) (n=10)	2.57±0.37	1.83±0.27	23.88±2.88***	20.08±3.74***
Urinary Ang (1-7)/Creatinine ratio (ng/mg) (n=10)	3.45±0.62	3.22±0.58	0.91±0.11**	2.58±0.39^{+}

Table 4. Physiological and histological measurements

	WT	Akita	Akita+Trig	Akita+Trig+Olz
Blood glucose (BG, mM) (n=9)	8.87±0.44	32.86±0.19***	31.72±1.11***	32.69±0.39***
Systolic blood pressure (SBP, mmHg) (n=9)	111.0±0.50	136.0±1.43***	124.1±0.84^{***+++}	133.4±0.52$^{***\#\#\#}$
Body weight (BW, g) (n=9)	32,75±0.60	22.65±0.84***	22.88±0.54***	21.14±0.40***
Kidney weight (KW, mg) (n=9)	364.3±9.83	549.8±9.15***	514.7±9.43***	528.3±4.77***
Tibia length (TL, mm) (n=9)	22.56±0.46	21.87±0.41	21.32±0.52	20.95±0.28
KW/BW (mg/g) (n=9)	11.12±0.40	24.24±0.89***	21,51±0.42^{***+}	24.92±0.64$^{***\#\#}$
KW/TL (mg/mm) (n=9)	16.19±0.34	26.04±0.68***	23.01±0.82^{***++}	25.95±0.51$^{***\#\#}$
GFR/BW (µl/min/g) (n=9)	9.92±0.80	22±1.96***	21.15±1.12***	20.25±0.58***
ACR (µg/mg) (n=9)	22.22±1.64	105.10±19.87**	82.19±20.79	123.0±32.91*
Urinary AngII/Creatinine ratio (ng/mg) (n=9)	1.27± 0.15	25.92±1.93***	16.79±1.87**	23.84±3.03***
Urinary Ang (1-7) /Creatinine ratio (ng/mg) (n=9)	2.61± 0.55	0.83±0.15*	2.65±0.39^{++}	2.4±0.33^{+}

*p < 0.05, **p < 0.01, ***p < 0.001 vs WT; $^{+}$p < 0.05, $^{++}$p < 0.01, $^{+++}$p < 0.001 vs Akita; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs Akita+Trig

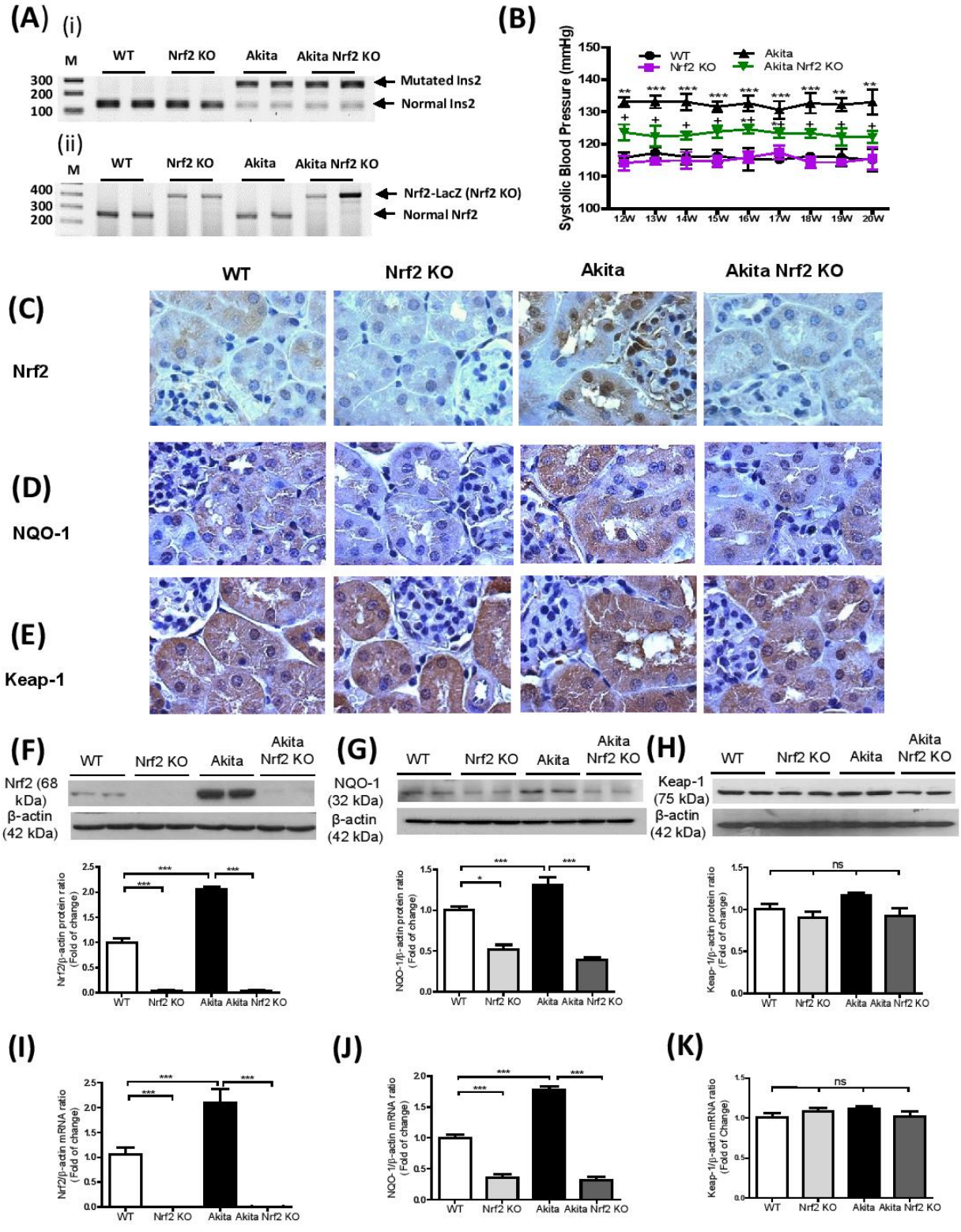

(A)
(i)
M
WT
Nrf2 KO
Akita
Akita Nrf2 KO
300
200
100
Mutated Ins2
Normal Ins2
(ii)
400
300
200
Nrf2-LacZ (Nrf2 KO)
Normal Nrf2
(B)
Systolic Blood Pressure (mmHg)
12W
13W
14W
15W
16W
17W
18W
19W
20W
(C)
Nrf2
(D)
NQO-1
(E)
Keap-1
(F)
Nrf2 (68 kDa)
β-actin (42 kDa)
Nrf2/β-actin protein ratio (Fold of change)
(G)
NQO-1 (32 kDa)
β-actin (42 kDa)
NQO-1/β-actin protein ratio (Fold of change)
(H)
Keap-1 (75 kDa)
β-actin (42 kDa)
Keap-1/β-actin protein ratio (Fold of change)
ns
(I)
Nrf2/β-actin mRNA ratio (Fold of change)
(J)
NQO-1/β-actin mRNA ratio (Fold of change)
(K)
Keap-1/β-actin mRNA ratio (Fold of Change)

Figure 1

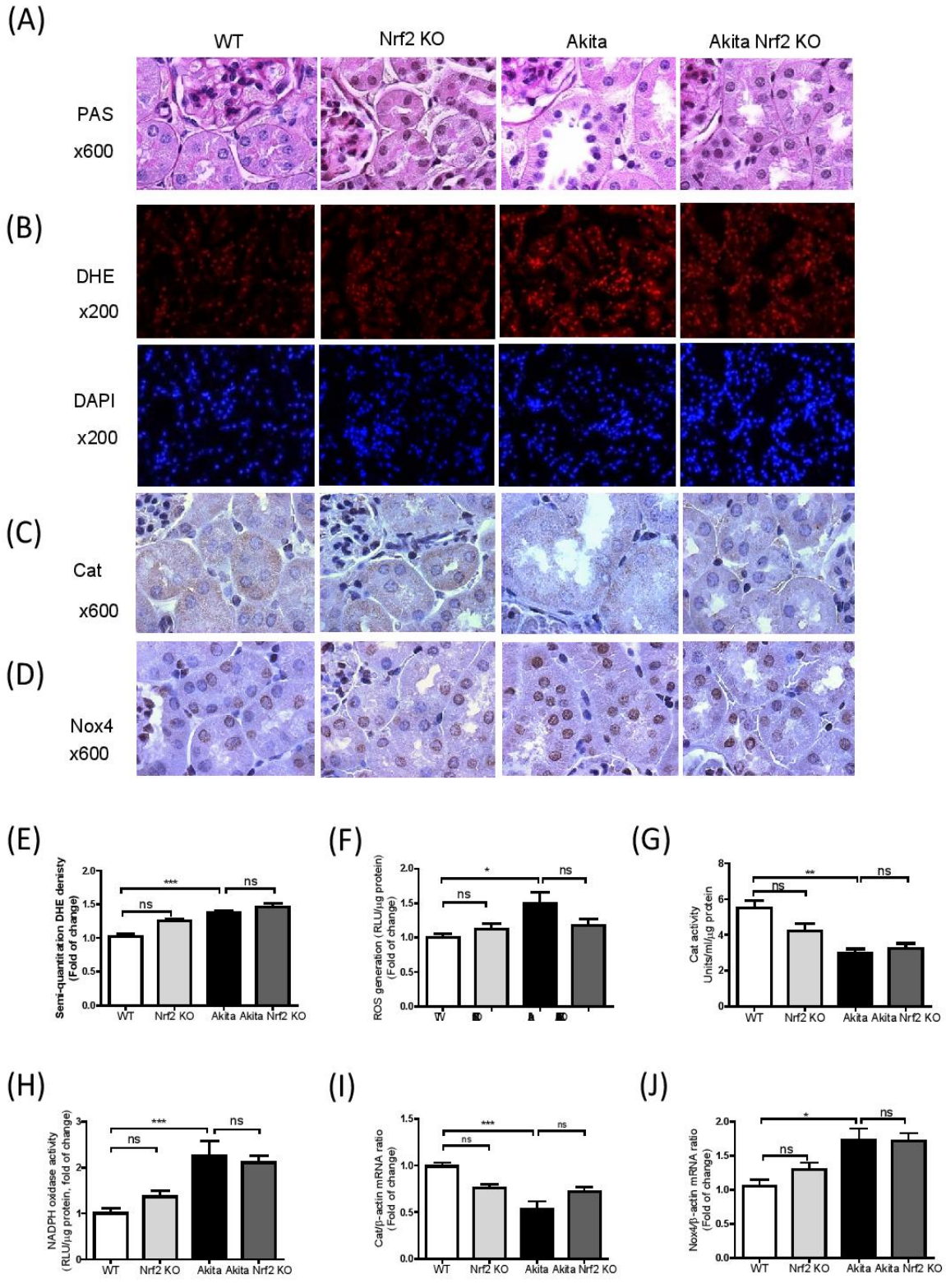

(A)
WT
Nrf2 KO
Akita
Akita Nrf2 KO
PAS
x600
(B)
DHE
x200
DAPI
x200
(C)
Cat
x600
(D)
Nox4
x600
(E)
Semi-quantitation DHE denisty (Fold of change)
(F)
ROS generation (RLU/μg protein) (Fold of change)
(G)
Cat activity Units/ml/μg protein
(H)
NADPH oxidase activity (RLU/μg protein, fold of change)
(I)
Cat/β-actin mRNA ratio (Fold of change)
(J)
Nox4/β-actin mRNA ratio (Fold of change)
ns
WT
Nrf2 KO
Akita
Akita Nrf2 KO

Figure 2

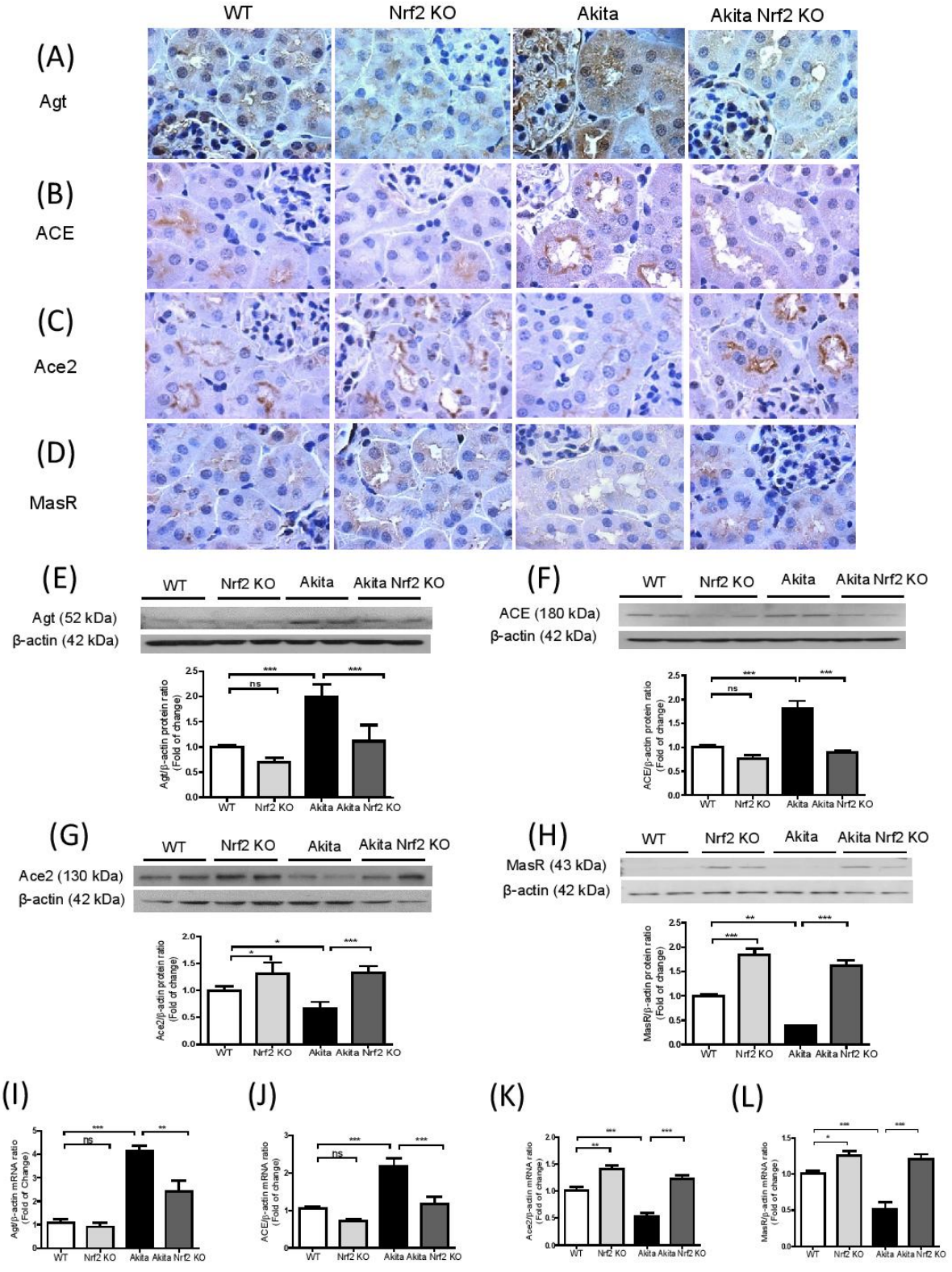

WT
Nrf2 KO
Akita
Akita Nrf2 KO
(A)
Agt
(B)
ACE
(C)
Ace2
(D)
MasR
(E)
Agt (52 kDa)
β-actin (42 kDa)
Agt/β-actin protein ratio (Fold of change)
(F)
ACE (180 kDa)
β-actin (42 kDa)
ACE/β-actin protein ratio (Fold of change)
(G)
Ace2 (130 kDa)
β-actin (42 kDa)
Ace2/β-actin protein ratio (Fold of change)
(H)
MasR (43 kDa)
β-actin (42 kDa)
MasR/β-actin protein ratio (Fold of change)
(I)
Agt/β-actin mRNA ratio (Fold of Change)
(J)
ACE/β-actin mRNA ratio (Fold of change)
(K)
Ace2/β-actin mRNA ratio (Fold of change)
(L)
MasR/β-actin mRNA ratio (Fold of change)
ns

Figure 3

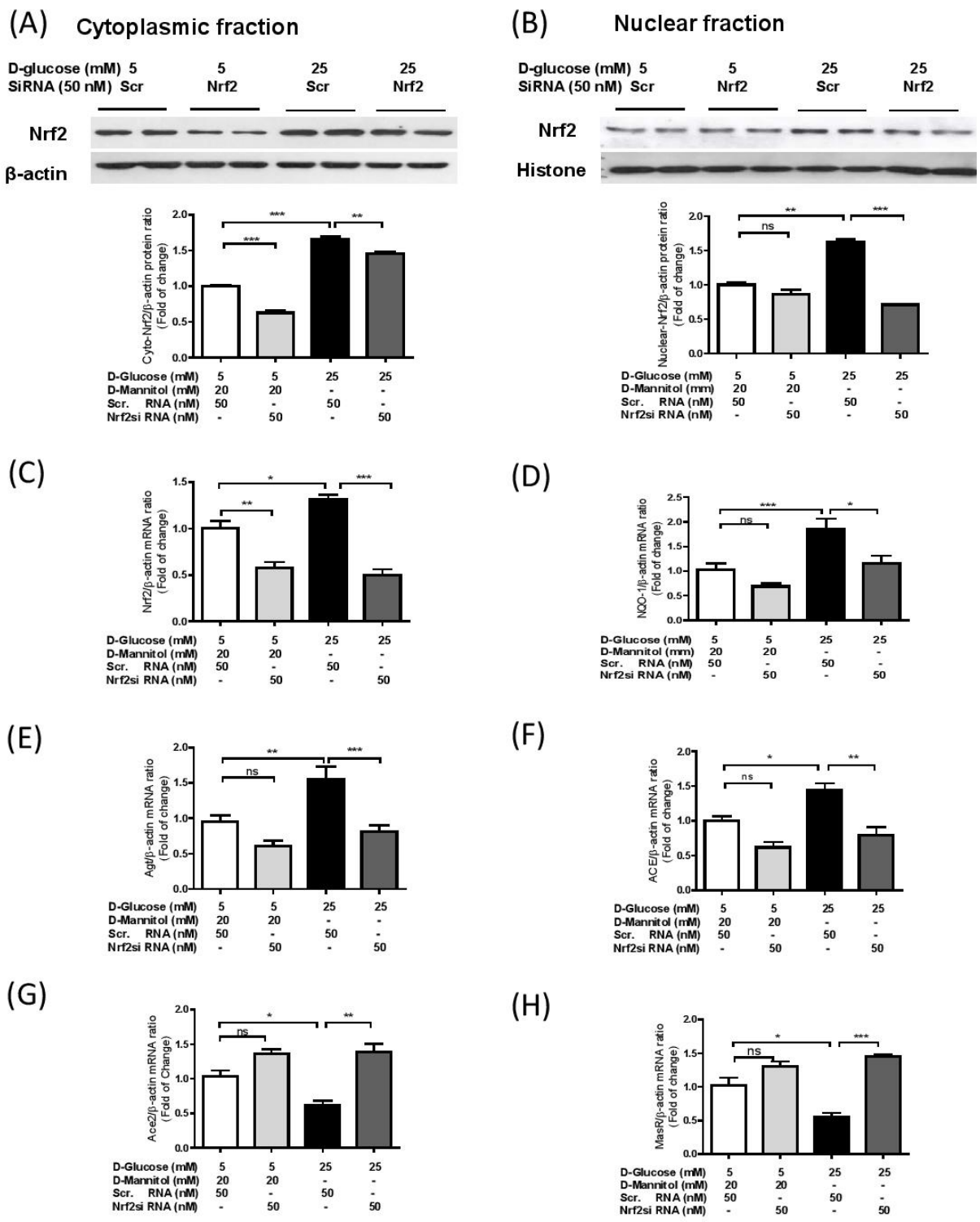

(A) Cytoplasmic fraction
D-glucose (mM) 5 5 25 25
SiRNA (50 nM) Scr Nrf2 Scr Nrf2
Nrf2
β-actin
Cyto-Nrf2/β-actin protein ratio (Fold of change)
D-Glucose (mM) 5 5 25 25
D-Mannitol (mM) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50
(B) Nuclear fraction
D-glucose (mM) 5 5 25 25
SiRNA (50 nM) Scr Nrf2 Scr Nrf2
Nrf2
Histone
Nuclear-Nrf2/β-actin protein ratio (Fold of change)
ns
D-Glucose (mM) 5 5 25 25
D-Mannitol (mm) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50
(C)
Nrf2/β-actin mRNA ratio (Fold of change)
D-Glucose (mM) 5 5 25 25
D-Mannitol (mM) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50
(D)
NQO-1/β-actin mRNA ratio (Fold of change)
ns
D-Glucose (mM) 5 5 25 25
D-Mannitol (mm) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50
(E)
Agt/β-actin mRNA ratio (Fold of change)
ns
D-Glucose (mM) 5 5 25 25
D-Mannitol (mM) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50
(F)
ACE/β-actin mRNA ratio (Fold of change)
ns
D-Glucose (mM) 5 5 25 25
D-Mannitol (mM) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50
(G)
Ace2/β-actin mRNA ratio (Fold of Change)
ns
D-Glucose (mM) 5 5 25 25
D-Mannitol (mM) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50
(H)
MasR/β-actin mRNA ratio (Fold of change)
ns
D-Glucose (mM) 5 5 25 25
D-Mannitol (mM) 20 20 - -
Scr. RNA (nM) 50 - 50 -
Nrf2si RNA (nM) - 50 - 50

Figure 4

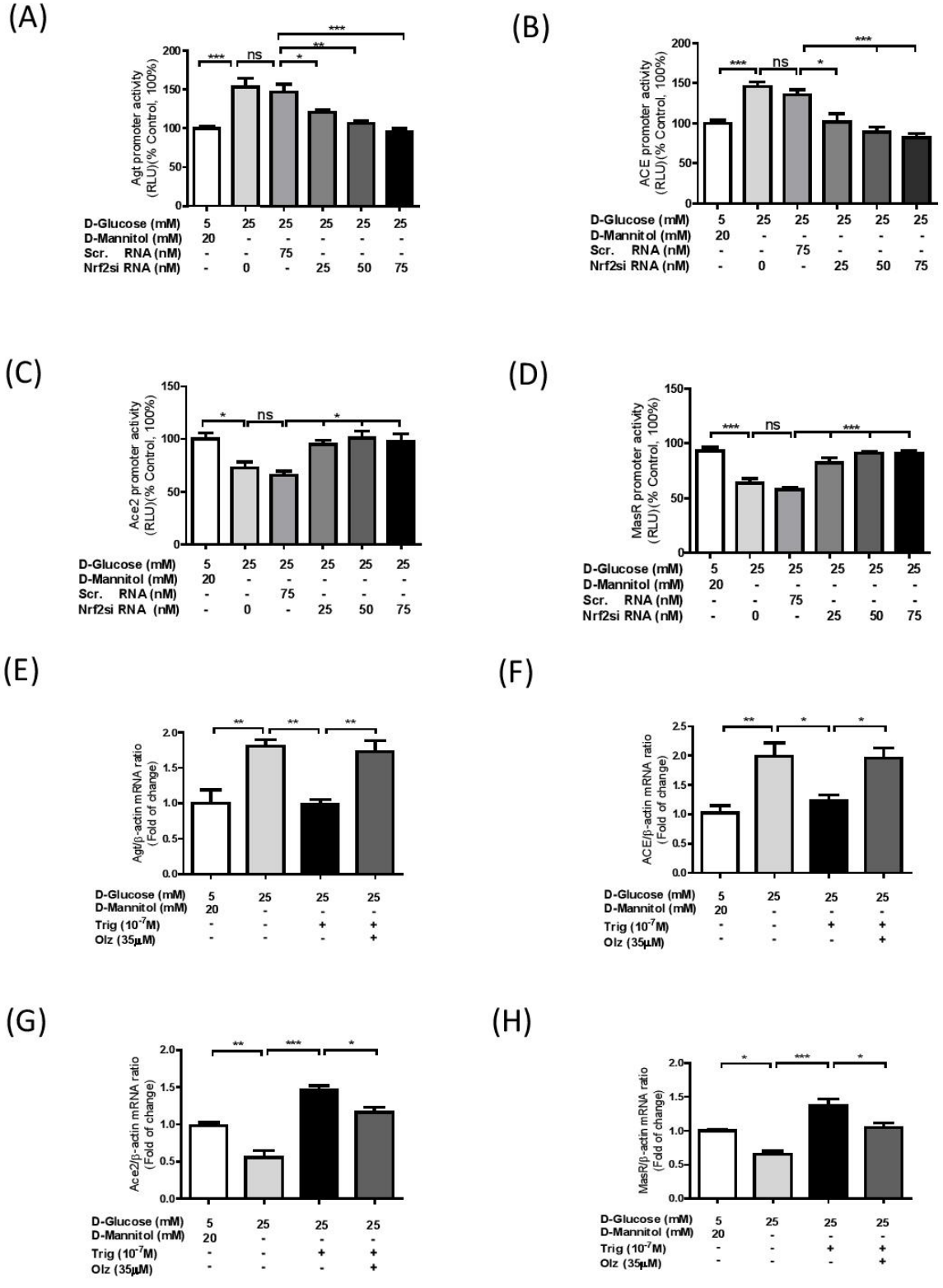

(A)
Agt promoter activity (RLU)(% Control, 100%)
D-Glucose (mM) 5 25 25 25 25 25
D-Mannitol (mM) 20 - - - - -
Scr. RNA (nM) - - 75 - - -
Nrf2si RNA (nM) - 0 - 25 50 75
(B)
ACE promoter activity (RLU)(% Control, 100%)
D-Glucose (mM) 5 25 25 25 25 25
D-Mannitol (mM) 20 - - - - -
Scr. RNA (nM) - - 75 - - -
Nrf2si RNA (nM) - 0 - 25 50 75
(C)
Ace2 promoter activity (RLU)(% Control, 100%)
D-Glucose (mM) 5 25 25 25 25 25
D-Mannitol (mM) 20 - - - - -
Scr. RNA (nM) - - 75 - - -
Nrf2si RNA (nM) - 0 - 25 50 75
(D)
MasR promoter activity (RLU)(% Control, 100%)
D-Glucose (mM) 5 25 25 25 25 25
D-Mannitol (mM) 20 - - - - -
Scr. RNA (nM) - - 75 - - -
Nrf2si RNA (nM) - 0 - 25 50 75
(E)
Agt/β-actin mRNA ratio (Fold of change)
D-Glucose (mM) 5 25 25 25
D-Mannitol (mM) 20 - - -
Trig (10^{-7}M) - - + +
Olz (35μM) - - - +
(F)
ACE/β-actin mRNA ratio (Fold of change)
D-Glucose (mM) 5 25 25 25
D-Mannitol (mM) 20 - - -
Trig (10^{-7}M) - - + +
Olz (35μM) - - - +
(G)
Ace2/β-actin mRNA ratio (Fold of change)
D-Glucose (mM) 5 25 25 25
D-Mannitol (mM) 20 - - -
Trig (10^{-7}M) - - + +
Olz (35μM) - - - +
(H)
MasR/β-actin mRNA ratio (Fold of change)
D-Glucose (mM) 5 25 25 25
D-Mannitol (mM) 20 - - -
Trig (10^{-7}M) - - + +
Olz (35μM) - - - +

Figure 5

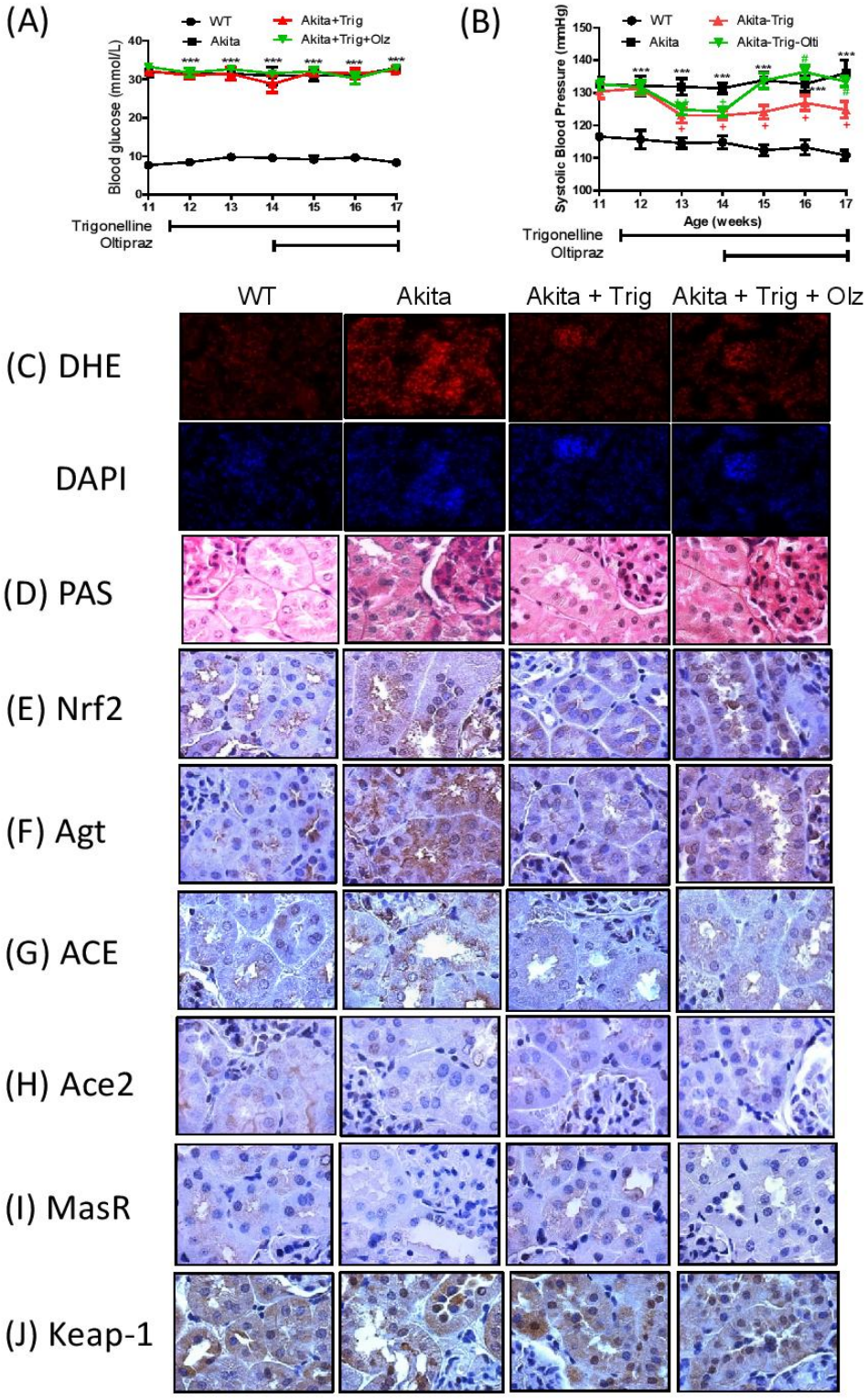

(A)
WT
Akita
Akita+Trig
Akita+Trig+Olz
Blood glucose (mmol/L)
Trigonelline
Oltipraz
(B)
WT
Akita
Akita-Trig
Akita-Trig-Olti
Systolic Blood Pressure (mmHg)
Age (weeks)
Trigonelline
Oltipraz
WT
Akita
Akita + Trig
Akita + Trig + Olz
(C) DHE
DAPI
(D) PAS
(E) Nrf2
(F) Agt
(G) ACE
(H) Ace2
(I) MasR
(J) Keap-1

Figure 6

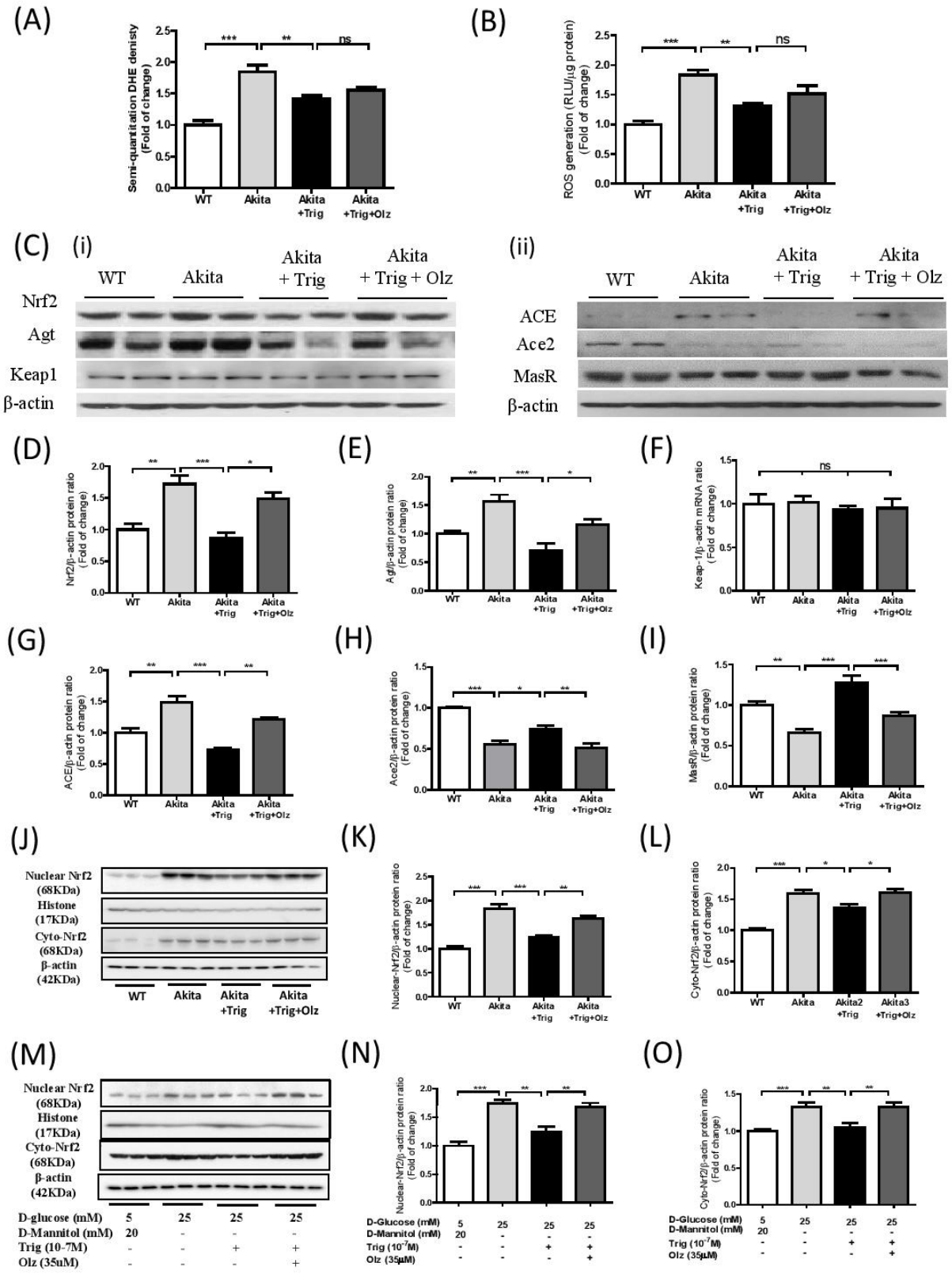

(A)
Semi-quantitation DHE denisty (Fold of change)
WT
Akita
Akita +Trig
Akita +Trig+Olz
(B)
ROS generation (RLU/μg protein) (Fold of change)
(C) (i)
WT
Akita
Akita + Trig
Akita + Trig + Olz
Nrf2
Agt
Keap1
β-actin
(ii)
ACE
Ace2
MasR
β-actin
(D)
Nrf2/β-actin protein ratio (Fold of change)
(E)
Agt/β-actin protein ratio (Fold of change)
(F)
Keap-1/β-actin mRNA ratio (Fold of change)
(G)
ACE/β-actin protein ratio (Fold of change)
(H)
Ace2/β-actin protein ratio (Fold of change)
(I)
MasR/β-actin protein ratio (Fold of change)
(J)
Nuclear Nrf2 (68KDa)
Histone (17KDa)
Cyto-Nrf2 (68KDa)
β-actin (42KDa)
(K)
Nuclear-Nrf2/β-actin protein ratio (Fold of change)
(L)
Cyto-Nrf2/β-actin protein ratio (Fold of change)
Akita2 +Trig
Akita3 +Trig+Olz
(M)
Nuclear Nrf2 (68KDa)
Histone (17KDa)
Cyto-Nrf2 (68KDa)
β-actin (42KDa)
D-glucose (mM) 5 25 25 25
D-Mannitol (mM) 20 - - -
Trig (10-7M) - - + +
Olz (35uM) - - - +
(N)
Nuclear-Nrf2/β-actin protein ratio (Fold of change)
D-Glucose (mM) 5 25 25 25
D-Mannitol (mM) 20 - - -
Trig (10-7M) - - + +
Olz (35μM) - - - +
(O)
Cyto-Nrf2/β-actin protein ratio (Fold of change)
D-Glucose (mM) 5 25 25 25
D-Mannitol (mM) 20 - - -
Trig (10-7M) - - + +
Olz (35μM) - - - +

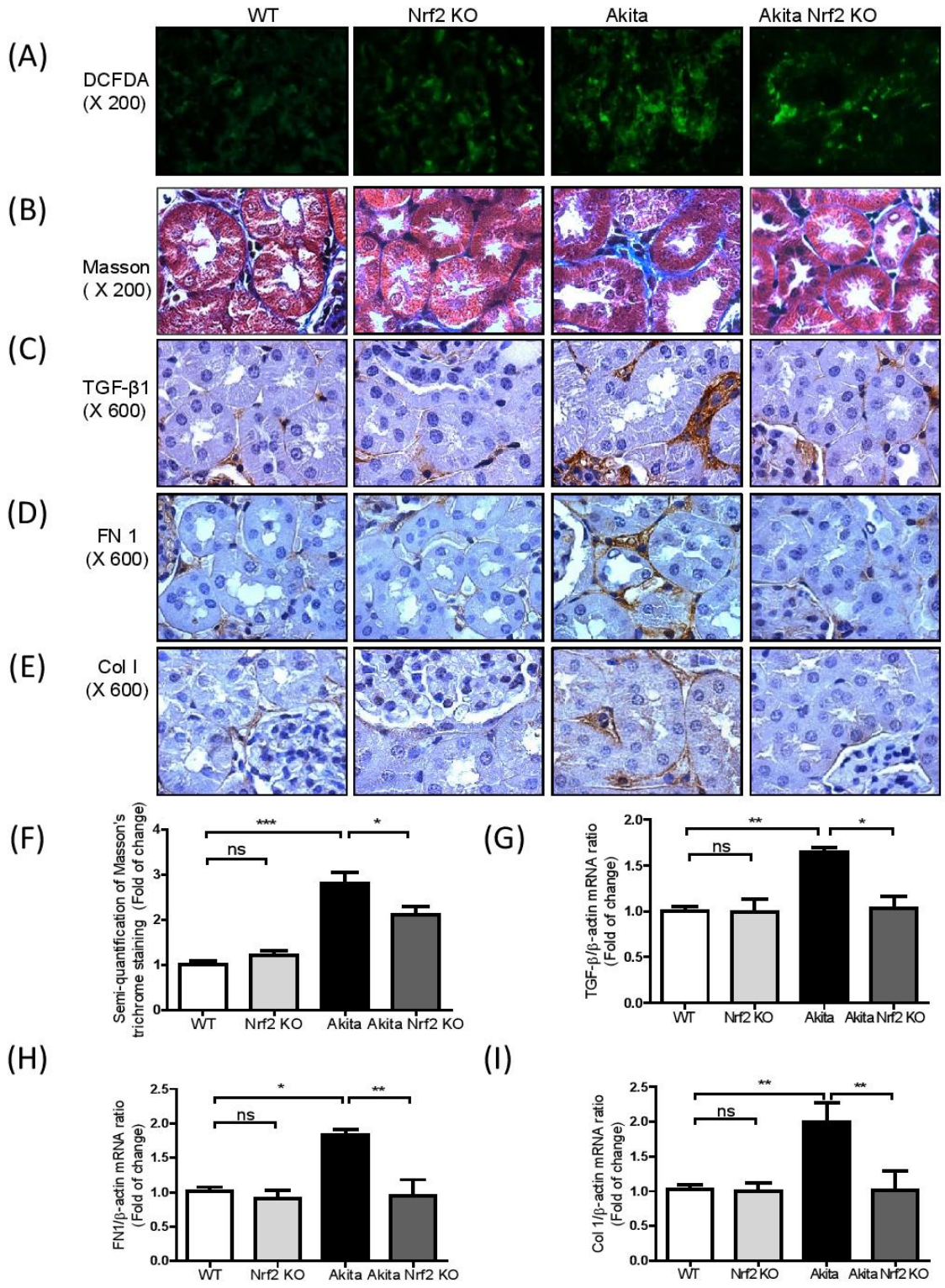
Figure 7

Caption for supplementary figures

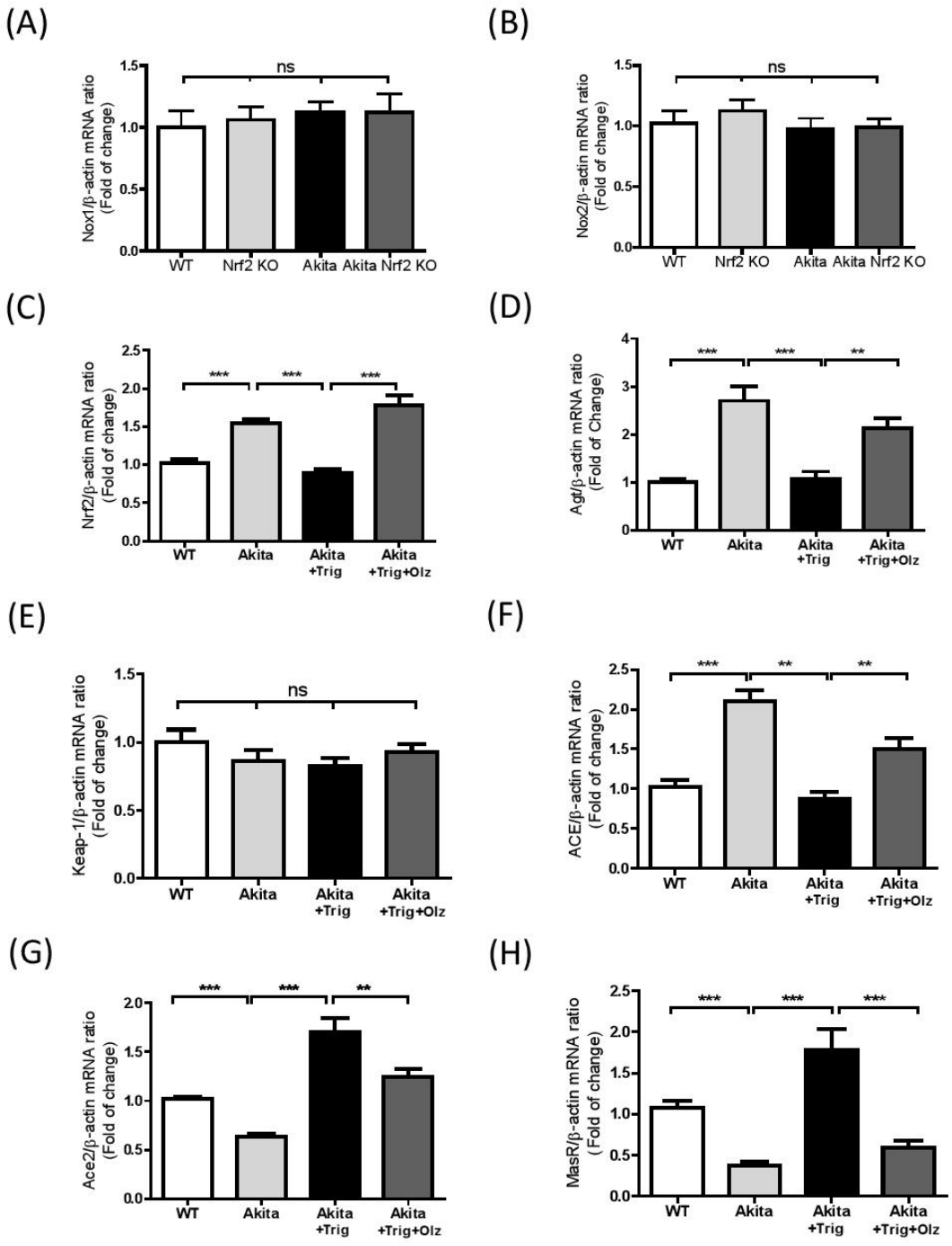
Supplementary Figure 1. Oxidative Stress, tubulointerstitial fibrosis and pro-fibrotic gene expression in mouse kidneys at age 20 weeks. (A) DCFDA (green) (magnification x200), (B) Masson's trichrome staining (magnification x200), (C) Transforming growth factor-beta 1 (TGF-β1), (D) Fibronectin 1 (FN1) (E) Collagen I (Col I) immunostaining in kidney sections (Magnification x600) from WT, *Nrf2* KO, Akita and Akita *Nrf2* KO mice. (F) Quantification of extracellular matrix component accumulation (Masson's trichrome staining) and RT-qPCR of *TGF-β1* (G), *FN1* (H) and *Col I* (I) mRNA in freshly isolated RPTs of WT, *Nrf2* KO, Akita, and Akita *Nrf2* KO mouse kidneys. Values are means ± SEM, n=8. *p<0.05; **p<0.01; ***p<0.005; ns, not significant. WT (empty bars), *Nrf2* KO mice (light grey bars), Akita (solid black bars) and Akita *Nrf2* KO mice (dark grey bars).

Supplementary Figure 2. *Nox1 and Nox2* mRNA expression and the effect of trigonelline on *Nrf2*, *Agt, ACE*, *Ace2* and *MasR* expression in Akita mice. RT-qPCR of *Nox1* mRNA (A) and *Nox2* mRNA (B) expression in RPTs of WT controls, *Nrf2* KO, Akita and Akita *Nrf2* KO mice. RT-qPCR of *Nrf2* (C), *Agt* (D), *Keap 1* (E), *ACE* (F), *Ace2* (G) and *MasR* (H) mRNA expression in RPTs of WT and Akita mice ± trigonelline and oltipraz. Values are expressed as means ± SEM (n=6 per group). *p<0.05; **p<0.01; ***p<0.005; ns, not significant. WT (empty bars), Akita mice (light grey bars), Akita mice treated with trigonelline (Trig, solid black bars) and Akita mice treated with Trig and oltipraz (Olz, dark grey bars).

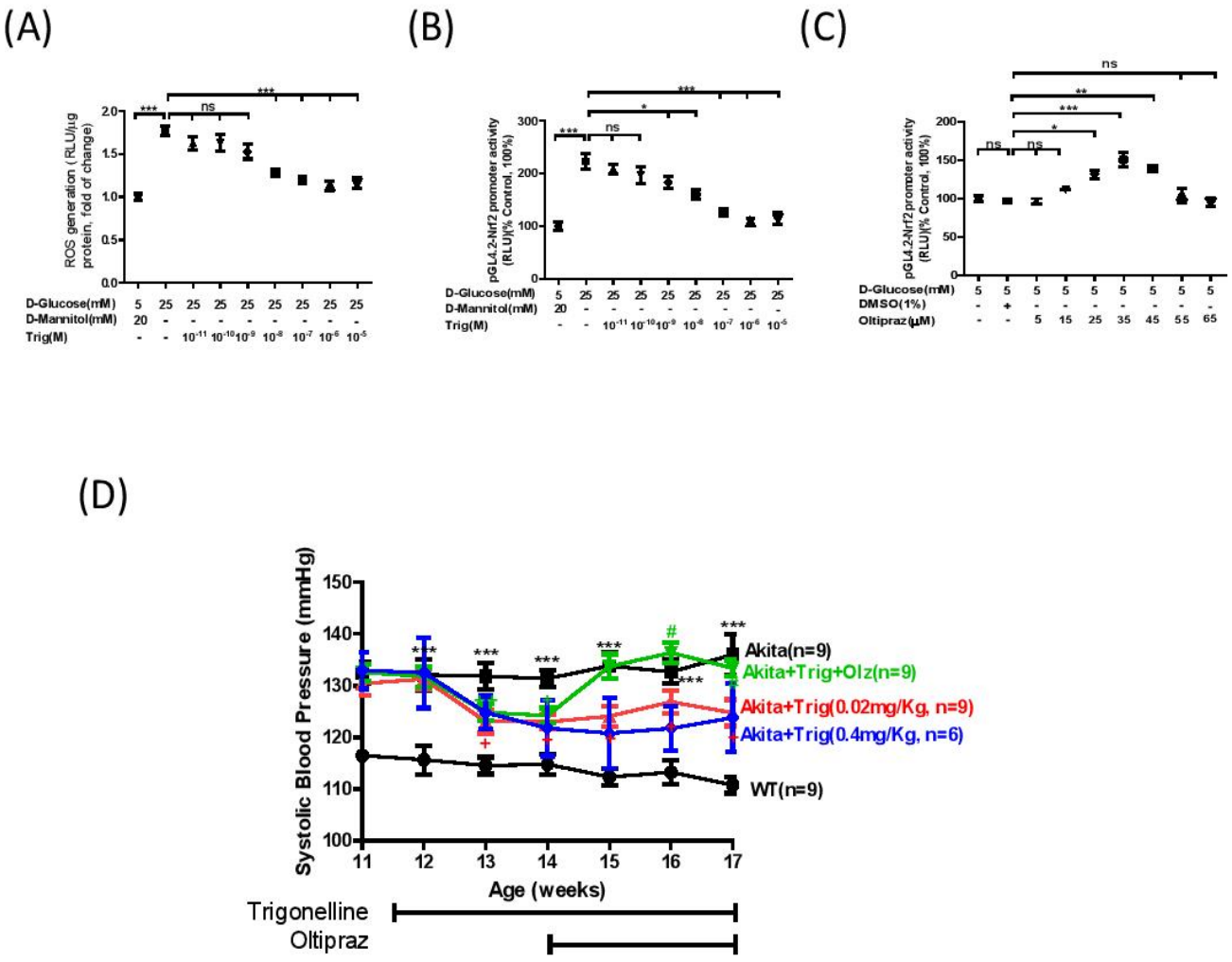
Supplementary Figure 3. Effect of trigonelline and oltipraz on ROS generation and Nrf2 promoter activity in IRPTCs and the effect of trigonelline on systolic blood pressure (SBP) in Akita mice. (A) Dose-dependent inhibitory effect of trigonelline on ROS generation in IRPTCs incubated in HG medium. Dose-dependent effect of trigonelline (B) and oltipraz (C) on Nrf2 promoter activity in stably transfected IPTCs incubated in respective HG and normal glucose medium and quantified by luciferase activity assay. Longitudinal changes in mean SBP (D) in male WT (●), Akita (■), Akita + trigonelline (Trig) (▲) and Akita + Trig + Oltipraz (Olz) (▼) mice. Values are means ± SEM, n = 9 for each group with trigonelline at 0.02 mg/kg except n=6 for group with trigonelline at 0.4 mg/kg. ***p<0.005 Akita compared to WT mice; $^{+}$P<0.05 Akita compared to Akita + Trig mice; # P<0.05 Akita + Trig compared to Akita + Trig + Olz mice.



Supplementary figure 1



Supplementary figure 2



Supplementary figure 3.

Chapter 3 – Article 2

Manuscript under revision at Diabetes (ID DB20-1126)

Overexpression of Nrf2 in Renal Proximal Tubular Cells Stimulates Sodium-Glucose Co-Transporter 2 Expression and Exacerbates Dysglycemia and Kidney Injury in Diabetic Mice

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Running Title: Nrf2, Sglt2 and nephropathy

Keywords: Nrf2, Sglt2, transgenic mice, kidney injury

Subject categories: Pathophysiology of renal disease and progression

Title: 22 words; Abstract: 200 words; Text: 4,000 (including Methods)

Abstract

We investigated the impact of nuclear factor erythroid 2-related factor 2 (Nrf2) overexpression in renal proximal tubular cells (RPTCs) on blood glucose, kidney injury and sodium-glucose co-transporter 2 (Sglt2) expression in diabetic Akita $Nrf2^{-/-}$/$Nrf2^{RPTC}$ transgenic (Tg) mice. Immortalized human RPTCs (HK2) stably transfected with plasmid containing the SGLT2 promoter, human kidneys from patients with diabetes were also studied. Nrf2 overexpression increased blood glucose, glomerular filtration rate, urinary albumin-creatinine ratio, tubulointerstitial fibrosis and Sglt2 expression in Akita $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg mice compared to their Akita $Nrf2^{-/-}$ littermates. In vitro, oltipraz or transfection of NRF2 cDNA stimulated SGLT2 expression and SGLT2 promoter activity in HK2, and these effects were inhibited by trigonelline or small interfering RNA of NRF2. The deletion of the NRF2-responsive element (NRF2-RE) in the SGLT2 promoter abolished the stimulatory effect of oltipraz on SGLT2 promoter activity. NRF2 binding to the NRF2-RE of the SGLT2 promoter was confirmed by gel mobility shift assay and chromatin immunoprecipitation assay. Kidneys from patients with diabetes exhibited higher levels of NRF2 and SGLT2 in the RPTCs than kidneys from patients without diabetes. These results identify a novel mechanism by which NRF2 mediates hyperglycemia-stimulation of SGLT2 expression and exacerbates blood glucose and kidney injury in diabetes.

Introduction

Under physiological conditions, sodium-glucose co-transporter 2 (SGLT2) in the S1/S2 segments of renal proximal tubules (RPT) mediates resorption of more than 90% of the glucose filtered by the glomerulus; SGLT1 in the late RPT (S2/S3 segments) resorbs the remaining glucose (1,2). In diabetes, excessive glucose uptake via SGLT2 may contribute to glucose toxicity, hyperfiltration and glomerular injury via a tubulo-glomerular feedback (TGF) mechanism (3). Thus, SGLT2 inhibition has the potential to reduce glucose toxicity, hyperfiltration and renal injury in diabetes. Indeed, the cardio- and reno-protective effects of SGLT2 inhibition have now been documented in large clinical trials in patients with diabetes, irrespective of whether they have chronic kidney disease (CKD) (4-7).

In addition to lowering sodium and glucose resorption, SGLT2 inhibition attenuates oxidative stress, inflammatory and fibrotic pathways and improves renal oxygenation and glomerular hyperfiltration in the diabetic kidney (8-12). Elevated SGLT2 expression and activity in RPTs were found in preclinical models of diabetes (13,14) and in patients with diabetes (15,16). However, the mechanisms underlying SGLT2 up-regulation have not been fully elucidated.

Nuclear factor erythroid 2-related factor 2 (NRF2) functions as a master regulator of redox balance and is important in cellular cytoprotective responses (17). The effect of NRF2 activation, however, is controversial in animals and humans with diabetes (18-23). We reported that global knockout of Nrf2 ($Nrf2^{-/-}$) lowered systolic blood pressure (SBP), urinary albumin creatinine ratio (ACR) and inhibited angiotensinogen (Agt) expression in RPTs of Akita $Nrf2^{-/-}$ mice (24). We have identified a putative NRF2-responsive element (NRF2-RE) in the mouse and human SGLT2 promoter.

In the present study, we investigated whether Nrf2 overexpression in renal proximal tubular cells (RPTCs) would stimulate Sglt2 expression and exacerbate blood glucose, SBP and kidney injury in Akita $Nrf2^{-/-}$ /$Nrf2^{RPTC}$ Tg mice and validated the presence of a putative NRF2-RE in the mouse and human SGLT2 promoter.

Methods

Chemicals and Constructs

D-glucose, D-mannitol, oltipraz and trigonelline were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Normal glucose (NG, 5 mM D-glucose) Dulbecco's Modified Eagle's Medium (DMEM, catalogue No. 12320), fetal bovine serum (FBS) and the expression vector pcDNA 3.1 were procured from Invitrogen, Inc. (Burlington, ON, Canada). HK-2 (an immortalized human RPTC line) (Cat. No. CRL-2190) was purchased from American Tissue Cell Collection (ATCC) (Manassas, VA, USA) (http://www.atcc.org). pGL4.20 vector containing a Luciferase reporter was obtained from Promega (Sunnyvale, CA, USA). The plasmid, pCI-HA NRF2 containing human NRF2 cDNA was obtained from Dr. Donna D Zhang (University of Arizona, Tucson, Arizona, USA). The NRF2 cDNA was subcloned into pcDNA 3.1 plasmid via Kpn1 and Not1 enzyme restriction sites. Mouse Sglt2 promoter (N-1,952/N+684) was amplified by PCR with specific primers (Supplemental Table 1) from pGEM-Sglt2–5pr-mut plasmid (25) (obtained from Dr. Isabelle Rubera, University of Nice-Sophia Antipolis, Nice, France), confirmed by DNA sequencing, and then inserted into pGL4.20 plasmid at KpnI and Xho1 restriction sites. Human SGLT2 promoter (N-1,986/N+17) was amplified from HK-2 genomic DNA by PCR with specific primers as previously described (26). QuickChange II Site-Directed Mutagenesis kits and LightShift Chemiluminescent Electrophoretic Mobility Shift Assay (EMSA) kits were procured from Agilent Technologies (Santa Clara, CA, USA) and Thermo Scientific (Life Technologies Inc., Burlington, ON, Canada), respectively. Primer biotin-labeling kits were supplied by Integrated DNA Technologies, Inc. (Coralville, IA, USA). Oligonucleotides were synthesized by Integrated DNA Technologies. Scrambled Silencer Negative Control siRNA (sc-37007) and NRF2 siRNAs (309757) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Dharmacon (Ottawa, ON, Canada), respectively. Restriction and modifying enzymes were purchased from Invitrogen, Roche Biochemicals, Inc. (Dorval, QC, Canada) and GE Healthcare Life Sciences (Baie d'Urfé, QC, Canada). The antibodies used are listed in Supplemental Table 2.

Chromatin immunoprecipitation (ChIP) assay was performed using a kit from SIMPLECHIP® PLUS SONICATION CHROMATIN IP KIT (Cell Signaling #56383) with slight

modification as previously described (27). Briefly, 70%-80% confluent HK2 cells were transfected with or without pcDNA 3.1/NRF2 cDNA plasmid for 24h and then cross-linked with formaldehyde. Chromatin was fragmented by sonication and incubated with ChIP grade anti-Nrf2 or anti-Histone H3 antibody or rabbit IgG (Supplemental Table 2). DNA was purified by spin column and used to amplify the SGLT2 promoter region containing the putative NRF2-RE or the RPL30 exon3 (internal control) by PCR with specific primers (Supplemental Table 1).

Generation of Akita Nrf2$^{-/-}$ /NRF2^{RPTC} Tg Mice

Tg mice specifically overexpressing rat Nrf2-Flag in their RPTCs were generated using a strategy similar to the method that we described previously for the generation of Agt RPTC, Cat RPTC, hnRNP F RPTC and Bmf RPTC Tg mice using the kidney-specific androgen regulated (KAP) promoter (28-31). Then, Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice were generated by cross-breeding female Nrf2^{RPTC} Tg mice (C57BL6) with male Akita Nrf2$^{-/-}$ mice (C57BL6) (24).

Physiological Studies

Male (12-week-old) non-Akita wild type (WT), Akita, Akita Nrf2$^{-/-}$, Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice (9 mice per group) were studied. All animals received standard mouse chow and water ad libitum. Animal care and procedures were approved by the CRCHUM Animal Care Committee and followed the Principles of Laboratory Animal Care (NIH Publication No. 85-23, revised 1985: http://grants1.nih.gov/grants/olaw/references/phspol.htm).

SBP (systolic blood pressure) was monitored with a BP-2000 tail-cuff pressure monitor (Visitech Systems, Apex, NC, USA) in the morning, at least 2-3 times per week, for 8 weeks (24,28-31). Each animal was accustomed to the procedure for at least 15-20 min per day for 5 days before the first SBP measurement at week 10. SBP values are presented as means ± SEM of 2 to 3 determinations per mouse per group.

Glomerular filtration rate (GFR) was estimated with fluorescein isothiocyanate inulin, as recommended by the Animal Models of Diabetic Complications Consortium (http://www.diacomp.org/) with slight modifications (24,30,31).

Blood glucose levels were measured in mice with the Accu-Chek Performa System (Roche Diagnostics, Laval, QC, Canada) throughout the study period and with a glucose colorimetric detection kit (Cayman Chemical) at age 20 weeks. The mice were housed individually in metabolic cages for 6 h for urine collection. Urine samples were assayed for albumin and creatinine by albumin enzyme-linked immunosorbent assay (ELISA, Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA) (24,30,31).

Following euthanasia, the kidneys were removed, decapsulated and weighed. Left kidneys were processed for histology and immunostaining. Right kidneys were harvested for isolation of RPTs by Percoll gradient (24,30,31). Aliquots of freshly isolated RPTs from individual mice were immediately processed for total RNA and protein analysis.

Histology

Immunohistochemical staining was performed using the standard avidin-biotin-peroxidase complex method in 4-5 sections (4 µm thick) per kidney and 4-6 mouse kidneys per group (ABC Staining System; Santa Cruz Biotechnology). Periodic acid Schiff (PAS) staining and sirius red staining were performed to assess tubulointerstitial fibrosis as previously described (24,30,31). Oxidative stress in RPTs was assessed by dihydroethidium (DHE, Sigma-Aldrich Canada Ltd) staining of frozen kidney sections (24,32). Semi-quantitation of the relative staining was done by NIH Image J software (http://rsb.info.nih.gov/ij/).

Immunofluorescence (IF) staining for Sglt2 was performed on 4-µm tissue sections from mouse kidney fixed in formalin and embedded in paraffin followed by staining with ALEXA FLUOR-594-labeled secondary antibody (Invitrogen). Proximal tubules were identified by fluorescein-labeled lotus tetragonolobus lectin (LTL, a marker of renal proximal tubule (33) (Vector Labs, Burlingame, CA). Image quantification and merge were assessed by ImageJ software

(http://rsb.info.nih.gov/ij/). To quantify the amount of Sglt2 expression, the pixel intensity of Sglt2 was divided by LTL intensity. To calculate the average ratio, 6 sections per mouse, 4-6 mice per group were analyzed.

Cell Culture

Immortalized human RPTCs (HK2) were cultured as described (26,34). Plasmids pGL4.20 or pGL4.20 containing mouse Sglt2 (N-1952/N+684) or human SGLT2 promoter (N-1,986/N+17) were transiently or stably transfected into HK2 as described (24,35,36). HK2 or stable transformants at 75-85% confluence were synchronized overnight in DMEM containing 5 mmol/l D-glucose and 1% depleted FBS, and then cultured with various concentrations of oltipraz ± trigonelline for the indicated time periods for up to 24 h. SGLT2 expression was assessed with IF in DAPI-stained HK2. In separate experiments, HK2 stable transformants were transiently transfected with pcDNA 3.1/NRF2 cDNA.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

The mRNA levels of selected genes in RPTs were quantified by RT-qPCR with forward and reverse primers as previously reported (Supplemental Table 1) (24,26,30-32)

Western Blotting (WB)

WB was performed, as previously reported (24,26,30-32). Relative densities of Agt, Nrf2, Sglt2 and β-actin bands were quantified by computerized laser densitometry (ImageQuant software, version 5.1; Molecular Dynamics, Sunnyvale, CA, USA).

Immunostaining of Kidney Specimens from Patients with or without Diabetes

Nephrectomy specimens (paraffin sections) for immunostaining were obtained from the Department of Pathology, CHUM. The study was approved by the CHUM Clinical Research Ethics Committee. All patients provided written informed consent for this research use of their kidney tissue. The clinical characteristics of 6 patients (3 non-diabetic and 3 with T2D) had been published previously (37) and are shown in Supplementary Table 3. All patients had undergone nephrectomy for kidney cancer.

Statistical Analysis

Data are expressed as mean±SEM. Statistical analysis was performed with Student`s t-test or one-way analysis of variance with the Bonferroni correction as appropriate, using Graphpad Software, Prism 5.0 (http://www.graphpad.com/prism/Prism.htm). $p<0.05$ values were taken as statistically significant.

Data and Resource Availability

All data generated or analyzed during this study are included in the published article (and its online supplementary files). No datasets were generated or analyzed during the current study.

Results

RPTC-specific Expression of Nrf2 Transgene in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg Mice

A schema for generating the Nrf2 transgenic mice is depicted in Figure 1A. RT-qPCR analysis confirmed selective expression of the Nrf2-Flag transgene in the kidney and RPTs of male Nrf2RPTC Tg mouse but was undetectable in non-Tg mouse (Figure 1B). The Nrf2-Flag transgene was detected in isolated RPTs of Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice but not in wild-type (WT), Akita or Akita Nrf2$^{-/-}$ mice (Figure 1C). A mutated ins2 gene was detected in Akita, Akita Nrf2$^{-/-}$/ Nrf2-/- and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice but not in WT mice (Figure 1D). Nrf2-Flag mRNA expression was detected in RPTs from Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice but not in WT, Akita and Akita Nrf2-/- mice

by RT-qPCR (Figure 1E). These results confirm that the KAP gene promoter directs Nrf2-Flag transgene expression in RPTs of Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice.

Nrf2 expression was higher in Akita and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice than in WT and Akita Nrf2-/- mice, respectively, by WB (Figure 1F) and immunostaining (Figure 1G). NAD(P)H:quinone oxidoreductase 1 (NQO-1) immunostaining was also higher in RPTCs from Akita and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice than in WT mice and Akita Nrf2$^{-/-}$ mice, respectively (Figure 1H), indicating that Nrf2 overexpression stimulates the expression of its downstream target gene, NQO-1 (17). In contrast, no differences in Keap 1 (Kelch-like ECH-associated protein 1) immunostaining were detected in these groups (Figure 1).

Physiological Parameters in WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg Mice

We detected significantly lower serum glucose levels in Akita Nrf2-/- mice as compared to Akita mice, and elevated glucose levels in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice as compared to Akita Nrf2$^{-/-}$ mice at age 20 weeks using a colorimetric kit (Table 1) but not by a glucometer (Supplemental Figure 1a). Longitudinal (Supplemental Figure 1b) and cross-sectional SBP measurements (Table 1) documented significantly higher SBP in Akita than in WT mice, whereas SBP was significantly lower in Akita Nrf2$^{-/-}$ mice than in Akita mice. Although SBP were higher in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice than in Akita Nrf2$^{-/-}$ mice, these differences were not statistically significant. BW of WT mice was significantly higher than those of Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice at the age of 20 weeks, with no statistically significant differences between these latter three groups (Table 1, Supplemental Figure 1c). Kidney weight and the KW/TL and GFR/BW ratios were also significantly increased in Akita mice as compared to WT, whereas these parameters were lower in Akita Nrf2$^{-/-}$ mice. GFR/BW was significantly higher in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg than in Akita Nrf2$^{-/-}$ mice (Table 1). Akita mice had significantly elevated urinary ACR levels as compared to WT mice, whereas ACR levels were lower in Akita Nrf2$^{-/-}$ than in Akita mice. Nrf2 overexpression significantly increased ACR in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg as compared to Akita Nrf2$^{-/-}$ mice (Table 1).

Nrf2 Overexpression Increased Sglt2 and Agt Expression in Tg Mice

Double immunofluorescence (IF) of kidney sections with an anti-Sglt2 antibody and LTL-FITC antibody, confirmed significantly higher Sglt2 expression in isolated RPTs from Akita mice than from WT mice (Figure 2A). Akita mice showed higher expression of Agt in isolated RPTs than WT mice (Figure 2B). In contrast, Sglt2 and Agt expression were significantly lower in isolated RPTs from Akita Nrf2$^{-/-}$ mice than Akita mice, and this was reversed in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. WB for Sglt2 and Agt (Figures 2C and 2D, respectively) and RT-qPCR of their respective mRNAs from isolated RPTs (Figures 2E and 2F) confirmed these changes.

Oxidative Stress and Tubulointerstitial Fibrosis in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg Mouse Kidneys

PAS staining revealed more pronounced proximal tubular cell atrophy, tubular luminal dilatation with accumulation of cell debris in Akita mice than in WT mice (Figure 3A). These abnormalities were attenuated in Akita Nrf2$^{-/-}$ mice and partially reversed in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice.

Staining for DHE (Figure 3B) was significantly increased in RPTs from Akita mice as compared to WT, but it did not differ from that in Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. Increased sirius red (Figure 3C) staining and transforming growth factor-beta 1 (TGF-β1) immunostaining (Figure 3D) were noted in glomeruli and tubules in Akita mice. Interstitial fibrosis and TGF-β1 immunostaining were less pronounced in Akita Nrf2$^{-/-}$ mice than in Akita mice and further increased in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice as compared to Akita Nrf2-/- mice. These changes were confirmed by semi-quantification of staining of DHE (Figure 3E) and sirius red (Figure 3F) and of TGF-β1 mRNA expression by RT-qPCR (Figure 3G). These changes were also associated with significant increases in fibronectin 1 (Fn1) mRNA expression (Figure 3H) by RT-qPCR in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice as compared to Akita Nrf2$^{-/-}$ mice. Cat mRNA expression was significantly lower in Akita mice than in WT mice, whereas Nrf2 overexpression failed to affect Cat expression in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice as compared to Akita Nrf2$^{-/-}$ mice (Figure 3I). In contrast, Nox4 mRNA expression was higher in Akita and Akita Nrf2$^{-/-}$ mice than in WT mice but was similar between Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice and Akita Nrf2$^{-/-}$ mice (Figure 3J). .

Oltipraz and NRF2 Overexpression Increased SGLT2 Expression and SGLT2 Promoter Activity in HK2

Oltipraz increased SGLT2 expression in HK2 and this was inhibited by trigonelline (Figure 4A and 4B). Oltipraz treatment also increased expression of both NRF2 and SGLT2 mRNA in a concentration-dependent manner (Supplementary Figure 2A and 2B, respectively). Transfection with NRF2 siRNA inhibited the stimulatory effect of oltipraz on NRF2 and SGLT2 mRNA expression (Figures 4C and 4D, respectively), whereas scrambled siRNA had no effect. Furthermore, oltipraz stimulated mouse and human SGLT2 promoter activity and its stimulatory effect were inhibited by trigonelline (Figures 4E and 4F, respectively). Transiently transfection of the plasmid pcDNA-NRF2 cDNA significantly stimulated NRF2 and SGLT2 mRNA expression (Figures 4G and 4H, respectively) as well as mouse and human SGLT2 promoter activity in HK2 (Figures 4I and 4J, respectively).

Localization of Nrf2-RE in Mouse and Human SGLT2 Promoter

To validate effects of the putative Nrf2-RE on the mouse Sglt2 promoter (N-1527/N-1516, 5'-CTGACACTGCT-3') and human SGLT2 promoter (N-1316/N-1305, 5'-GTGACACAGCA-3'), different lengths of Sglt2 promoters or SGLT2 promoters were transiently transfected into HK2 and then cultured ± oltipraz in NG medium. Sglt2 promoter (N-1,952/N+684) (Figure 5A) and SGLT2 promoter (N-1,986/N+17) (Figure 5B) exhibited 29-fold and 28-fold increases compared to control plasmid pGL4.20 in HK2, respectively. Deletion of nucleotides N-1952 to N-1248 in Sglt2 promoter and N-1986 to N-1285 in SGLT2 promoter, respectively, reduced the promoter activity to 24-fold and 14-fold increase as compared to pGL4.20 empty vector. Further deletion of nucleotides N-1952 to N-235 in Sglt2 promoter and N-1986 to N-194 in SGLT2 promoter, respectively, reduced the promoter activity to 16-fold and 4-fold increase as compared to pGL4.20 empty vector. Interestingly, the activity of Sglt2 promoter and SGLT2 promoter was further increased by 1.7-fold and 1.4-fold in HK2 in the presence of oltipraz (Figure 5C and 5D, respectively). Oltipraz did not increase the promoter activity of other fusion genes. Furthermore,

deletion of the putative NRF2-RE, N-1527 to N-1516 in Sglt2 promoter (Figure 5E) and N-1316 to N-1305 in SGLT2 promoter (Figure 5F), abolished the stimulatory effect of oltipraz.

EMSA revealed that the double-strand DNA fragment nucleotides N-1535 to N-1505 containing the core Nrf2-RE (N-1527 to N-1516) of Sglt2 promoter (Figure 6A) and N-1323 to N-1296 containing the core NRF2-RE (N-1316 to N-1305) of SGLT2 promoter (Figure 6B) bind to nuclear proteins from HK2 cells, which can be displaced by respective WT DNA but not by mutated DNA fragments.

ChIP assays were used to test whether endogenous NRF2 interacts with the NRF2-RE of the SGLT2 promoter in vitro. Figure 6C displays the PCR product of pulled-down DNA by anti-NRF2 antibody with primers specific to the SGLT2 promoter in HK2 cells. A ~224-bp DNA fragment was generated in naïve HK2 (lane 2) but no similar DNA fragment was generated with pull-down by anti-Histone3 (lane 3) and rabbit IgG (lane 4). The ~224-bp DNA fragment was further enhanced in HK2 when transiently transfected with pcDNA 3.1/NRF2 cDNA (lane 6). Again, no similar DNA fragment was generated with pull-down by anti-Histone3 (lane 7) and rabbit IgG (lane 8). On the other hand, a ~ 161 bp of hRPL was generated with DNA pull-down by anti-Histone3 (lanes 3 and 7) but not by rabbit IgG (lanes 4 and 8).

NRF2 and SGLT2 Expression in Kidney Sections from Patients with or without Diabetes

We detected more pronounced immunostaining for NRF2 and immunofluorescence for SGLT2 in normal areas from nephrectomy specimens from patients with kidney cancer with diabetes as compared to patients without diabetes (Figures 7). Keap 1 expression appeared to be similar in kidney specimens from patients with and without diabetes (Supplemental Figure 3). These observations are consistent with the changes observed in RPTCs of Akita mice.

Discussion

Our results demonstrate that selective overexpression of Nrf2 in RPTCs of Akita Nrf2-/- mice effectively upregulates Sglt2 expression, resulting in elevation of blood glucose levels, GFR, ACR and tubulointerstitial fibrosis. Consistently, in cultured HK2, pharmacological stimulation of NRF2 with oltipraz or transfection with NRF2 cDNA stimulated SGLT2 expression and its promoter activity, and their effects were reversed by trigonelline and NRF2 siRNA. NRF2 binds to NRF2-RE in the SGLT2 promoter as revealed by EMSA and ChIP assay. Furthermore, specimens from kidneys of patients with diabetes exhibited higher expression of NRF2 and SGLT2 in RPTs than from kidneys of patients without diabetes. Our findings identify a novel mechanism by which NRF2 activation by oxidative stress (secondary to hyperglycemia) stimulates SGLT2 expression and activation, leading to further elevations in blood glucose, hyperfiltration and progression of kidney injury in diabetes.

Akita mice, an autosomal dominant model of spontaneous type 1 diabetes (T1D) with a mutated Ins2 gene develop hyperglycemia as early as 3-4 weeks of age and closely mimic human T1D with renal and cardiac morphological changes characteristic of early to moderately advanced human T1D (38,39).

Global Nrf2 KO mice have increased susceptibility to various kidney diseases (40,41). Some studies in diabetic rodents with bardoxolone methyl (BM) analogs reported anti-diabetic effects (18,19). Other studies, however, found that BM analogs increased albuminuria and blood pressure and weight loss in Zucker obese rats with diabetes (20). At high doses, BM analogs increased atherosclerosis and kidney injury in diabetic apoE$^{-/-}$ mice (21).

We found significantly lower blood glucose levels in Akita Nrf2$^{-/-}$ mice compared with Akita mice. However, there were no differences between Akita mice and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice at 20 weeks of age, suggesting that the presence of Nrf2 in the proximal tubules prevented this amelioration.

We previously reported that Nrf2 stimulates RPTC Agt transcription through binding to an Nrf2-RE in the Agt promoter (42). While the higher RPTC Agt expression in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg compared to Akita Nrf2$^{-/-}$ mice was associated with a modest increase in SBP (3.5 mm Hg), it

did not reach statistical significance. Similar urinary Ang II levels in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg and Akita Nrf2$^{-/-}$ mice (Table 1) were also detected, which is compatible with no significant SBP effect.

In the present study, we detected increased Nrf2 and NQO-1 expression in RPTs of 20-week-old Akita mice as compared to WT mice. These were associated with marked increases in DHE staining (a marker of ROS generation). DHE staining, however, was not different in Akita Nrf2$^{-/-}$ mice compared to Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. We do not presently understand why Nrf2 overexpression did not lead to attenuation of oxidative stress in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice as compared to Akita Nrf2$^{-/-}$ mice. Previous studies from our group (42,43) and others (44,45) consistently showed enhanced NADPH oxidase activity and Nox4 expression with reduced Cat expression and activity in diabetic rodents, indicating that hyperglycemia would alter relative expression and activity of Nox4 and Cat, thereby enhancing ROS generation in the kidney (46,47). This possibility is supported by our findings of up-regulation and down-regulation of Nox4 and Cat expression, respectively, in Akita mice. However, Nox4 and Cat expression was similar in Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice.

Akita mice exhibited increased tubulointerstitial fibrosis as compared to WT mice, which was less apparent in Akita Nrf2-/- mice. Increased tubulointerstitial fibrosis was also observed in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice as compared Akita Nrf2$^{-/-}$ mice. The functional relation between Nrf2 and tubulointerstitial fibrosis is incompletely understood. One possibility is that Nrf2 stimulates Agt expression and Ang II production, which, in turn, would stimulate TGF-β1 and subsequently enhance the expression of extracellular matrix proteins and profibrotic genes in RPTCs, resulting in tubulointerstitial fibrosis. Indeed, we detected higher Agt, TGF-β1 and Fn1 expression in RPTs of Akita mice than in WT. These increases were mitigated in Akita Nrf2$^{-/-}$ mice and then augmented in Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice, linking Nrf2 with intrarenal RAS activation and upregulation of TGF-β1 expression in RPTs and consequently to tubulointerstitial fibrosis in Akita and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. Most recently, Rush et al (48) reported that genetic or pharmacologic Nrf2 activation increases proteinuria and kidney injury in several mouse models of CKD, consistent with our observation that Nrf2 overexpression or activation may exacerbate kidney injury in diabetic mice.

The mechanism by which NRF2 overexpression leads to upregulation of renal SGLT2 gene expression in diabetes remains unclear. One possibility is that hyperglycemia/ROS can augment NRF2 activation by promoting its dissociation from Keap 1 and translocation into the nucleus. NRF2 will then bind to NRF2-RE in the SGLT2 promoter region and promote gene transcription. This possibility is supported by our findings that oltipraz and NRF2 cDNA transfection increased SGLT2 promoter activity. Deletion of the putative NRF2-RE markedly reduces oltipraz up-regulation of mouse and human SGLT2 promoter activity in HK2. Moreover, biotin-labeled mouse and human NRF2-RE specifically binds to nuclear proteins. Importantly, our ChIP assays confirmed NRF2 interaction with SGLT2 promoter loci. Taken together, these data demonstrate that NRF2 binds to NRF2-RE to stimulate SGLT2 transcription in vivo.

Our results may have clinical implications that also may help explain the harmful effects of NRF2 activation observed with BM in T2D patients with CKD in the BEACON trial (23). We speculate that this adverse action may be attributed to SGLT2 and AGT upregulation by NRF2 in RPTs, leading to hyperfiltration, hypertension and exacerbation of nephropathy and to adverse cardiac effects. Thus, the critical question of whether NRF2 activation may be harmful in T2D patients with CKD warrants further investigations. Of note, the safety and efficacy of BM are currently being tested in ongoing phase 2/3 clinical trials on T1D/T2D (49).

In summary, our findings document that selective Nrf2 overexpression in RPTCs leads to upregulation of renal Sglt2 and Agt expression with subsequent increases in blood glucose and fibrotic gene expression, leading to kidney injury in mice with diabetes. Our results imply an important role of oxidative stress (hyperglycemia)-induced NRF2 expression and SGLT2 activation in the exacerbation of hyperglycemia and renal injury in diabetes. Our observations raise the possibility that selective targeting of NRF2 might provide a potentially novel approach for the prevention and reversal of diabetes-associated nephropathy.

Abbreviations

Agt, angiotensinogen; Ang II, angiotensin II; BG, blood glucose; BW, body weight; Cat, catalase; DHE, dihydroethidium; DMEM, Dulbecco's Modified Eagle's Medium; DN, diabetic nephropathy; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift

assay; Fn1, fibronectin 1; GFR, glomerular filtration rate; HG, high glucose; HK2, immortalized human renal proximal tubular cells; KW, kidney weight; NG, normal glucose; Nox4, NADPH oxidase 4; PAS, periodic acid-Schiff; RAS, renin-angiotensin system; RE, responsive element; ROS, reactive oxygen species; RPTs, renal proximal tubules; RPTCs, renal proximal tubular cells; RT-qPCR, real time-quantitative polymerase chain reaction; SBP, systolic blood pressure; SGLT2, human sodium-glucose cotransporter 2; Sglt2, mouse sodium-glucose cotransporter 2; Tg, transgenic; TGF-β1, transforming growth factor-beta 1; WB, Western blotting; WT, wild type

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The authors declare that there is no potential conflict of interest relevant to this article.

Principal investigators JSDC and SLZ are co-guarantors of these data and responsible for study conception and design; as such, they had full access to all study data, take responsibility for data integrity and vouch for the accuracy of data analysis. JSDC drafted the manuscript, SLZ contributed to data research and discussion the manuscript. CSL, KNM, AG, XZ, IC, JFC, JE and JBL contributed to the in vivo and in vitro experiments and data collection. KNM, JGF and JRI contributed to the discussion, and reviewed/edited the manuscript. All authors approved the final version for publication. No financial arrangements were made with commercial companies regarding this manuscript.

Disclosure

None

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Figure Legends

Figure 1. Generation of Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg Mice. (A) Schematic map of the kidney androgen-regulated promoter (KAP2)-rat Nrf2-Flag construct. Nrf2 transgenic mice were generated by inserting rat Nrf2 cDNA fused with Flag-tag including the stop codon into a construct containing the KAP promoter and exons 2 to 5 of human Agt gene, including non-coding DNA at the 3' terminal. The isolated 17-kb KAP2-rNrf2 transgene (digested with SpeI and NdeI) was microinjected into 1-cell fertilized mouse embryos obtained from C57Bl6 mice (Cyagen Biosciences Inc. (www.cyagen.com). (B) PCR product showing tissue expression of rNrf2-Flag mRNA in male Tg and non-Tg mice. rNrf2-Flag (404 bp) and β-actin (350 bp) fragments are indicated. Br, brain; H, heart; Ki, kidney; Li, liver; Lu, lung; Pan, pancreas; Spl, spleen; T, testis; PT, isolated proximal tubule; Pos, positive Nrf2 plasmid control. Specific PCR analysis of rNrf2-Flag transgene (C) and mouse ins 2 and mutated ins2 (D) gene in genomic DNA of offspring of WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. Ins2, insulin 2. (E) RT-qPCR of Nrf2-Flag mRNA levels in WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC}Tg mice. (F) WB of Nrf2 and β-actin protein expression in WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC}Tg mice. Representative IHC for Nrf2 (G), NQO-1 (H) and Keap 1 (I) expression in kidneys of WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice (x600). P: proximal tubule, G: glomerulus. n=9 per group for mRNA expression; n=4 per group for WB and IHC.

Figure 2. Effect of Nrf2 Overexpression on Sglt2 and Agt Gene Expression in Tg Mouse Kidneys at 20 weeks of age. (A) Representative colocalization of immunofluorescence staining for Sglt2 and LTL (marker of renal proximal tubule) (x100) and (B) immunostaining of Agt (x600) in kidney sections from WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. Representative WB of Sglt2 (C) and Agt (D) protein and RT-qPCR of Sglt2 mRNA (E) and Agt mRNA (F) in freshly isolated RPTs from WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. Values are means $\pm$ SEM, n=9 per group for mRNA expression; Statistics were done by one-way Anova; n=3-4 per group for IF and WB. *p<0.05; **p<0.01; ***p<0.005; ns, not significant.

Figure 3. Oxidative Stress and Tubulointerstitial Fibrosis in Mouse Kidneys at Age 20 Weeks. (A) PAS staining (x600), (B) DHE staining (x100), (C) Sirius red staining (x200) and (D) TGF-β1 immunostaining in kidney sections (x600) from WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mouse kidneys at the age of 20 weeks. Semi-quantification of DHE fluorescence (E), Sirius red staining (F), TGF-β1 mRNA (G), Fn1 mRNA (H), Cat mRNA (I) and Nox4 mRNA (J) expression in RPTs of WT controls, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice. Values are expressed as means ± SEM, n=9 per group for mRNA expression; Comparison were done by one-way Anova. n=4-6 per group for staining. *p<0.05; **p<0.01; ***p<0.005; ns, not significant.

Figure 4. Effect of Oltipraz and NRF2 cDNA Transfection on NRF2 and SGLT2 Gene Expression in HK2 with or without NRF2 siRNA. Immunofluorescence staining (IF) of SGLT2 expression in HK2 incubated in NG medium in the absence or presence of oltipraz ± trigonelline (x600) (A) and semi-quantitation of positive SGLT2 stained cells/total cells ratio (B). n=3 per group. Each dot indicates 20 cells, total 120 cells were randomly counted. Effect of NRF2 siRNA or scrambled (Scr) siRNA on NRF2 (C) and SGLT2 (D) mRNA expression in HK2 incubated in NG medium ± oltipraz and quantified by RT-qPCR (n=4). Effect of oltipraz on mouse (E) and human (F) SGLT2 promoter activity in HK2 in the absence or presence of trigonelline and quantified by luciferase activity assay (n=4). Effect of NRF2 cDNA transfection on NRF2 mRNA (G), SGLT2 mRNA (H) (n=6), mSglt2 promoter activity (I) and hSGLT2 promoter activity (J) in HK2 in NG medium (n=6). Promoter activity and mRNA levels in cells incubated in NG medium are expressed as arbitrary unit 1. The results are reported as fold changes of control values (means ± SEM), one-way Anova for figure B-F, unpaired t-test for figure G-I. *p<0.05; **p<0.01; ***p<0.005; ns, not significant.

Figure 5. Identification of putative NRF2-RE in mouse and human SGLT2 Promoter. Luciferase activity of plasmids containing various lengths of the mouse Sglt2 (A) and human SGLT2 (B) gene promoter in HK2 after 24-hr culture in NG medium. Luciferase activities were normalized by co-transfecting pRC/RSV vector containing beta-galactosidase cDNA. Luciferase activity of plasmids containing various lengths of the Sglt2 (C) and SGLT2 (D) gene promoter in HK2 after 24-hr culture

in NG medium with or without oltipraz stimulation. (E) pGL4.20-Sglt2 promoter (N-1,952/+684) activity with or without deletion of putative Nrf2-RE (N-1,527 to N-1,516; 5'-CTGACACTGCT-3') in HK2 after 24-hr culture in NG medium with or without oltipraz stimulation. (F) pGL4.20-SGLT2 promoter (N-1,986/+17) activity with or without deletion of putative NRF2-RE (N-1,316 to N-1,305; 5'-GTGACACAGCA-3') in HK2 after 24-hr culture in NG medium with or without oltipraz stimulation. Values are means $\pm$ SEM, n=4 per group. Statistics were done by *unpaired t-test* between control and oltipraz group for each promoter. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.005$; ns, not significant.

Figure 6. Identification of putative NRF2-RE in mouse and human SGLT2 Gene Promoter by Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay. (A) EMSA of putative biotinylated mouse double strand oligo (N-1535 to N-1505) containing Nrf2-RE (N-1,527 to N-1,516) with HK2 nuclear proteins with or without excess unlabeled WT Nrf2-RE or mutated Nrf2-REs. (B) EMSA of putative biotinylated human double strand oligo (N-1323 to N-1296) containing NRF2-RE (N-1,316 to N-1,305) with HK2 nuclear proteins with or without excess unlabeled WT NRF2-RE or mutated NRF2-REs. Arrow indicates the putative binding. The results are representative of 3 independent experiments. ChIP analysis with anti-SGLT2 antibody in HK2 (C). The HK2 with or without transient transfection with pcDNA3.1/NRF2 cDNA were lysed, and nuclei were isolated and then sonicated. NRF2 was immunoprecipitated without (-, lanes 1 and 5) or with (+, lanes 2 and 6) anti-NRF2 antibody or with anti-Histone3 (lanes 3 and 7) or rabbit IgG (lanes 4 and 8). Complexes were eluted, cross-linking was reversed, and purified DNA was used as a template in PCR with primers specific to the SGLT2 gene promoter and hRPL30 gene. DNA was separated by agarose gel electrophoresis and visualized.

Figure 7. Oxidative Stress, NRF2 and SGLT2 Expression in Nephrectomy Specimens from Patients with or without Diabetes. Samples were obtained from areas without tumor from patients who underwent nephrectomy for carcinoma of the kidney. This figure shows immunostaining for NRF2 and SGLT2 from 4 patients without diabetes (a, patient with papillary variant carcinoma; b,

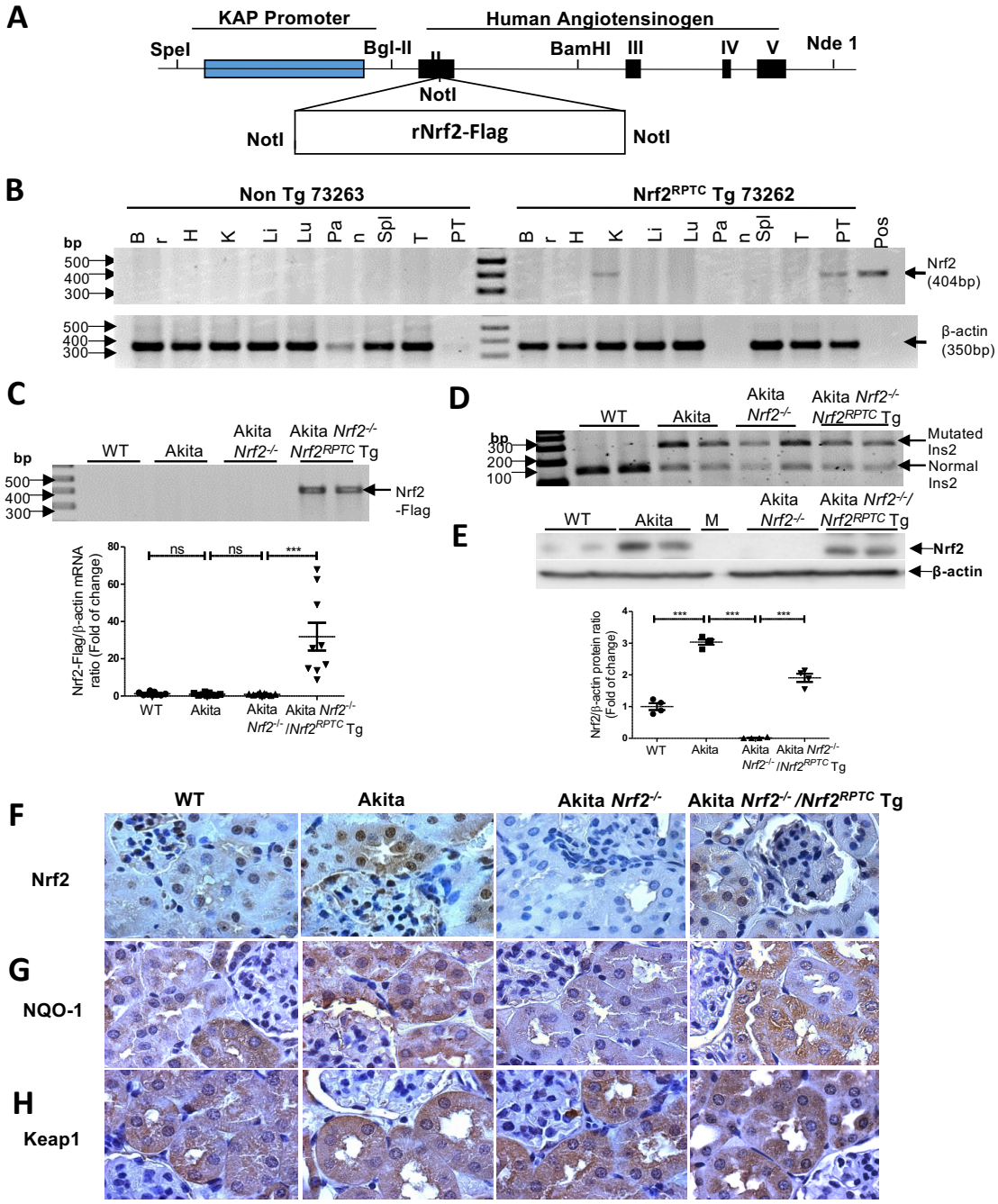
patient with clear cell carcinoma; c, patient with renal carcinoma; d, patient with clear cell carcinoma) and from 4 patients with diabetes (e, diabetic patient with papillary variant carcinoma; f, diabetic patient with clear cell carcinoma; g, diabetic patient with clear cell carcinoma; h, diabetic patient with clear cell carcinoma). NRF2 magnification x200; SGLT2 magnification x100. G, glomerulus; and P, proximal tubule.

Table 1 (Physiological parameters of WT, Akita, Akita Nrf2$^{-/-}$ and Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg mice)

	WT	Akita	Akita Nrf2$^{-/-}$	Akita Nrf2$^{-/-}$/Nrf2^{RPTC} Tg
Body weight (BW, g)	31.02±0.66	24.50±0.90***	23.30±1.27***	23.19±0.47***
Serum glucose level (mg/dL)[1]	248.2±33.2	976.1±49.81***	676.1±40.43***###	916.8±64.57***††
Systolic blood pressure (SBP, mmHg)	113.3±2.10	133.6±3.23***	121.2±0.97#	124.7±3.2*
Kidney weight (KW, mg)	346.2±7.43	548.9±11.11***	425.6±16.68*##	443.0±28.9**
KW/tibial length (KW/TL, mg/mm)	13.92±0.41	26.99±0.76***	19.04±0.59***###	21.64±1.19***
GFR/BW (ml/min/g)	7.76±0.68	19.69±1.46***	18.06±1.07***	24.00±1.88***†
ACR (μg/mg)	23.45±0.86	102.50±9.94***	53.29±6.49***###	92.08±10.75***††
Urinary Ang II/Creatinine (ng/mg)	2.60±0.31	26.59±2.16***	20.47±3.01***	21.62±2.59***

All data are expressed as means ± SEM. ***P < 0.005, **P < 0.01, *P < 0.05 vs. WT; ### P < 0.005, ## P < 0.01, # P < 0.05 vs. Akita; †† P < 0.01, † P < 0.05 vs. Akita Nrf2$^{-/-}$ (n=9).

[1]Measured with a glucose colorimetric detection kit (Cayman Chemical)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BamHI
III
IV
V
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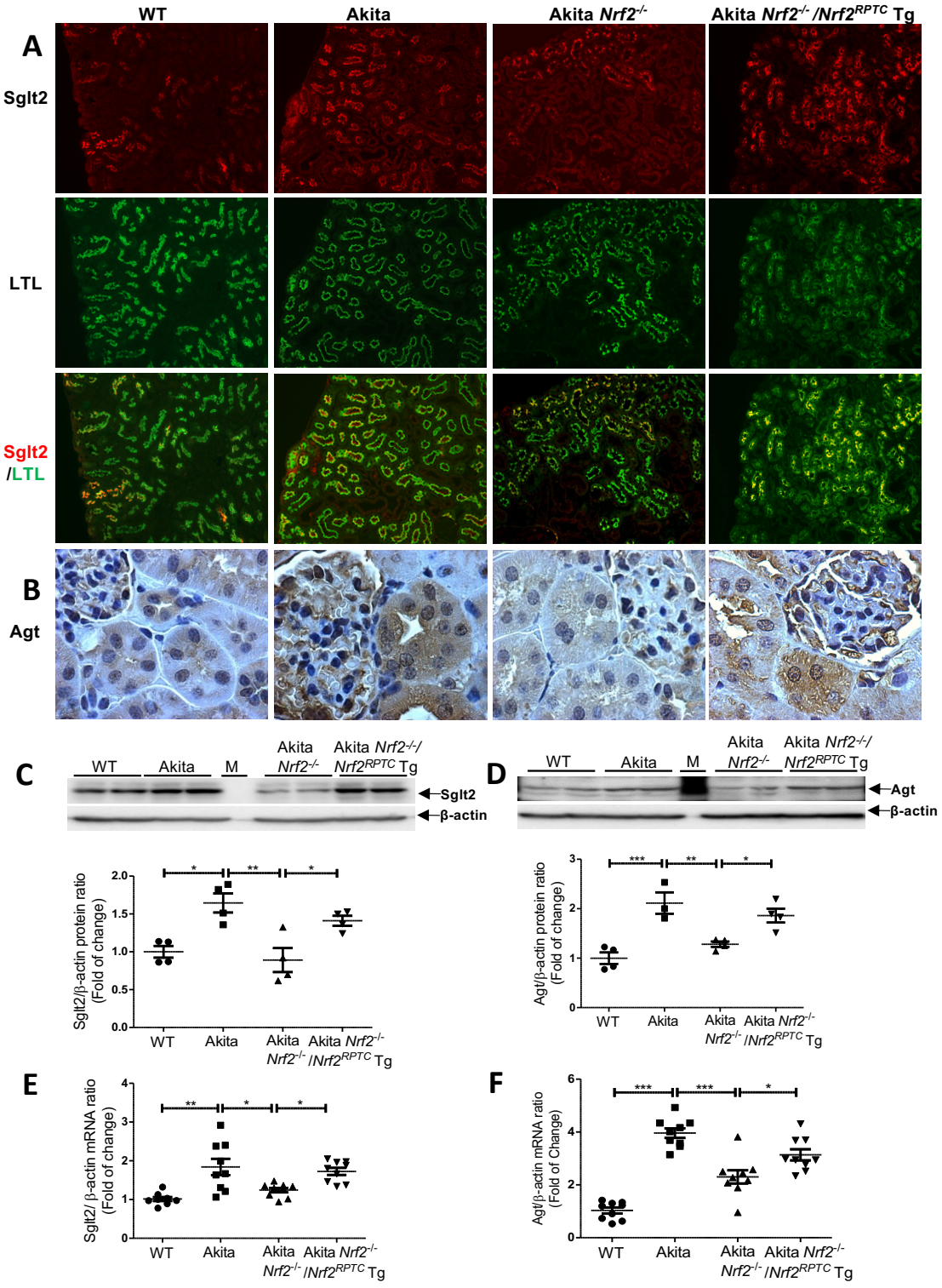
NotI
B
Non Tg 73263
Nrf2RPTC Tg 73262
B r H K Li Lu Pa n Spl T PT
B r H K Li Lu Pa n Spl T PT Pos
bp
500
400
300
Nrf2 (404bp)
β-actin (350bp)
C
WT
Akita
Akita Nrf2-/-
Akita Nrf2-/- Nrf2RPTC Tg
Nrf2 -Flag
Nrf2-Flag/β-actin mRNA ratio (Fold of change)
ns
ns
***
D
Mutated Ins2
Normal Ins2
E
M
Nrf2
β-actin
Nrf2/β-actin protein ratio (Fold of change)
F
Nrf2
G
NQO-1
H
Keap1
WT
Akita
Akita Nrf2-/-
Akita Nrf2-/- /Nrf2RPTC Tg

Figure 1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Akita Nrf2-/-
Akita Nrf2-/- /Nrf2RPTC Tg
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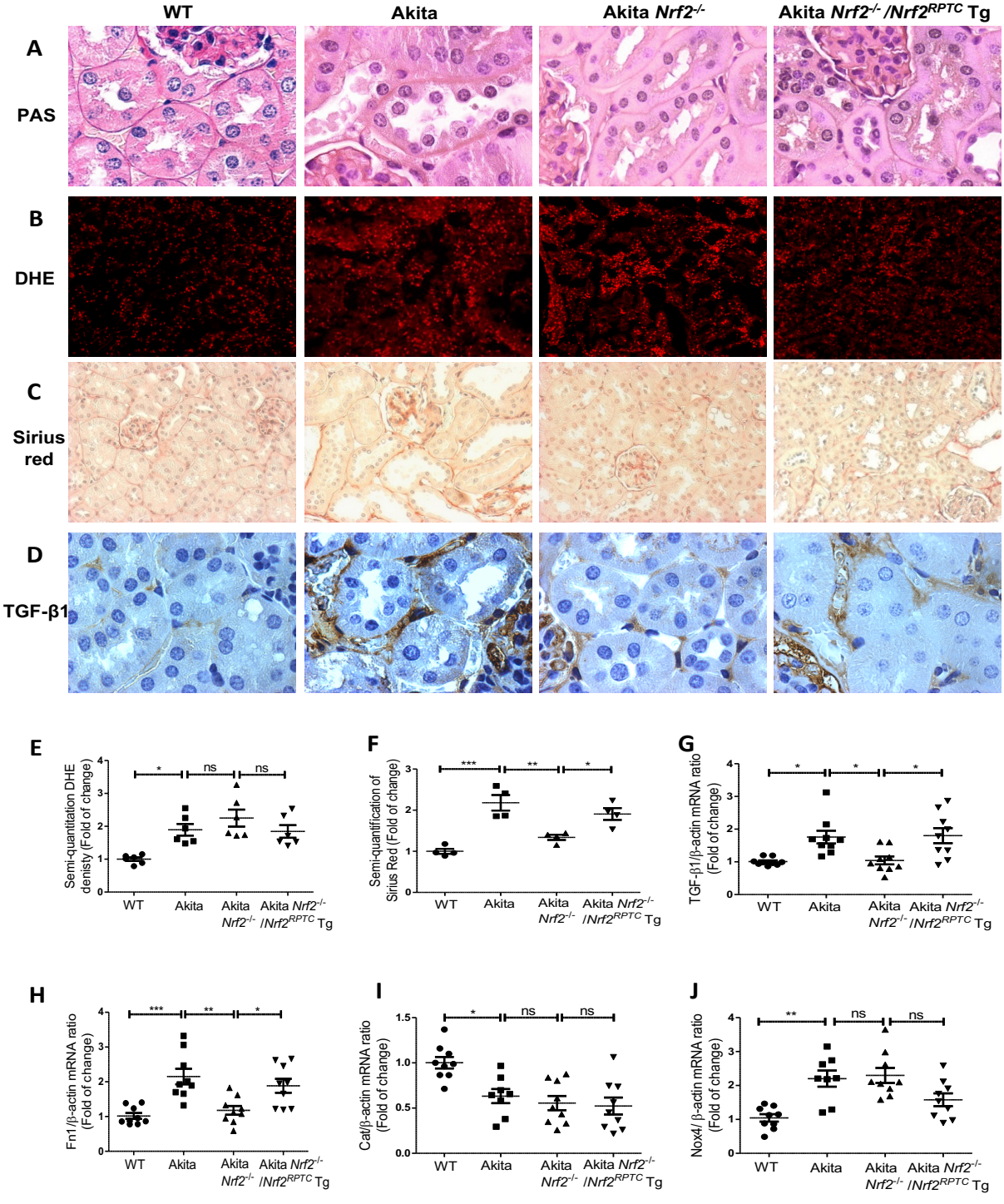
Sglt2
LTL
Sglt2
/LTL
B
Agt
C
WT
Akita
M
Akita Nrf2-/-
Akita Nrf2-/- /Nrf2RPTC Tg
Sglt2
β-actin
D
Agt
β-actin
Sglt2/β-actin protein ratio (Fold of change)
Agt/β-actin protein ratio (Fold of change)
E
Sglt2/ β-actin mRNA ratio (Fold of change)
F
Agt/β-actin mRNA ratio (Fold of Change)

Figure 2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PAS
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DHE
C
Sirius red
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TGF-β1
E
Semi-quantitation DHE denisty (Fold of change)
F
Semi-quantification of Sirius Red (Fold of change)
G
TGF-β1/β-actin mRNA ratio (Fold of change)
H
Fn1/β-actin mRNA ratio (Fold of change)
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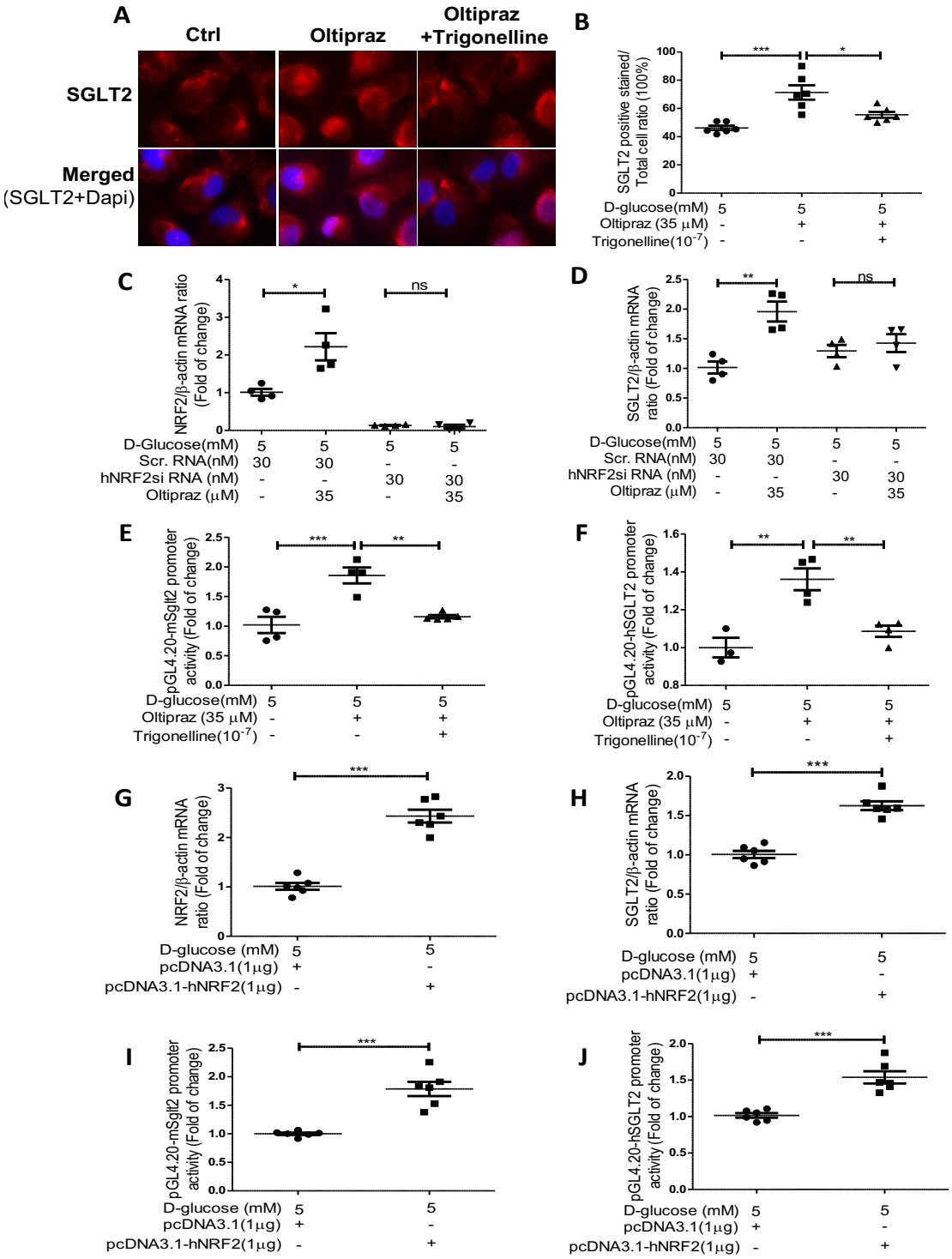
J
Nox4/ β-actin mRNA ratio (Fold of change)
ns

Figure 3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Oltipraz
Oltipraz +Trigonelline
SGLT2
Merged (SGLT2+Dapi)
B
SGLT2 positive stained/ Total cell ratio (100%)
D-glucose(mM) 5 5 5
Oltipraz (35 μM) - + +
Trigonelline(10-7) - - +
C
NRF2/β-actin mRNA ratio (Fold of change)
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D-Glucose(mM) 5 5 5 5
Scr. RNA(nM) 30 30 - -
hNRF2si RNA (nM) - - 30 30
Oltipraz (μM) - 35 - 35
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ns
D-Glucose(mM) 5 5 5 5
Scr. RNA(nM) 30 30 - -
hNRF2si RNA (nM) - - 30 30
Oltipraz (μM) - 35 - 35
E
pGL4.20-mSglt2 promoter activity (Fold of change)
D-glucose(mM) 5 5 5
Oltipraz (35 μM) - + +
Trigonelline(10-7) - - +
F
pGL4.20-hSGLT2 promoter activity (Fold of change)
D-glucose(mM) 5 5 5
Oltipraz (35 μM) - + +
Trigonelline(10-7) - - +
G
NRF2/β-actin mRNA ratio (Fold of change)
D-glucose (mM) 5 5
pcDNA3.1(1μg) + -
pcDNA3.1-hNRF2(1μg) - +
H
SGLT2/β-actin mRNA ratio (Fold of change)
D-glucose (mM) 5 5
pcDNA3.1(1μg) + -
pcDNA3.1-hNRF2(1μg) - +
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pGL4.20-mSglt2 promoter activity (Fold of change)
D-glucose (mM) 5 5
pcDNA3.1(1μg) + -
pcDNA3.1-hNRF2(1μg) - +
J
pGL4.20-hSGLT2 promoter activity (Fold of change)
D-glucose (mM) 5 5
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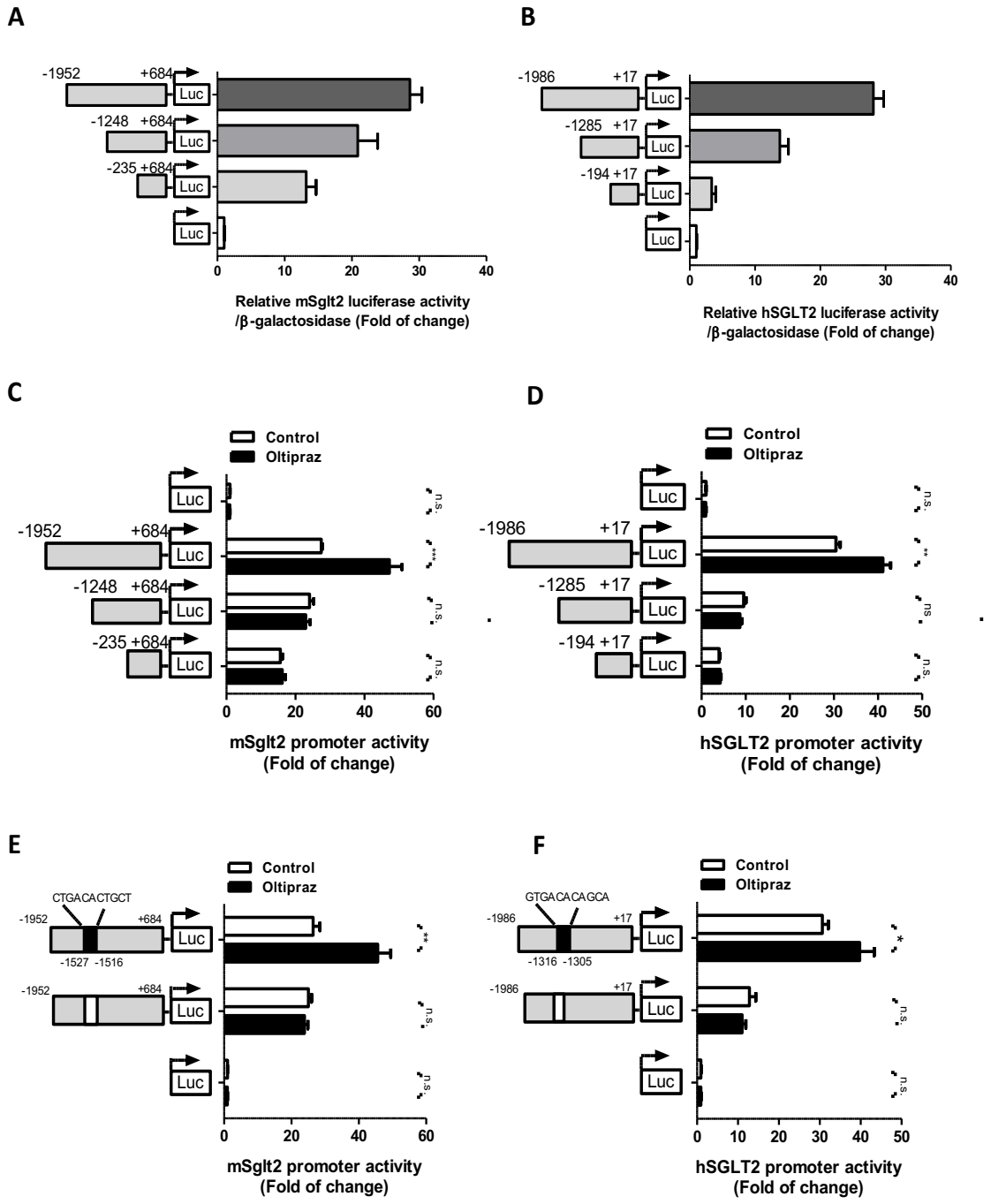
pcDNA3.1-hNRF2(1μg) - +

Figure 4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Relative mSglt2 luciferase activity
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+17
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+17
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+17
Relative hSGLT2 luciferase activity
/β-galactosidase (Fold of change)
C
Control
Oltipraz
n.s.
***
mSglt2 promoter activity
(Fold of change)
D
**
hSGLT2 promoter activity
(Fold of change)
E
CTGACACTGCT
-1527
-1516
F
GTGACACAGCA
-1316
-1305
*

Figure 5

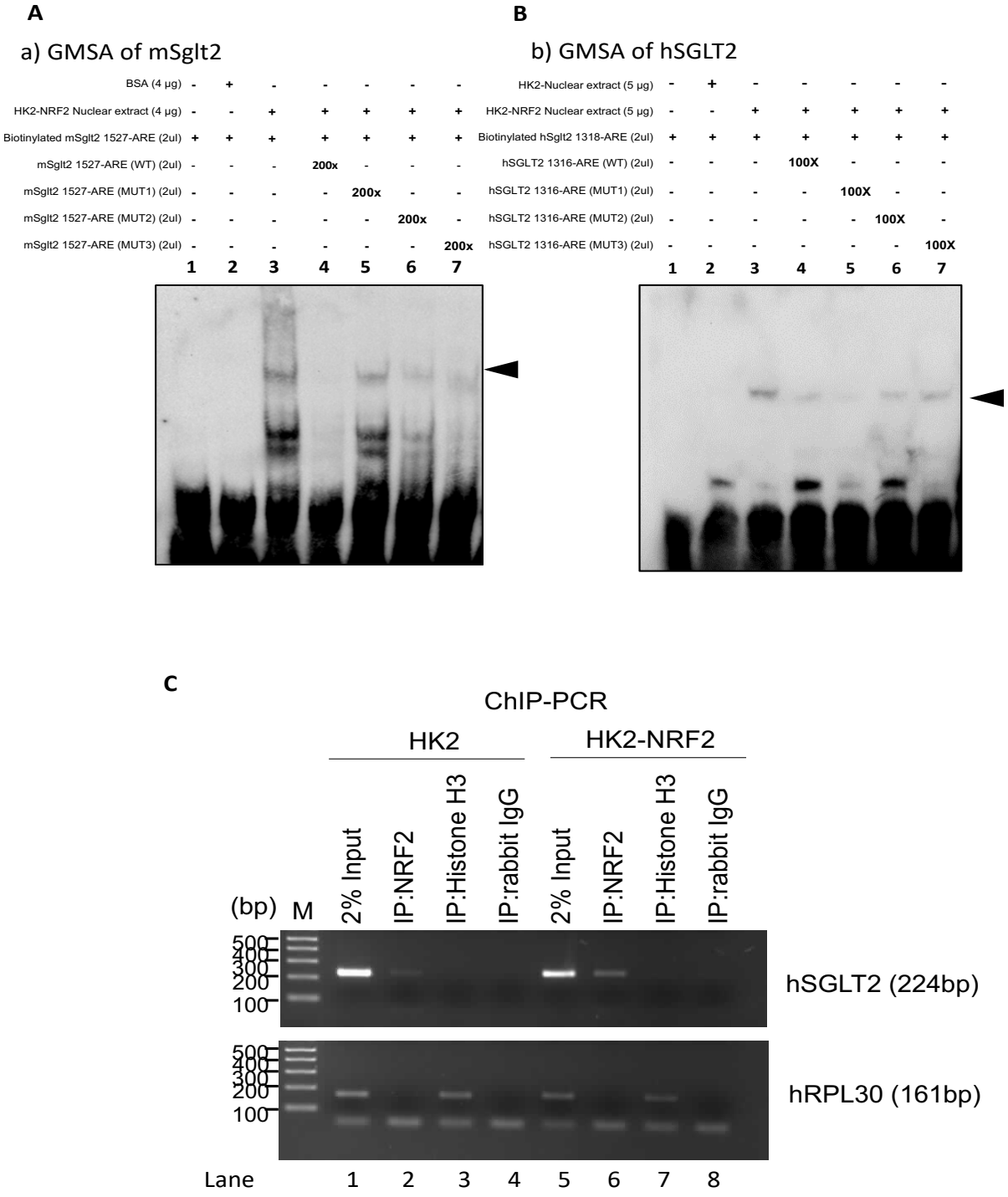
A
a) GMSA of mSglt2
BSA (4 µg)
HK2-NRF2 Nuclear extract (4 µg)
Biotinylated mSglt2 1527-ARE (2ul)
mSglt2 1527-ARE (WT) (2ul)
mSglt2 1527-ARE (MUT1) (2ul)
mSglt2 1527-ARE (MUT2) (2ul)
mSglt2 1527-ARE (MUT3) (2ul)
200x
1 2 3 4 5 6 7
B
b) GMSA of hSGLT2
HK2-Nuclear extract (5 µg)
HK2-NRF2 Nuclear extract (5 µg)
Biotinylated hSglt2 1318-ARE (2ul)
hSGLT2 1316-ARE (WT) (2ul)
hSGLT2 1316-ARE (MUT1) (2ul)
hSGLT2 1316-ARE (MUT2) (2ul)
hSGLT2 1316-ARE (MUT3) (2ul)
100X
1 2 3 4 5 6 7
C
ChIP-PCR
HK2
HK2-NRF2
2% Input
IP:NRF2
IP:Histone H3
IP:rabbit IgG
(bp)
M
500
400
300
200
100
hSGLT2 (224bp)
hRPL30 (161bp)
Lane
1 2 3 4 5 6 7 8

Figure 6

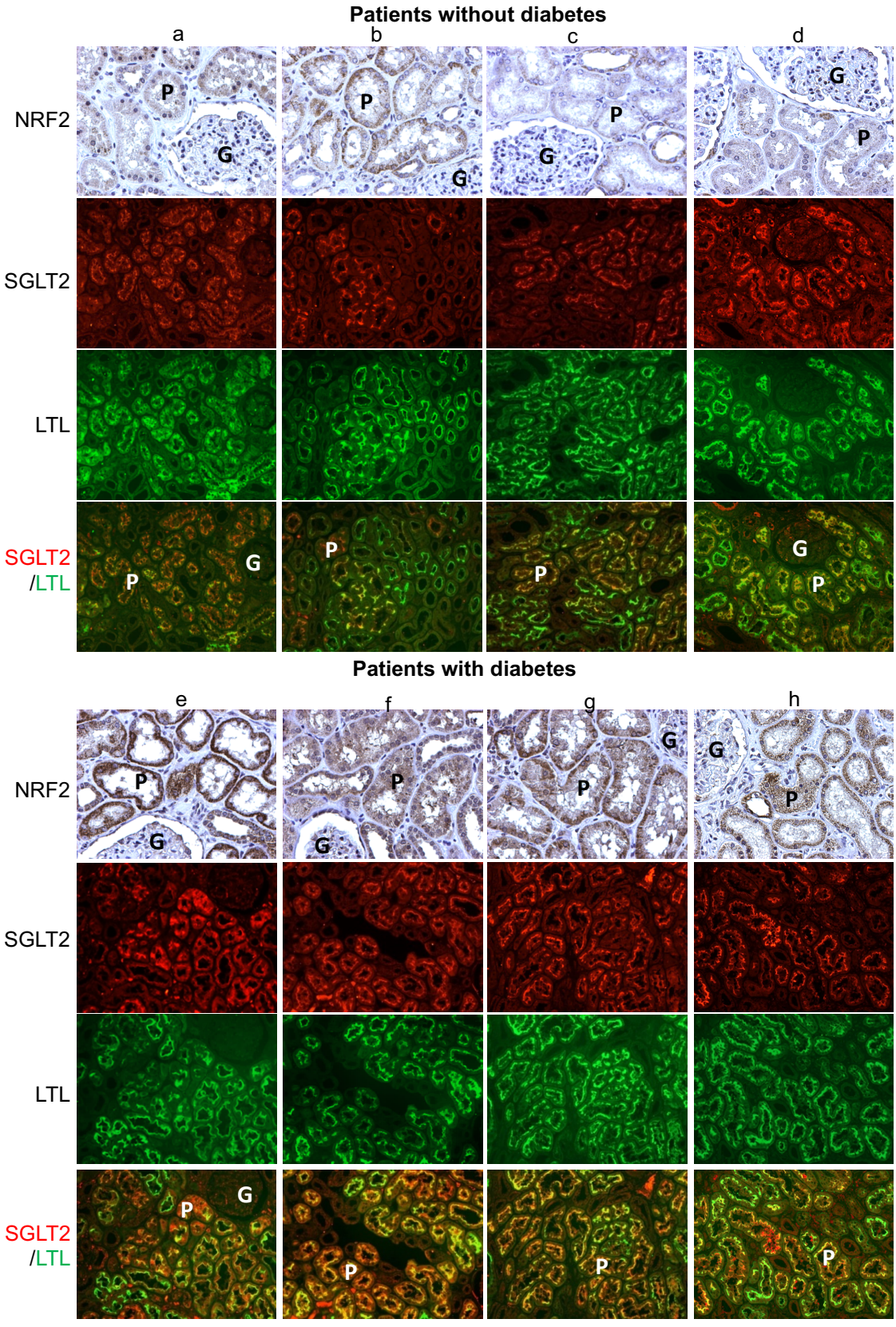
Patients without diabetes
a
b
c
d
NRF2
SGLT2
LTL
SGLT2
/LTL
P
G
Patients with diabetes
e
f
g
h
NRF2
SGLT2
LTL
SGLT2
/LTL
P
G

Figure 7

Supplemental method 1: ChIP assay

Briefly, 70%-80% confluent HEK293 cells were transfected with or without pcDNA 3.1/NRF2 plasmid for 24h. Samples were cross-linked in 1% formaldehyde for 15min. Cells were lysed and nuclei were pelleted by centrifugation. Chromatin fragmentation was sonicated. Chromatin was immunoprecipitated with 10µl of anti-Nrf2 (ChIP grade, Abcam AB62352), 10µl positive control Histone H3 (D2B12) (ChIP grade, Cell signaling, CST4620S) and 10µl negative control rabbit IgG (Cell signaling, CST2729P) antibody. The reversal cross-links by 5M NaCl and proteinase K were incubated at 65°C for 2h. DNA was purified by spin columns. Human SGLT2 promoter region containing putative antioxidant responsive element (ARE) bound to Nrf2 was amplified from DNA by PCR with specific primers (Supplemental Table 1). Human RPL30 PCR was performed as an internal control. Primers used for human RPL30 exon3 (Cell signaling, CST7014) were provided by the kit. PCR products were analyzed in 2% agarose gel. The expected size of the PCR product is 224 bp for SGLT2 promoter and 161 bp for human RPL30.

Supplemental method 2: Generation of Nrf2-Tg mice

In brief, rat Nrf2 cDNA was cloned from rat kidney total RNA by RT-PCR, fused with Flag tag at 3' end and inserted into a plasmid containing the kidney androgen regulated promoter (KAP2) at Not1 site. Isolated KAP2-Nrf2-Flag transgene was then microinjected into one cell fertilization mouse embryos by a standard procedure (performed by Cyagen Biosciences Inc. (www.cyagen.com)). Positive founders were then crossed with WT C56BL/6 mice for F1 and further generation. Plasmid pKAP2 was a gift from Dr. Curt D. Sigmund (University of Iowa, Iowa City, IA).

Supplemental Table 1. Primers

Gene (Species; Accession number)	**Forward and Reverse Primers**
Primers for mice genotyping	
Ins2 (mouse; NT_039437.7)	F: TGCTGATGCCCTGGCCTGCT
	R: TGGTCCCACATATGCACATG
$Nrf2^{-/-}$ (genotyping)	Common-F: GCCTGAGAGCTGTAGGCCC
	WT-R: GGAATGGAAAATAGCTCCTGCC
	Mutant-R: GACAGTATCGGCCTCAGGAA
$Nrf2^{RPTC}$ Tg (genotyping)	F: GCTGGAAAACATTGTAGAGCTG
	R: CTGATCTCAGCTACACATTGGA
Primers for RT-qPCR	
Nrf2 (mouse/rat; NM_010902.3)	F: CGCCGCCTCACCTCTGCTGCCAGTAG
	R: AGCTCATAATCCTTCTGTCG
Nrf2 (human; NM_006164.5)	F: ACACGGTCCACAGCTCATC
	R: TGTCAATCAAATCCATGTCCTG
Sglt2 (mouse; NM_133254.4)	F: TTGGTGTTGGCTTGTGGTCTAT
	R: ATGTTGCTGGCGAACAGAGA
SGLT2 (human; NM_003041.4)	F: GACACGGTACAGACCTTCGTCAT
	R: CTCCCAGGTATTTGTCGAAGAGA
Agt (mouse/rat; NM_031144.3)	F: CCACGCTCTCTGGATTTATC
	R: ACAGACACCGAGATGCTGTT
Tgf-β1 (mouse; NM_011577)	F: CCAAACTAAGGCTCGCCAGTC
	R: GGCACTGCTTCCCGAATGTC
FN1 (mouse; NM_001276413.1)	F: TAGCAGGCTACCGACTGACCG
	R: CACCCAGCTTGAAGCCAATCC
Catalase (mouse; NM_009804.2)	F: CGACCAGATGAAGCAGTGGA
	R: CCACTCTCTCAGGAATCCGC
Nox4 (mouse; NM_015760.4)	F: TGGCCAACGAAGGGGTTAAA
	R: GATGAGGCTGCAGTTGAGGT
β-actin (human/mouse/rat; NM_031144.3)	F: ACGATTTCCCTCTCAGCTT
	R: TACAATGAGCTGCGTGTGGC

Supplemental Table 1. Primers (continued)

Gene (Species; Accession number)	**Forward and Reverse Primers**
Primers for Sglt2 promoter site-directed mutation	
del hSGLT2 (-1316)-ARE	F: ACTGTACTCCAGCTTTGGAAGACCCTGTCTCAAAAC
	R: GTTTTGAGACAGGGTCTTCCAAAGCTGGAGTACAGT
del mSglt2 (-1527)-ARE	F: AGGTTCTCAGGCATTTTGGGATGGGTCCCATTTC
	R: GAAATGGGACCCATCCCAAAATGCCTGAGAACCT
Primers for truncated Sglt2 promoters	
mSglt2 (-1952)-F	GGACATCACATGGTACCAAAC
mSglt2 (-1248)-F	AAATTGGTACCGATGCAGGTTCTATACAGGAG
mSglt2 (-235)-F	AAATTGGTACCGCTTGGAACACTGGGTATAG
mSglt2 (-684)-(xhoI)-R	TTTTCTCGAGCACCAACTGTGCCTCTATT
hSGLT2 (-1285)-F	GAAACACATGCTTTGTTGG
hSGLT2 (-194)-F	TAAGGCCCAGGAAAGAGTGC
hSGLT2 (-1986)-(XhoI)-F	ttaaCTCGAGGTCTGTAACACACACGTGTC
hSGLT2 (+17)-(BglII)-R	aattAGATCTCATTCTCCCCAGGATCTGC
Primers for EMSA	
hSGLT2 promoter	
BIO-GS-hSGLT2 (-1316)	F: AGCTTTGGTGACACAGCAAGACCCTGT
	R: ACAGGGTCTTGCTGTGTCACCAAAGCT
hSGLT2 (-1316) WT	F: AGCTTTGGTGACACAGCAAGACCCTGT
	R: ACAGGGTCTTGCTGTGTCACCAAAGCT
hSGLT2 (-1316) MUT1	F: AGCTTTGGaatCACAGCAAGACCCTGT
	R: ACAGGGTCTTGCTGTGATTCCAAAGCT
hSGLT2 (-1316) MUT2	F: AGCTTTGGaatCACAaaAAGACCCTGT
	R: ACAGGGTCTTTTTGTGATTCCAAAGCT
hSGLT2 (-1316) MUT3	F: AGCTTTGGaataaaaaaAAGACCCTGT
	R: ACAGGGTCTTTTTTTTATTCCAAAGCT
mSglt2 promoter	
BIO-GS-mSglt2 (-1527)	F: GACCCATCCCTGACACTGCTAAAATGCCTGA
	R: TCAGGCATTTTAGCAGTGTCAGGGATGGGTC
mSglt2 (-1527) WT	F: GACCCATCCCTGACACTGCTAAAATGCCTGA
	R: TCAGGCATTTTAGCAGTGTCAGGGATGGGTC
mSglt2 (-1527) MUT1	F: GACCCATCCCaataaaaaaTAAAATGCCTGA
	R: TCAGGCATTTTAttttttattGGGATGGGTC
mSglt2 (-1527) MUT2	F: GACCCATCCCaatCACTaaTAAAATGCCTGA
	R: TCAGGCATTTTAttAGTGattGGGATGGGTC
mSglt2 (-1527) MUT3	F: GACCCATCCCaatCACTGCTAAAATGCCTGA
	R: TCAGGCATTTTAGCAGTGattGGGATGGGTC
Primers for ChIP assay	
ChIP-SGLT2 (177)	F: CCAGCTATTTGGGAAGCTGA
ChIP-SGLT2 (400)	R: TGTGCAACGACTTTCCTGAG

Supplemental Table 2. Antibodies for Western Blotting

Antibody (Host)	**WB/IHC** (Dilution)	**Suppliers**
Nrf2 (mouse monoclonal, ab89443) (rabbit polyclonal, ab31163) (rabbit monoclonal, ab62352)	WB (1:2000); IHC (1:400) Chip grade (1)	Abcam
NQO-1 (mouse monoclonal, ab28947)	IHC (1:400)	Abcam
Keap 1 (rabbit polyclonal, ab66620)	IHC (1:400)	Abcam
Sglt2 (D-6, mouse monoclonal, sc-393350) (rabbit polyclonal, ab85626)	WB (1:2000) IF (1:400)	Santa Cruz Biotechnology Abcam
Agt (rabbit polyclonal, 2)	WB (1:2000); IHC (1:200)	Generated in our lab
Tgf-β1 (rabbit polyclonal, sc-146)	IHC (1:200)	Santa Cruz Biotechnology
Histone H3 (rabbit monoclonal, CST4620s)	Chip grade	Cell signaling
IgG (rabbit polyclonal, CST2729P)	Chip grade	Cell signaling
β-actin (mouse monoclonal, a5441)	WB (1:10000)	Sigma

(1) Use of this antibody is as descripted in the supplemental methods.
(2) Wang L, Lei C, Zhang et al. Synergistic effect of dexamethasone and isoproterenol on the expression of angiotensinogen in immortalized rat proximal tubular cells. Kidney International 53:287-295, 1998.

Supplemental Table 3. Patient Characteristics

Patient ID	a	b	c	d	e	f	g	h
Diabetes (Non/Yes)	Non	None	None	None	Yes (Type 2)	Yes (Type 2)	Yes (Type 2)	Yes (Type 2)
Age (y)	38	50	63	64	67	53	80	65
Sex (M or F)	F	F	F	M	M	M	M	M
Serum creatinine (mg/dL)	0,54	0,76	0,74	0,80	1,71	1,02	1,73	1,74
Serum glucose (mg/dL)	97,3	100,9	102,7	97,0	232,4	195,6	228,8	200,0
Urine glucose	0	0	0	0	6 mmol/L	3 mmol/L	17 mmol/L	56 mmol/L
Urine protein*	0	0	0	0	2,65 g/L (2)	0	1,5 g/L	0,73 g/L
GFR (mL/min)**	140	86	85	92	40	81	38	73
Known drugs used	ARB	None	None	ARB/HCT2	***	****	*****	*****
Hypertension (Yes or No)	Yes	No	No	Yes	Yes	Yes	Yes	Yes
Diagnosis	Papillary variant carcinoma	Clear cell carcinoma	Renal carcinoma (1)	Clear cell carcinoma	Papillary variant carcinoma	Clear cell carcinoma	Clear cell carcinoma	Clear cell carcinoma

*Urine protein is determinated on dipstick.

**GFR is calculated with MDRD equation.

(1) with changes suggestive of renal carcinoma, thyroid-like

(2) obtained with urinary collect

***Diuretic, ARB, insulin, Beta-blocker, Calcium Channel Blocker

****Diuretic, Angiotensin Receptor Blocker, antineoplasic agent, oral hypoglycemic agent, Beta-blockers, Calcium Channel Blockers

***** ACEI, Oral hypoglycemic agent, Beta-blocker

Supplemental Figures

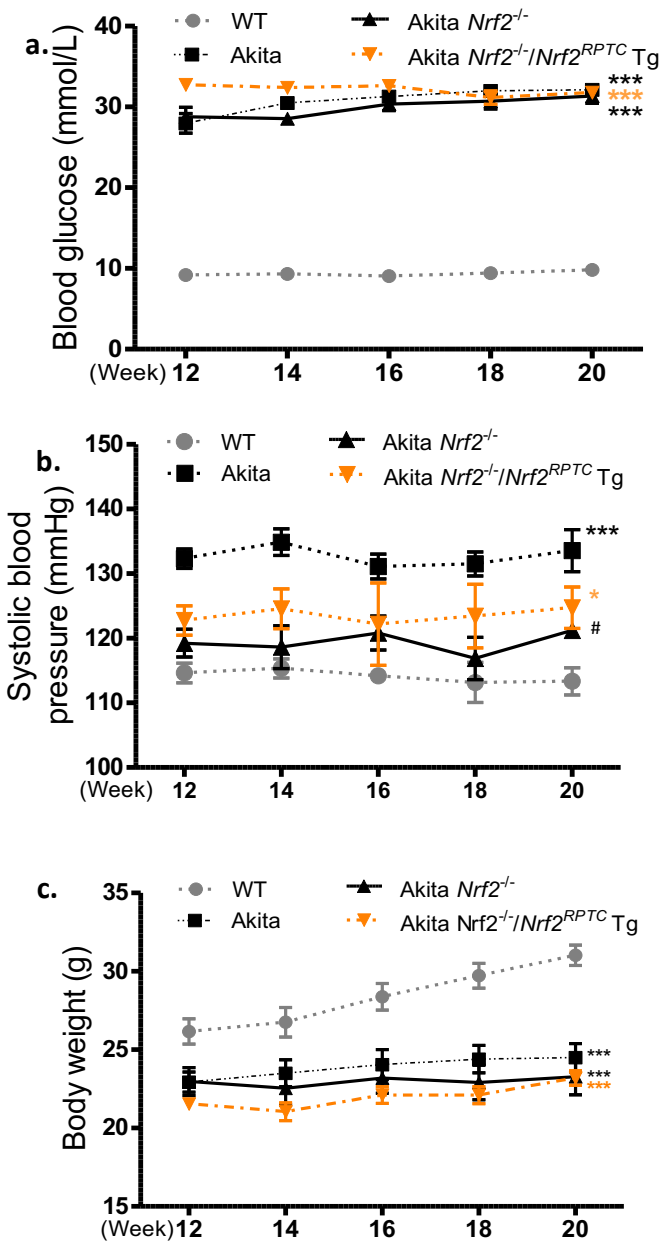

Figure 1. Longitudinal blood glucose (BG, A), systolic blood pressure (SBP, B) and body weight (BW, C) of WT, Akita, Akita *Nrf2*$^{-/-}$ and Akita *Nrf2*$^{-/-}$/*Nrf2*^{RPTC} Tg mice. BG level, SBP and BW were recorded every two weeks starting week 12 to week 20. BG and SBP were measured by glucometer (Accu-Chek Performa System) and BP-2000 Tail-cuff Pressure Monitor, respectively. All data are expressed as means ± SEM. *P < 0.05 ***P < 0.005 vs. WT at 20 weeks, #P < 0.05 vs. Akita Nrf2$^{-/-}$ (n=9); #P < 0.05 vs. Akita at 20 weeks.

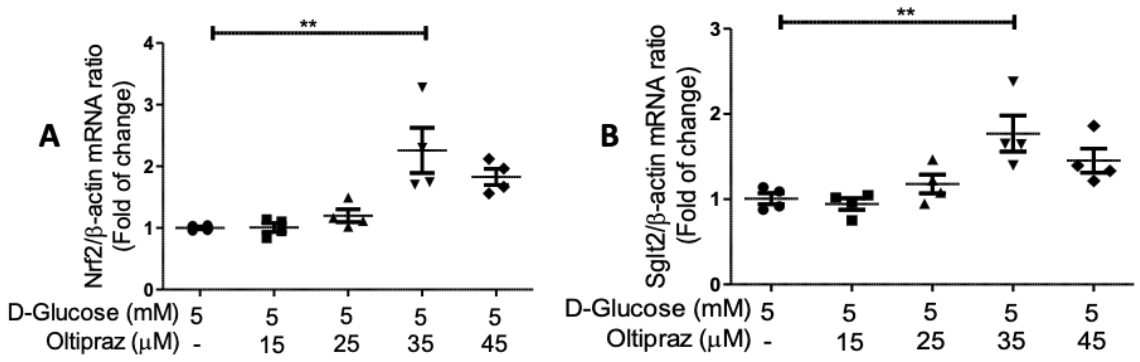

Figure 2. Oltipraz stimulates the expression of Nrf2 mRNA and Sglt2 mRNA in a dose-dependent manner in HK2 cells. Cells were incubated with Oltipraz for 24 hours in normal glucose (5 mM). All data are expressed as means ± SEM. **P < 0.01 (n=4).

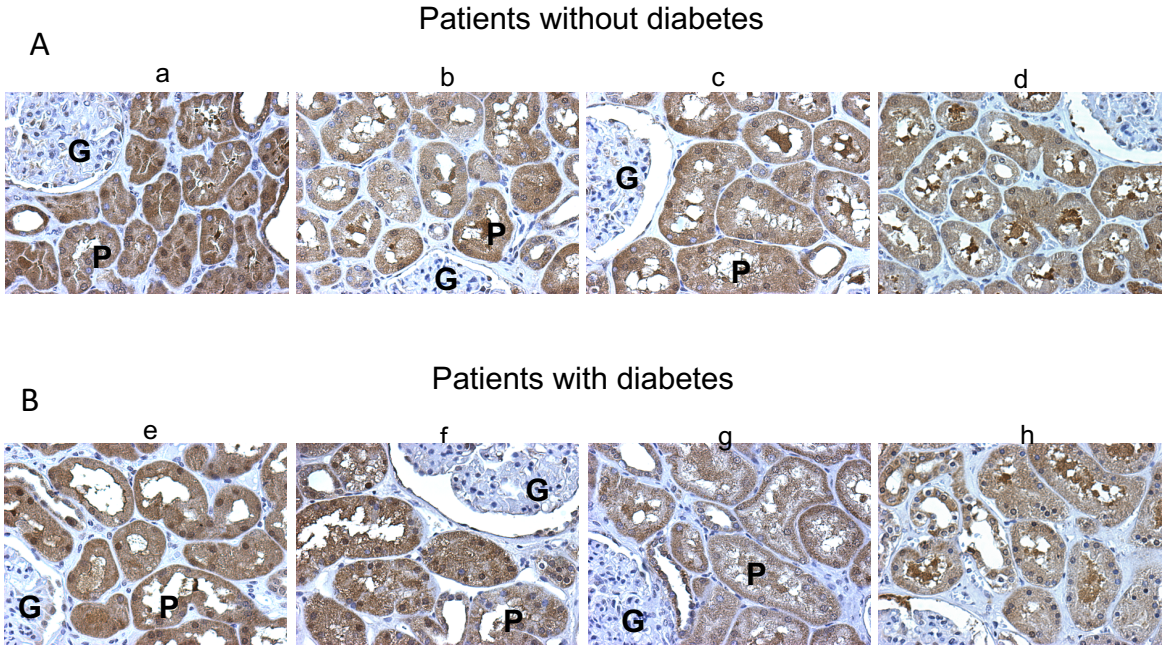

Figure 3. KEAP 1 IHC staining in patients without (A) and with diabetes (B) (G, glomerulus; and P, proximal tubule; X200) .

.

Chapter 4 – Discussion

4.1 Nrf2 in hyperglycemia-induced oxidative stress

4.1.1 Activation of Nrf2 in DN

DN is one of leading causes of ESRD and characterized by albuminuria, hypertension, decreased GFR and renal function. Hyperglycemia-induced oxidative stress is one of the major pathogenesis in the progression of DN. Nrf2 is an antioxidant factor that abundantly expresses in the kidneys especially in the cortical tubules [148, 190]. In diabetic condition, oxidative stress causes the conformation change of Keap 1, which results in translocation of Nrf2 into nucleus. Nrf2 then binds to AREs sequences in the promoter of various genes such as NQO-1 and HO-1 and initiates their transcription [300]. Consistent with these findings [189, 301], our studies found that Nrf2 pathway and its downstream gene, NQO-1, were significantly activated in both type 1 (Akita) and type 2 (db/db) diabetic mice model at week 20 and 16, respectively.

However, several studies reported a decrease of Nrf2 expression in long-term diseases condition. For example, in chronic kidney diseases induced by 5/6 nephrectomy in Sprague-Dawley rats, Nrf2 with its downstream genes were mildly decreased at 6 weeks and significantly diminished at 12 weeks [302]. In diabetic condition, it has been suggested that Nrf2 was adaptively activated to overcome hyperglycemia-induced oxidative stress at the early stage, whereas expression of Nrf2 and its downstream antioxidants were decreased at the late stage of diabetes [301, 303]. Other studies have reported that Nrf2 protein level and its downstream genes, NQO1 and HO-1, were increased at 3 months but decreased at 6 months in the heart of STZ-induced diabetic mice [304, 305]. Jiang's group found that high glucose can induce ROS production, activate Nrf2 and its downstream genes in human renal mesangial cells [189], whereas Sireesh *et al.* reported a decreased level of Nrf2 in peripheral blood mononuclear cells in diabetic patients with poor glycemic control, compared to diabetic patients with good glycemic control [306]. In our studies, we found that Nrf2 and its downstream genes were increased in type 1 diabetic Akita mice model at the age of 20 weeks. In db/db mice model, Nrf2 and its downstream gene, NQO-1, were increased at week 12 and 16 and then mildly decreased at week

20 in isolated RPTCs, indicating that the expression of Nrf2 may vary at at different stage of diabetes.

4.1.2 Nrf2 deficiency in diabetic condition

Several studies have shown that activation of Nrf2 protected diabetic kidney from oxidative stress. However, our studies yielded different results. First, Nrf2 deficiency in 20-week Akita mice did not exert significantly increased oxidative stress markers (though there is a trend of increase). Second, overexpression of Nrf2 in RPTCs by transgene in Akita $Nrf2^{-/-}$ showed only slight decrease of oxidative stress compared to Akita $Nrf2^{-/-}$.

We cannot presently understand why loss of Nrf2 did not dramatically increase the level of ROS and overexpression of Nrf2 in RPTCs failed to attenuate oxidative stress. One possible explanation might be that hyperglycemia enhances nuclear factor-κB (NF-κB) activation which competes for Nrf2 stimulation via competitively binding to the same domain of CREB-binding protein (CBP), resulting in a primarily increased oxidative stress and inflammation in diabetic condition. To be specific, NF-κB is a family of transcription factor that respond to inflammation, cell proliferation and infections as well as redox sensitive factors. Persistent hyperglycemia significantly activated NF-κB and its downstream genes such as monocyte chemo-attractant protein-1 (MCP-1) in RPTCs, leading to macrophage infiltration, renal injury and increased microalbuminuria in DN [307, 308]. One of the most common subunits of NF-κB, p65, was demonstrated that competed with Nrf2 for a transcriptional co-activator CBP-p300 complex and subsequently inhibits the expression of Nrf2, resulting in activation of inflammation and kidney damage [309, 310].

Interestingly, a ‘glycemic memory’ has been reported regarding of the oxidative stress in diabetic condition. In diabetes, the term ‘glycemic memory’, also named ‘hyperglycemia memory’, refers to the profound transcriptional changes by the epigenetics regulation even in transient hyperglycemia [311]. Hyperglycemia seems to be remembered in many organs such as the kidneys, eyes and heart. Even after correction of hyperglycemia, tissue damages are progressively exacerbated due to persistent epigenetic changes. This theory has been used to explain the persistent mitochondrial superoxide production induced by hyperglycemia [312]. In

human aortic endothelial cells, transient hyperglycemia is sufficient to induce long-lasting activation NF-κB subunit p65 and increase mitochondrial ROS production. The function of Nrf2 might be partially suppressed by the NF-κB pathway, and not sufficient to lower down the mitochondria-generated ROS production, especially in long term diabetes. However, these hypotheses have not been tested [115].

Another possible explanation for the un-changed oxidative stress in Akita Nrf2 KO might be that oxidative stress is strongly associated with catalase and NADPH oxidase activity in diabetic condition. Consistent to other reports [313, 314], we observed a significantly reduced catalase activity and enhanced Nox4 expression in diabetic mice. Knockout of Nrf2 did not significantly change these parameters, indicating that Nrf2 might not be a strong mediator of these genes [144, 194]. Indeed, studies have shown that knockout of Nox4 in significantly decreased Nrf2 expression. Moreover, Nox4-related H_2O_2 production controlled Keap 1 oxidation and mediated activation of Nrf2 in renal tubular cells [315]. These data suggested that Nox4 may mainly act in the upstream of Nrf2, a possible reason that we did not observe change of Nox4 expression in Nrf2 knockout and transgenic mice.

Furthermore, we cannot exclude that in the progression of diabetic nephropathy, oxidative stress is rather overall consequence of metabolic disorders than the primary mechanism. Knockout or overexpression of Nrf2 may have a major impact on ROS. However, this impact was masked by other effects of Nrf2. For example, hyperglycemia and SBP were significantly decreased in Akita Nrf2 KO mice, which may release metabolic pressure, decreased AGEs and attenuated overall ROS. Similarly, overexpression of Nrf2 in Akita $Nrf2^{-/-}$ /$Nrf2^{RPTC}$ Tg mice was associated with higher serum glucose level, which may enhance overall ROS level.

Hence, we propose that the level of oxidative stress in mice is an overall result of complex effect. Nrf2 possesses multiple functions rather than just an antioxidant factor and the role of Nrf2 in diseases is more complicated [316]. A microarray analysis identified 2561 transcripts and 240 proteins that were differentially expressed in the kidneys of Nrf2 knockout mice, compared to WT controls [190]. Furthermore, in addition to the kidneys, Nrf2 also participates in the regulation of other organs such as the fat and liver, via FGF21 and peroxisome proliferator-

activated receptor γ (PPARγ) pathways, respectively [183, 317]. Taken together, the role of Nrf2 on ROS may vary from different disease models at different stages and further studies are clearly needed to elucidate.

4.2 The activators and inhibitors of Nrf2

As discussed in 1.3.2.1, chemicals that are electrophilic or reactive to thiol groups can induce covalent modifications of Keap 1 and disassociate it from Nrf2. As one of the most well-known Nrf2 activators, BM and its analogs were used in clinical trials including BEAM and BEACON [211]. However, BM could not be used in animal studies because long-term administration of BM undergoes toxic metabolites in rodent animals [203, 318]. An activator of Nrf2, oltipraz, was reported that it could release Nrf2's translocation into nucleus via interacting with the Keap 1 [165]. Indeed, our previous and current studies have shown that oltipraz serves as an activator of Nrf2 both *in vivo* and *in vitro* [144, 193].

In contrast to Nrf2 activators, most Nrf2 inhibitors are not specific. For example, a class of nuclear receptors such as retinoic X receptor alpha (RXRa) were reported to repress Nrf2 activation by binding to the Neh7 domain of Nrf2 and preventing its binding to ARE. Knockdown of RXRα increased ARE-driven genes by the Nrf2 activator *tert*-butylhydroquinone (tBHQ), while overexpression of RXRα decreased ARE-driven gene expression in HEK293 cells [157]. However, the nuclear receptors regulate multiple factors, which limit their specification as Nrf2 inhibitors. The second class of Nrf2 inhibitors are a variety of natural compounds, such as the quassinoid brusatol (extracted from Brucea javanica, a shrub was found in Sri Lanka, India and other countries), ascorbic acid and the coffee alkaloid trigonelline. One of the main concerns about these natural compounds is that the specific mechanisms of their inhibition on Nrf2 have not been conclusively demonstrated [319]. Some other chemical compounds have also been reported that can inhibit Nrf2 activation. For example, a first-in-class compound, ML385, was recently found by screening of a chemical library. It can block Nrf2 transcriptional activity via interacting with the DNA-binding domain of Nrf2 and preventing its binding to ARE. However, given the similarity of ARE and other DNA-binding site of bZip transcription factors such as the activator protein 1 (AP-

1, binding consensus sequence: 5'-TGAG(C)TCA-3'), additional studies are needed to determine whether ML385 could be a selective Nrf2 inhibitor [319, 320].

Our studies demonstrated that the coffee alkaloid trigonelline could significantly decrease expression of Nrf2. The underlying mechanisms by which trigonelline decreases Nrf2 protein levels in RPTCs of Akita mice were still not known. Van Raaij's group found that incubation of trigonelline significantly decreased Nrf2 and NQO-1 protein expression in human proximal tubule cells [321]. Nazir's s group has reported that in Human Dermal Fibroblasts (Hs68 cells) and Balb/C mice, treatment of Trigonelline significantly attenuated oxidative stress, restored cellular calcium homeostasis and prevented endoplasmic reticulum stress [322].

Similarly, our group have found that trigonelline decreased oxidative stress and Nrf2 expression in the kidneys of Akita mice. In IPRTCs, it prevented Nrf2 nuclear translocation and decreased Nrf2 promoter activity. These data suggest that trigonelline inhibited Nrf2 expression probably by lowering oxidative stress in diabetic RPTCs. These data are consistent with our previous report that trigonelline inhibited Nrf2 expression in WT mice [144, 193]. In human, inhibition of Nrf2 by trigonelline yielded positive results in overweight men with low toxicity. Administration of trigonelline to overweight men at least for 6 days, in a double-blind way, significantly reduced glucose and insulin concentrations at 15 min after the start of OGTT compared with placebo. This findings indicate that trigonelline might be one of the putative targets in the therapy of type 2 diabetes [323].

4.3 The paradox of Nrf2 study in DN

Bardoxolone methyl (BM) is one of most robust Nrf2 activators. The effect of BM on animals were not consistent. For example, administration of RTA 405, an analog of the BM, led to an increase of GFR, higher blood pressures, higher serum cholesterol levels, deteriorative proteinuria and glomerulosclerosis in a type II diabetic nephropathy animal model [206], But a variant of RTA 405, dh404, did not display beneficial effects on proteinuria, glomerulosclerosis, and interstitial inflammation in ZDF rats. Rather, kidneys from rats on dh404 showed the presence of a granulomatous and inflammatory process reminiscent of a pseudo-tumor. Followed Zoja's study with RTA 405and dh404, Tan's group treated the STZ-induced ApoE($^{-/-}$) diabetic mice with

dh404 at three dose: 3, 10, or 20 mg/kg in sesame oil by gavage. Results showed that dh404, at lower but not higher doses, significantly lessens oxidative stress, renal injury, diabetes-associated atherosclerosis with and proinflammatory markers. Higher dose of dh404 were associated with increased expression of proinflammatory mediators MCP-1 and NF-κB [204]. This finding was consistent with the BEAM clinical trial that administration CKD patients with type 2 diabetes with BM increased the GFR dose-dependently at the 25 mg and 75 mg group but not in 150 mg group [208]. Implied by Tan's study, the mechanisms may be related to the increased expression of proinflammatory genes and production at higher drug doses. These works have been summarized in Hall's review, which concluded that there may be a narrow therapeutic window associated with the use of BM in DN [206, 318].

As discussed in 1.3.7, multiple underlying mechanisms have been suggested to explain the failed BEACON clinical trial.

First, an analysis indicated that BM treated patients have a clinically meaningful reduction in urine volume and sodium excretion at week 4 relative to baseline, which was similar to that observed with endothelin receptor antagonists in advanced CKD patients. This analysis suggested that BM may pharmacologically promote acute sodium and volume retention and increase blood pressure in patients with more advanced CKD via modifying endothelin signaling [210]. Further analysis showed that patients with BM treatment had an increase of the risk factor of blood brain natriuretic peptide (BNP), a cardiac hormone that serves as a chronic heart failure monitor [324]. Second, it has been suggested that the BM may upregulate the iRAS via stimulating renal Agt expression, which further increased hypertension and renal injury [144]. Last, it was reported that BM can downregulate megalin, a protein that involves in albumin reabsorption and subsequently result in enhanced ACR in BM treated patients [203]. However, it has been argued that the baseline characteristics of the population in BEACON clinical trial represent a somewhat 'healthier' type 2 diabetes population with stage 4 CKD than seen in clinical practice. Therefore, the frequently observed laboratory abnormalities and co-morbidities in BEACON clinical trial may underestimate the function of BM in tested population [325].

Of note, there seems a lack of reports on Nrf2/NQO-1 expression related to these BEAM and BEACON clinical trial. It might be because that BM has been widely accepted and applied as a robust Nrf2 activator in a variety of studies. However, since BM is likely to yield off-target effect besides an Nrf2 activator, Nrf2 and other genes that relate to BM should be considered in further clinical trials.

Overall, BEACON clinical trial has become a landmark in the study of Nrf2 in diabetes and diabetic complications. Criticisms have been risen from different aspects including rush to move forward with the Phase III trial, largely depending on animal data, ignoring negative reports of Nrf2 and the side effects of BM [326]. As clinical trials of BM and its analogs are still going, the role of Nrf2 and BM in diabetes need further studies urgently.

4.4 The Nrf2/iRAS pathway

Activation of iRAS is one of the most important pathways that contribute to the development of DN. Our previous study showed that overexpression of catalase in RPTCs curbed systemic hypertension, RPTC apoptosis, decreased oxidative stress and Nrf2-stimulation of angiotensinogen (Agt) gene expression in diabetic Akita Cat-Tg mice [144]. Furthermore, our recent work demonstrated that genetic deletion of Nrf2 or pharmacological inhibition of Nrf2 attenuated hypertension, tubulointerstitial fibrosis, ACR and upregulated Ace2/MasR/Ang 1-7 while downregulated of Agt/ACE/Ang II [193]. Our work has been highlighted by Shoemaker Ashley H in journal of 'Science Translational Medicine', which emphased that action of Nrf2 on iRAS could be important for the prevention of diabetes complications [327]. In chapter 3, we showed that overexpression of Nrf2 in renal proximal tubular cells on a Nrf2-null background (Akita $Nrf2^{-/-}$ /$Nrf2^{RPTC}$ Tg mice) significantly increased the Agt level, confirming the Nrf2's role in upregulation of iRAS. However, the blood pressure was only slightly increased (3-5 mmHg) and not significant. This might be because Nrf2 deficiency is a global KO model and increase of Agt by Nrf2 in the proximal tubule is not sufficiently high enough to increase intrarenal Ang II and subsequent blood pressure [193].

In addition, although we found decreased expression of SGLT2 in Akita $Nrf2^{-/-}$ mice and this decrease was restored in Akita $Nrf2^{-/-}$ /$Nrf2^{RPTC}$ Tg mice, we do not know whether SGLT2 could

directly cause augmentation of blood pressure and iRAS. We anticipated that sodium reabsorption might be promoted by the increased SGLT2, which could exert enhancing effect on blood pressure, but it will need further experiments to investigate.

4.5 The Nrf2/SGLT2 pathway

4.5.1 The paradox of Nrf2's regulation in hyperglycemia

As an antioxidant regulator, most of the studies about Nrf2 targeted on its function against oxidative stress. One point that seems to be often neglected is that in diabetic condition, global knockout of Nrf2 is associated with decreased blood glucose, enhanced insulin signaling, and decreased fat and body weight in Nrf2 knockout mouse models [178, 182, 183, 328]. Consistent with these reports, we also found that Nrf2 deficiency significantly downregulated blood glucose level in both type 1 (Akita $Nrf2^{-/-}$) and type 2 diabetic model (db/db $Nrf2^{-/-}$, chapter 5, unpublished data) compared to their controls, Akita and db/db mice, respectively [193].

However, specific depletion of Nrf2 in pancreatic beta cell supressed the expression of cytoprotective antioxidant genes and exacerbated oxidative stress damage in pancreatic beta cell [329]. Moreover, upregulation of Nrf2 by Keap 1 knockout preserved beta cell function and decreased blood glucose in db/db mice at ICR background. Further studies showed that specific overexpression of Nrf2 by Keap 1 knockout in skeletal muscle seems to be responsible for amelioration of insulin sensitivity, while increase of Nrf2 specifically in liver can decrease the blood glucose level in an HFD-induced diabetic model on a C57BL/KsJ background. This study particularly mentioned the decreased blood glucose in global Nrf2 knockout mice, but the reasons were not known [179].

4.5.2 The regulation of nrf2 on SGLT2

The kidneys are responsible to filter and reabsorb glucose mainly by SGLT2. However, whether Nrf2 can interact with SGLT2 in the kidneys have never been studied. We first found that Nrf2 binding site presents in both human and mouse SGLT2 gene promoters and that Nrf2 activator can increase SGLT2 expression in HK-2 cells. To understand whether overexpression of Nrf2 in RPTCs has a direct effect on SGLT2 expression, specifically in diabetic condition, we

crossbred RPTC-specific Nrf2 transgenic mice with Akita Nrf2 KO mice. Indeed, increase of Nrf2 led to augment of SGLT2, accompanied by increased blood glucose level, tubulointerstitial fibrosis and renal injury in type 1 diabetic mouse model. Similarly, downregulated SGLT2 expression was observed in RPTCs of db/db Nrf2 KO mice, a type 2 diabetic mouse model. In BEACON clinical trials, however, although administration of BM significantly increased the blood pressure, ACR, heart failure and death, the blood glucose was not increased but rather slightly decreased (glucose baseline/change from baseline during the trial (mg/dL): Placebo: 156.4 ± 1.7/ 6.2 ± 1.4; BM: 155.5±1.7/ 4.1 ± 1.5; Hemoglobin A1c (%): Placebo: 7.1 ± 0.04/ -0.02 ± 0.03; BM: 7.15 ± 0.04/ -0.19 ± 0.04). We do not know how to explain this observation. One point that needs to address is that population in this trial received background conventional therapy including RAS inhibitors, insulin or other hypoglycemic agents [209]. In our study, administering Akita mice with Nrf2 inhibitor, trigonelline, significantly decreased hyperglycemia and SGLT2 expression, indicating that trigonelline might affect hyperglycemia via Nrf2/SGLT2 pathway (unpublished data) [193].

Lastly, we did not observe significant decrease of SFLT2 expression and glucosuria in $Nrf2^{-/-}$ (C57BL/6 background) or db/m $Nrf2^{-/-}$ (BKS background) in non-diabetic condition compared to WT and db/m, respectively. A few explanations might help us to understand this observation. First, most of the Nrf2 was degraded in non-diabetic condition. Therefore, its regulation on Sglt2 is limited. Second, a slight decrease of SGLT2 would not cause glucosuria since the SGLT1 can compensate the effect of Sglt2. Lastly, Nrf2 may serve as an 'enhancer' of SGLT2 expression but not indispensable as SLGT2 is also regulated by other genes such as HIF-1α, HNF-1α and TGF-β1, as discussed in 1.4.3.

4.5.3 The fluid regulation by Nrf2 and SGLT2

It has been reported that Nrf2 participates in the fluid regulation. Hyperactivation of Nrf2 in mice by knockout of Keap 1-null is juvenile lethal due to starvation, which is caused by the hyperkeratosis in the upper digestive tract and obstruction of oesophagus. Double knockout of Nrf2 and Keap 1-null rescued Keap 1-induced lethality [195]. Another study showed that deletion of oesophageal Nrf2 in Keap 1-null mice allowed survival until adulthood, whereas animals developed severe polyuria with low osmolality and bilateral hydronephrosis via reduced

aquaporin 2. Moreover, deletion of Keap 1 in renal tubules promoted nephrogenic diabetes insipidus features, confirming that Nrf2 activation in tubular cells causes a water reabsorption defect [196]. In the BEACON clinical trial, activation of Nrf2 on patients with BM treatment leads to a clinically meaningful reduction in urine volume and sodium excretion at week 4 relative to baseline, indicating that BM may pharmacologically promote acute sodium and volume retention and contribute to the increased blood pressure, heart failure and death [210].

A recent clinical trial revealed that administration of SGLT2 inhibitor, dapagliflozin, on patients with T2DM who had or were at risk for atherosclerotic cardiovascular disease, significantly lower the rate of cardiovascular death or hospitalization for heart failure. These promising results are believed to be associated with the well-documented downstream effects of SGLT2i, such as natriuresis and blood-pressure reduction [330]. Indeed, increase of urinary sodium excretion and urine volume with SGLT2i treatment have been reported both in animal and human with diabetes [331]. The diuretic actions of SGLT2i presumably play an important role in the protection of patients from cardiovascular disease.

Since both increase of Nrf2 by BM and inhibition of SGLT2 appear to be associated with regulation of sodium and urine volume, we anticipated that Nrf2 might also interreact with SGLT2 in fluid regulation at a post-translational level, or in an indirect way.

4.6 The relation of RAS and SGLT2

To date, although SGLT2 inhibitors were approved in the therapy of T2DM patients, their use for T1DM patients is not approved except in Japan and Europe due to the safety concerns such as the diabetic ketoacidosis (DKA) [332]. Combined use of SGLT2i with other therapies for T1D still lacks data. It has been already known that adding SGLT2 inhibitor on insulin therapy in T1DM patients achieved better glucose control without severe hypoglycemia or DKA [333]. However, use of SGLT2i as an add-on of RAS inhibitors in T1DM remains unclear. In chapter 3, our data showed that overexpression of Nrf2 in RPTCs both increased the SGLT2 and Agt level in T1DM mice model. In Dahl salt-sensitive (Dahl S) rats treated with streptozotocin (Dahl-STZ), combination therapy of SGLT2 inhibitor (luseogliflozin) and ACE inhibitor afforded greater renoprotection including reduction of blood pressure, blood glucose and renal injury, than

administration of either drug alone [334]. In T1DM patients, administration of empagliflozin increased both systemic and intrarenal RAS who were not on renin-angiotensin-aldosterone system (RAAS) blockers [246, 248, 249]. In contrast, our recent work found that despite reduction of blood glucose, GFR, oxidative stress and tubulointerstitial fibrosis, canagliflozin treatment did not lower SBP or renal Agt expression, as compared with insulin in Akita mice [246]. In surgery induced hypertensive Wistar rats, angiotensin II directly induced SGLT2 expression and activity via the AT1 receptor [335]. We are currently trying to understand whether Ang II could induce SGLT2 in diabetic condition. As shown in chapter 5.4, our unpublished data demonstrated that incubation of Ang II with HK2 cells can stimulate both Nrf2 and SGLT2 mRNA. In Ang II infused WT mice, together with our Agt-Tg mice, showed significant increase of SGLT2 expression and SBP. Furthermore, it is well established that Ang II is associated with increase of ROS level, which may further lead to the increase of Nrf2 and enhance SGLT2 expression. However, whether there is a positive feedback cycle between Nrf2 and Ang II with both increasing SGLT2 still needs further study to prove.

Overall, our work suggested that activation of Nrf2 and iRAS in diabetic condition might further lead to augment of SGLT2 expression and contribute to DN progression.

4.7 Nrf2 and non-diabetic kidney diseases

Despite controversial data on DN, the role of Nrf2 in other types of kidney diseases has been well-established. For instance, acute kidney injury (AKI) induced by ischemic–reperfusion (IR) is often companied with increased oxidative stress, inflammation, followed by tubular atrophy and apoptosis [336]. Studies have reported that Nrf2 activators ameliorated the renal IR. Administration Nrf2 activator (sulforaphane) on rats with experimental IR significantly induced phase 2 enzymes expression, ameliorated histological abnormalities and decreased renal injury [337]. In mice, several downstream genes of Nrf2 were significantly upregulated in the kidneys of WT but not Nrf2 knockout mice with renal ischemia. Renal function, histology, inflammation and survival were also significantly exacerbated in Nrf2 knockout mice in IR model. Furthermore, Nrf2 knockout mice were susceptible to cisplatin-induced nephrotoxicity and pre-treatment of antioxidant could blunt this toxicity [338]. Similarly, overexpression of Nrf2 by curcumin and BM,

reduced nephrotoxicity in AKI mice model induced by heavy metal [339, 340]. These data suggested the importance of Nrf2 as a potential therapeutic target in AKI [341].

In addition to AKI, Nrf2 have also been found to protect renal injury against non-diabetic induced CKD. Treatment of BM analog significantly ameliorated renal functions such as reduction of urinary protein, glomerular and tubulo-interstitial injury and reduced fibrosis in rats with 5/6 nephrectomy surgery [205]. In rat UUO model, treatment of Nrf2 activator (sulforaphane) suppressed oxidative stress, inflammation, fibrosis and apoptosis via preserving mitochondrial function [342]. In lupus nephritis, oxidative stress and inflammation were considered as the core pathologies. Knockout of Nrf2 was associated with lupus-like autoimmune nephritis, possibly involving NF-κB pathway [343, 344]. Furthermore, treatment of BM reduced proteinuria, blood urea nitrogen, glomerulonephritis and lupus nephritis in an induced lupus mice model via Akt/Mek-1/2/NF-κB pathway [345]. Hence, it has been suggested that despite failure of the BEACON clinical trial in T2DM and stage 4 CKD, there still remains a possibility that Nrf2 might be one of therapeutic targets in managing CKD [346].

4.8 Limitations of our studies

4.8.1 Mouse models

We studied the role of Nrf2 in DN mainly in the Akita mouse model. Although Akita mice is a widely used diabetic model, a primary limitation of this mouse model is that Akita female do not develop severe hyperglycemia [294]. Therefore, we do not know whether the Akita Nrf2$^{-/-}$ female mice would exhibit lower hypertension, hyperglycemia and decrease renal injury compared to female Akita mice. Another limitation of the Akita mouse model is the difficulties to mimic the exact human DN stage. For example, adult Akita mice exhibited higher blood pressure, GFR, modest levels of albuminuria and modest structural changes of increased mesangial matrix and basement membrane thickening [347]. In contrast, human patients with diabetic nephropathy develop slightly micro-albuminuria with hyperfiltration at stage 2, and this micro-albuminuria further progresses to a modest level at stage 3 with a drop of GFR [80, 348]. Zhou's group monitored the hearts form 12 weeks old Akita mice and found that type 1 diabetic Akita

mouse model is characterized by abnormal cardiac deformation during early stages of diabetic cardiomyopathy [349]. Similarly, Kitada's group concluded that the Akita mice is a useful model of early to moderately advanced renal morphological changes in DN [350]. Hence, it indicates that the Akita mice at 20 weeks may be only mimicking stage 2 to 3 of DN in human patients.

Db/db mice is a commonly used mouse model in the study of type 2 diabetes. Db/db mice on BKS background develop uncontrolled blood glucose level, severe depletion of insulin-producing beta-cells of the pancreatic islets and peripheral diabetic complications. The stage of diabetes in db/db mice was previously categorized 'pre-diabetic, early diabetic, late stage diabetic mice' at '5 weeks, 10 weeks and 24 weeks old', respectively, according to the proliferation and apoptosis of beta cells [351]. Our study showed that db/db mice exhibited increased blood glucose level, GFR, albuminuria and kidney hypertrophy but without significant blood pressure elevation, suggesting an early to moderately developed stage of DN. Furthermore, we studied that both male and female db/db $Nrf2^{-/-}$ mice. We found that deletion of Nrf2 attenuated hypertension, hyperglycemia and kidney morphology in both male and female mice without noticeable sex difference.

Another limitation of our study is that we have used Nrf2 general knockout model both in Akita and db/db mice. Although we provide in vitro data on Nrf2 silencer in iRPTC and HK-2 cells, we cannot exclude that Nrf2 deficiency in other organs might affect kidney function in vivo. However, we have successfully generated Nrf2-Tg mice for our second study and crossbred Nrf2-Tg mice with Akita $Nrf2^{-/-}$ mice, which allowed us to study the effect of overexpression of Nrf2 in RPTCs in diabetic condition. Furthermore, we are generating Akita-PaxNrf2KO mice, which specifically deleted Nrf2 in the renal tubules. Our pilot study revealed that expression of Nrf2 and SGLT2 were significantly decreased in Akita-PaxNrf2KO mice. Further studies are ongoing.

4.8.2 Lack of studies at post-translational level

Another limitation of our work is that we focused on the function of Nrf2 in DN at the transcriptional level. Nrf2 is regulated by a variety of pathways at the post-translational level. For instance, phosphorylation of Nrf2 at Ser-40 by activated PKC pathway in diabetes leads to the dislocation of Nrf2 from Keap 1 and contributes to the Nrf2 activation [352]. In addition to PKC,

multiple factors can lead to Nrf2 phosphorylation such as the mitogen-activated protein kinases (MAPKs), PKR-like endoplasmic reticulum kinase (PERK), phosphatidylinositol 3-kinase (PI3K), and glycogen synthase kinase-3 (GSK-3) [316, 353]. Furthermore, acetylation of Nrf2 in nucleus was crucial for the Nrf2's binding to ARE, whereas deacetylation disassociated it from ARE and resulted in Nrf2 nuclear export [354]. Hence, understanding the regulation of Nrf2 at the post-translational level is indispensable.

4.9 Conclusions

The main purpose of our study was to elucidate the Nrf2's role inDN. Our findings documented genetic deletion of Nrf2 or pharmacological inhibition of Nrf2 in type 1 diabetic mice attenuated hypertension, tubulointerstitial fibrosis, urinary ACR and upregulated Ace2/MasR/Ang1-7 while downregulated Agt/ACE/Ang II. Our study identified a novel mechanism, Nrf2-mediated stimulation of intrarenal RAS gene expression in hyperglycemia contributes to the hypertension and renal injury in diabetes.

Moreover, we found that Nrf2 overexpression in RPTCs increased blood glucose, glomerular filtration rate, urinary ACR, tubulointerstitial fibrosis, stimulated Agt and SGLT2 expression but without the significant ROS change in Akita $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg mice. In human, diabetic kidneys exhibited enhanced oxidative stress with higher expression of NRF2 and SGLT2 expression in RPTCs. Furthermore, the glucose-lowering effect of Nrf2 deletion also exhibited both in male and female db/db mice with SGLT2 downregulation. These results suggested that Nrf2 mediates hyperglycemia-stimulation SGLT2 expression and exacerbates blood glucose and kidney injury in diabetic kidney.

Overall, our work documented that hyperglycemia induced Nrf2 activation stimulated iRAS and SGLT2 expression, further contributing to the DN progression (Figure 23), suggesting that Nrf2 might be a potential therapeutic target in the treatment of diabetic nephropathy.

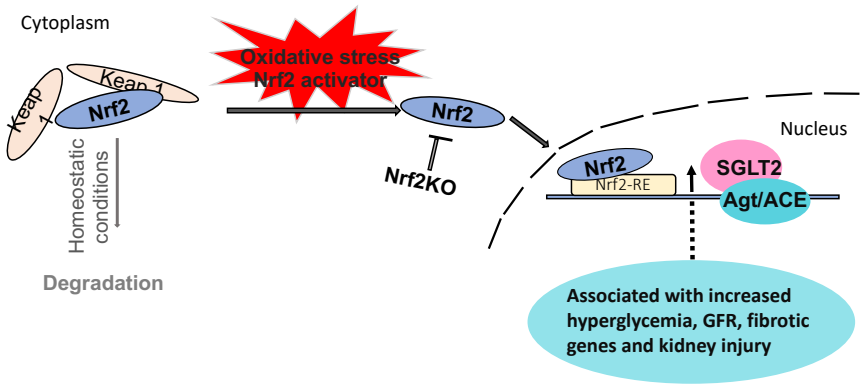

Figure 23. – Diagram of the role of Nrf2 in diabetic nephropathy.

Chapter 5

Unpublished Results and Research Perspectives

5.1 Studies in db/db Nrf2$^{-/-}$ mice

5.1.1 Establishment of db/db Nrf2$^{-/-}$ mice.

T2DM accounts for 90% of the diabetes that associated with hyperglycemia, insulin resistance, and insulin insufficiency. To better understand the role of Nrf2 in diabetic nephropathy, we crossbred and studied db/db Nrf2$^{-/-}$ on BKS background. Due to the background of Nrf2$^{-/-}$ mice (C57BL/6 background), db/m mice in BKS background were back crossed with Nrf2$^{-/-}$ to obtain the db/db Nrf2$^{-/-}$ mice on BKS background for several generation. Both male and female mice were monitored from week 12 to 16 and sacrificed at week 16. As shown in Figure 24, Nrf2, NQO-1 and SGLT2 mRNA were much elevated at week 16 in db/db mice compared to db/m.

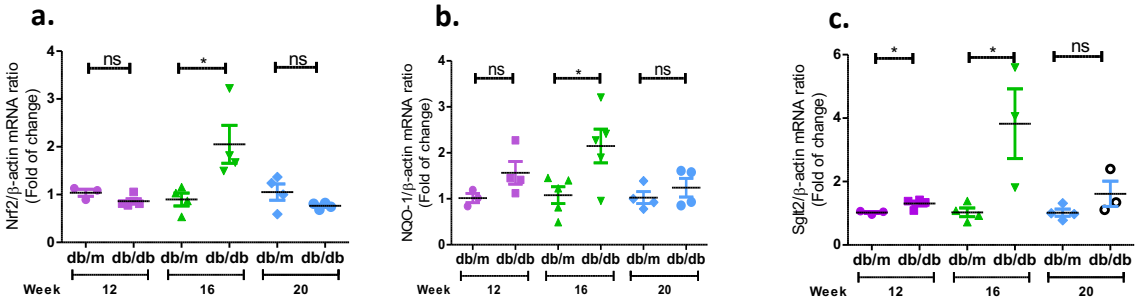

Figure 24. – Nrf2 (a.), NQO-1 (b.) and Sglt2 (c.) mRNA expression in male db/m and db/m at week 12, 16, and 20 in male mice. Statistics were done by unpaired *t-test* between each two groups.

5.1.2 Physiological measurements

Table 5. – Physiological measurements of db/m, db/m Nrf2$^{-/-}$, db/db and db/db Nrf2$^{-/-}$ at 16 weeks (male)

	db/m	db/m Nrf2$^{-/-}$	db/db	db/db Nrf2$^{-/-}$
Fasting blood glucose (FBG, mM)	8,78±0,36	9,31±0,64	27,96±0,38***	19,81±1,42***$^{\#\#\#}$
Systolic blood pressure (SBP, mmHg)	120,7.0±2,20	109,1±1.49**	122,6±3,26	110,4±2,67*$^{\#\#}$
Body weight (BW, g)	29,95±1,12	32,41±1,05	54,76±1,57***	55,04±2,39***
Kidney weight (KW, mg)	358,5±18,81	357,7±9,37	498,8±19,24***	393,9±17,13***$^{\#\#\#}$
KW/TL (mg/mm)	16,14±0.59	15,86±0,37	23,66±0,66***	17,93±0.86***$^{\#\#\#}$
HW/TL (mg/mm)	5,85±0.12	5,51±0.15	6,22±0.18	5,65±0.15
Fat/BW (mg/mg)	0,23±0.02	0,21±0.01	0,58±0.01***	0,55±0.02***
Muscle/BW (mg/mg)	0,65±0.02	0,67±0.01	0,34±0,02***	0,35±0.01***
Total water/BW (mg/mg)	0,53±0.0	0,52±0.02	0,26±0,02***	0,29±0,01***
GFR/BW (ml/min/g)	9,61±0,59	9,11±0,56	16,24±2,37*	10,19±1,09$^{\#}$
ACR (μg/mg)	89.97±0.59	64,93±12,41	418,3±54,64***	261,3±38,67*$^{\#}$

SBP was measured by BP-2000 tail-cuff; Fat/BW, muscle/BW and total water/BW were measured by EchoMRI. FBG, SBP, BW, KW, KW/TL, HW/TL n=9-12; Fat/BW, Muscle/BW, Total water/BW, GFR/BW and ACR n=6-9. Statistical analysis was done by one-way ANOVA. *p < 0.05, **p <0.01, ***p < 0.001 vs db/m; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs db/db.

Table 6. – Physiological measurements of db/m, db/m Nrf2$^{-/-}$, db/db and db/db Nrf2$^{-/-}$ at 16 weeks (female)

	db/m	db/m Nrf2$^{-/-}$	db/db	db/db Nrf2$^{-/-}$
Fasting blood glucose (BG, mM)	7,97±0,46	7,80±0,36	29,95±1,11***	19,97±1,33***$^{\#\#\#}$
Systolic blood pressure (SBP, mmHg)	119.0±1,28	110,3±1.94**	125,9±1,74	112,4±1,84$^{\#\#\#}$
Body weight (BW, g)	24,01±0,39	25,24±0,72***	54,92±2,14***	54,58±1,70***
Kidney weight (KW, mg)	236,2±9.31	231,2±17,41	426,0±26,1***	345,1±8,28**$^{\#}$
KW/TL (mg/mm)	11,17±0.44	10,33±0.54	20,71±1,14***	16,83±0.44***$^{\#}$
HW/TL (mg/mm)	4,62±0.20	4,41±0.20	6,11±0.16***	5,56±0.14**
Fat/BW (mg/mg)	0,27±0.01	0,27±0.03	0,60±0.01***	0,60±0.02***
Muscle/BW (mg/mg)	0,62±0.01	0,61±0.02	0,31±0,01***	0,32±0.01***
Total water /BW (mg/mg)	0,49±0.03	0,49±0.02	0,26±0,01***	0,25±0,01***
GFR/BW (ml/min/g)	7,59±1,37	7,67±0,44	13,94±0,91*	9,12±1,22$^{\#}$
ACR (µg/mg)	44,56±4,41	61,86±9,10	360,9±55,51***	237,4±35,9*$^{\#}$

SBP was measured by BP-2000 tail-cuff; Fat/BW, muscle/BW and total water/BW were measured by EchoMRI. FBG, SBP, BW, KW, KW/TL, HW/TL n=9-12; Fat/BW, Muscle/BW, Total water/BW, GFR/BW and ACR n=6-9. FBG: Fasting blood glucose; KW/BW: kidney weight/tibia length; KW/BW: kidney weight/body weight; ACR: Albumin/creatinine ratio. Statistical analysis was done by one-way ANOVA. *p < 0.05, **p <0.01, ***p < 0.001 vs db/m; $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001 vs db/db.

Table 3 and table 4 showed the results of physiological measurements in db/m, db/m Nrf2$^{-/-}$, db/db and db/db Nrf2$^{-/-}$ mice at the age of 16 weeks in male and female, respectively. As expected, fasting blood glucose (FBG), kidney weight/body weight (KW/BW), kidney weight/tibia length (KW/TL) and albumin/creatinine ratio (ACR) were significantly higher in db/db and db/db Nrf2$^{-/-}$ mice than in db/m or db/m Nrf2$^{-/-}$ mice, respectively. Deletion of Nrf2 in diabetic condition (but not in non-diabetic condition) significantly lowered the FBG, KW/BW and KW/TL while increased the insulin sensitivities (Figure 25) both in male and female mice. Although there were not obvious changes of SBP between db/m and db/db, mutation of Nrf2 significantly decreased

SBP level in both male and female mice. Moreover, deletion of Nrf2 in diabetic condition also attenuated the GFR compared to db/db control in both male and female mice.

In addition, BW and Fat/BW were significantly increased in db/db and db/db Nrf2$^{-/-}$ mice compared to respective db/m or db/m Nrf2$^{-/-}$ mice, whereas the Muscle/BW and Total water/BW were significantly decreased. Although previous report showed that Nrf2 was associated with BW and fat accumulation in obesity model [183], loss of Nrf2 in db/db mice did not change these parameters in both male and female mice. Lastly, the HW/TL exhibited a trend of increase in db/db mice compared to db/m and deletion of Nrf2 in diabetic condition slightly attenuated the HW/TL level without statistically differences. Taken together, these results indicated that knockout of Nrf2 in db/db mice, a type 2 diabetic model, ameliorated hyperglycemia and hyperglycemia-induced kidney dysfunction both in male and female mice.

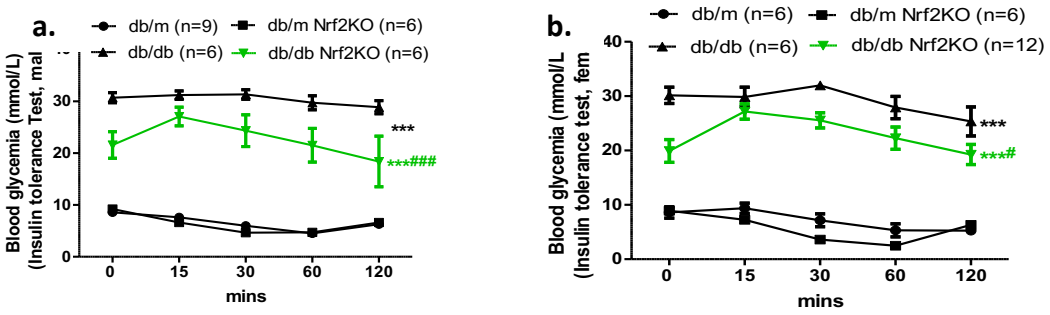

Figure 25. – Insulin tolerance test (ITT) in db/m, db/m Nrf2-/-, db/db and db/db Nrf2-/- mice in male (a) and female (b) mice. Briefly, ITT were performed at fasting status (6 hours). Insulin was given according to mice body weight by intraperitoneal injection. Blood glucose levels were measured at 0, 15, 30, 60 and 120 min after insulin injection. Statistical analysis was done by one-way ANOVA ($^{***}p < 0.001$ vs db/m; $^{\#\#\#}p < 0.001$ vs db/db).

5.1.3 Histology of db/m, db/m Nrf2$^{-/-}$, db/db and db/db Nrf2$^{-/-}$

The kidneys of db/db mice exhibited structural damage. As shown in Figure 26, tubular luminal dilatation with cell debris, proximal tubule cell atrophy and increased extracellular matrix proteins in glomeruli appeared both in male and female db/db mice compared to db/m mice. There were no noticeable changes between db/m and db/m Nrf2$^{-/-}$, whereas deletion of Nrf2 in diabetic condition ameliorated these abnormalities and protected kidney structures.

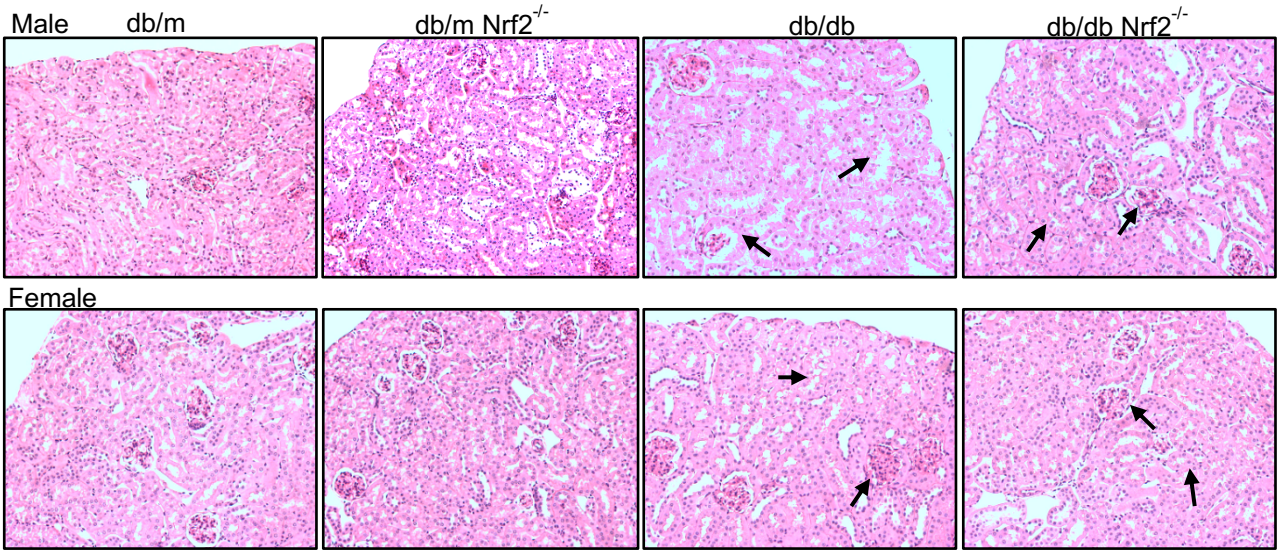

Figure 26. – PAS staining of db/m, db/m Nrf2$^{-/-}$, db/db and db/db Nrf2$^{-/-}$ mice in male (upper panel) and female (lower panel) mice (100x). Arrows indicate the structural changes in glomerulus or proximal tubules in db/db and db/db Nrf2$^{-/-}$ mice both in male and female mice.

5.1.4 Effect of Nrf2 deletion on SGLT2 expression

As shown in Figure 27a, immunostaining revealed significantly higher SGLT2 expression in RPTCs from db/db mice, whereas knockout of Nrf2 exhibited significantly lower expression of SGLT2 in both male and female mice. These changes were consistent with SGLT2 mRNA expressions in isolated RPTCs (Figure 27b & c) that deletion of Nrf2 in diabetic condition (but not in non-diabetic condition) decreased the expression of SGLT2.

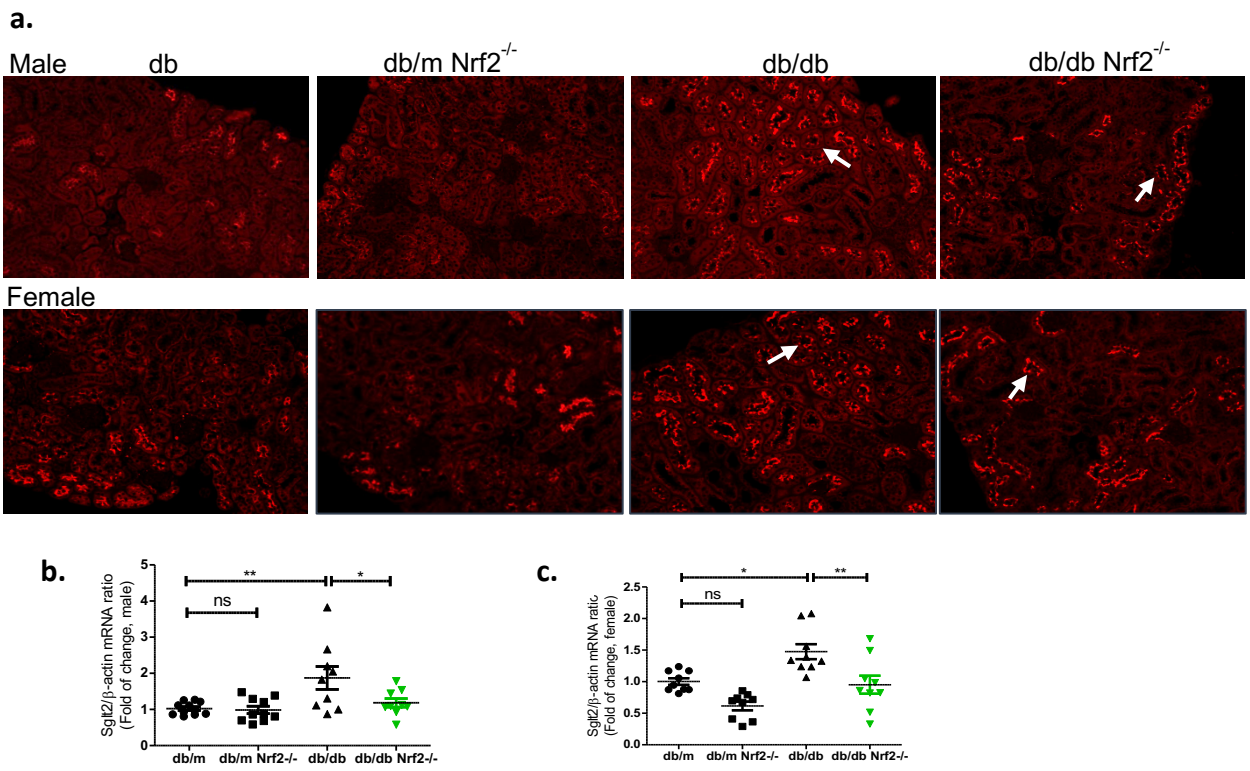

Figure 27. – SGLT2 expression in db/m, db/m Nrf2$^{-/-}$, db/db and db/db Nrf2$^{-/-}$ mice in male and female mice. (a) SGLT2 IF staining in male (upper panel) and female (lower panel) (100X); b) SGLT2 mRNA expression in male (b) and female mice (c). Statistical analysis was done by one-way ANOVA (*p < 0.05, **p <0.01).

Taken together, our data demonstrated that in db/db mice, a type 2 diabetic model, Nrf2 deficiency attenuated hyperglycemia, systolic blood pressure and kidney injury, at least in part, via down-regulation of SGLT2 expression. These results further confirmed that beyond type 1 diabetic mice model, the novel relation of Nrf2 and SGLT2 also exists in type 2 diabetic condition.

Furthermore, to mimic the failed clinical trial, we plan to administrate dh404 (analog of BM) with db/db mice and db/db Nrf2$^{-/-}$ mice. We expected to observe that dh404 could increase systolic blood pressure, blood glucose as well as Agt and SGLT22 expression in db/db mice and knockout of Nrf2 would reverse this effect.

5.2 SGLT2 promoter analysis

Nrf2 binding site has been previously reported [355]. As shown in Figure 28, the sequence TGACnnnGC exhibits high frequencies of Nrf2 binding.

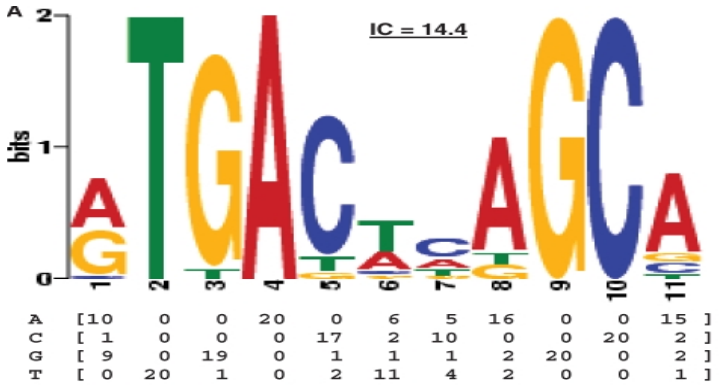

Figure 28. – The nucleotide profile of Nrf2-responsive element. The position Frequency Matrix (PFM) is created by counting the occurrences of each nucleotides at each position and indicates the frequency and importance of each nucleotide in specific position. The sequence TGACnnnGC exhibits high frequencies of Nrf2 binding [355].

As shown in a) & b), we have previously found the presence Nrf2 putative binding sites in both human (N-1316 to N-1305) and mouse SGLT2 promoter (N-1527 to N-1516). Additionally, putative binding sites of various transcription factors such as HNF1α, HNF4α, Smad2/3, SP1 and hnRNP F were identified both in hSGLT2 and mSGLT2 promoter. Although relation of SGLT2 with HNF1α, HNF4α, Smad2/3 and SP1 have been previously reported, Nrf2-RE and hnRNP F-RE have never been studied [356-359].

a) hSGLT2 promoter (NG_012892.1)

-1986 GTCTGTAACACACACGTGTCCTCTGACACTTGGCACTGGATTTCTCTCAGGCACTTTAAACTCATCACCCT**CTGAAGCAGACAC**TGTGGGTG**CCCCACCC**
SMAD2/3
sp1/hnRNP F
-1886 ATATACCTGCAT**GGCCCACC**TTGGAAGTCATATACTGTGACAGTTTCCAGATACCCTGACTTGCAGCTCCTTCTGGAGTTTC**TTGTCTGAGGCTG**TTCTT
SP1 SMAD2/3
-1786 GGGAGGAGGGAGTGTGCTCCGTGTTCTCAGCAGGCCTGAAGTGTGTGGACATTTCTGCAGCTTTTAGCCAGTGACAGACACAAATCAGGGAATAGATACC
SMAD2/3
-1686 CCAACCACCTGGCCCCTCAAGGAACATTGCTAT**GGGGTG**TTCTTCACAGTGTCTCAGAGTGTCCTTTTTGGGATGAGGCTCC**AACTGCTCTTTGTGG**TAA
SP1/hnRNP F. hnRNP F HNF4α
-1586 CCTGACAAATGACACACCCTTACGCCAGG**CGTGGTGGCTC**ACACCTGTAATCCCAGCACT**TCGGGAGGCTG**AGGCAGTTTGGCTCAGGATTTTGAGACCA
SP1 SP1
-1486 GCCTGGGCAACACAGTGAAACCCCATCTGTATAAAAAATACAAAAATTAGCCAAGTGTTGTGGCGTGCCCGTAGTTCCAGCTATTTGGGAAGCTGAGGTG

-1386 GGAGGATCCCTTGAGCCCAGGAGGTCGAGGCTGCAGTGAGCTGTGATCACACCACTGTACTCCAGCTTTG**GTGACACAGCA**AGACC**CTGTCTCAAAACA**A
Nrf2-RE(N-1316 to N-1305)
SMAD2/3
-1286 AGAAACACATGCTTTGTTGGTTTTTCTCCTTGTTTCATTTCTCTTCCCTACACCATCACAGTCCATACTGGAATCACC**TCCTCAGGAAAGTCGTTG**CACA
SP1 HNF4α
-1186 CAAATTCTCATCTCAGGGTCTCCTTTTAGAGGAATACAAGCTAAGGAACTCCTTCATCCAAACTCTGTCATATTACACTCAAAGCATTTTTCTCTGTTCC

-1086 CCATCTCCGGGATGACCATCGCTGCCATAATCAGGCAGGACTTTCCTTCGAC**TGTCTCCTGACATCCAAGTCTGGTCAA**TTCTTCTA**GAAAGGTAGACAT**
SMAD2/3 SMAD2/3
-986 **ACAGATATTAACC**AGCCTCTCCCTTCTTGAATCCAGTCCTGTGCTG**AGAAATGAGACTC**CAGGGATGAATCAGATCAGTCCTGCTCTTTTTAATTTTTAT
HNF1α SMAD2/3
-886 TTATTTATTTTTTAAGAGATGAGGTCTCACTCCGCTGCCCAGGCTGGTTTCAAACTCCTGAGCTCAAGT**GAGCCTCCTGC**TGCAGCCTCCCAAAGTGCTA
SP1
-786 GGATTACAGGCAT**GAGCCCCCACACC**CAGCCAGTCCTACT**CTTGAGGAGACAC**TAGTTT**GTTGGGAAGACAG**ACCTGGACCCAGGCAAGGGCCACACAGT
SP1/hnRNP F SMAD2/3 SMAD2/3
-686 GTGCTCAGAACTACATCAGAAATGTCAGAGAAGTCTTTCTTGAAGAGGCGACACAGGCTGGGTACTGAAAGCTGAGTAGGAGTTGGCCATTTGGGAGACA

-586 GGGAAGTGCTCTA**AACAGAAGACAC**AGTTTGTGCAAGGA**TTTGGTGGGGA**TAAAATATCTGGTCAAAAAAAGCAAAAAGCAAAAATCTGGGCTGGGTAGG
SMAD2/3 SP1
-486 TTAAAGGAGTGGGAAAGGATTTCTGATTTCCTCTAGATTTGGTTTGGAGAAG**CAGGGGGAAGG**ATGAGCGGGAATTGGGGCATGACCAGGATTGGAAATC
SP1/hnRNP F
-386 AGGCTGAAGAGCTTGTACTAAGAGC**TATGGAGGGTT**CC**TGAGGAGGGCG**AGTGA**CCCTGTCAGACTT**GGATTTGAAAATGATTCCTCTGGATTAGTTAAA
SP1 SP1 SMAD2/3
-286 TCCAGGGGTGCTAGCTTAGCTAAGGAAGCGATGCATTTTTAGGGAGTAAAAGAGTGATTTTGAGCCTGGAGCACA**GGGGAGAGG**GCGGATGCTAAGGCCC
hnRNP F
-186 AGGAAAGAGTGCTCTTGAACTTGGAAGGGCCCAGCTCCCCAAGACCAGCCTTCAGCCTTGATATGACCTGATTCAGCTAAACAAAGCTGGGGAGCGGGAA

-86 TGAGA**CCTGGGGGACT**TGTCGGCTCAGTGCCCCTG**AGGTAACCATTAATC**CTTCCCCTGGGGGAATCCAGGGGCTGGTTCCTGGATGGGGCAGATCCTGG
SP1 HNF1α
+15 GGAGAATG**AGATCT**
Bgl11

b) mSGLT2 promoter (AJ292928.1)

-1952 **GGACAT**CACATGGTACCAAACAAAAGACCCAGTACCAGGAATGAGTTACCAT**CTTATGAAGTCATTAACCAT**AGAGAGCTAATGGTAGCCTCAATCATAC
KPN1 HNF1α

-1854 AAAAGCTGTTGCCAAGGTTACTGGGTGCTCTCATACCCTGATGGTAAGACCCTATGGCTGAAGATGGAACTTATTGATATCCTTGAACATTGAGAAATTG

-1754 AGCTGTGTGACTAGAAGCTTCACCCCATCGACGATGGTCACAGTGCTGCAAAGTGCTATGTTCAGGAGGAAGGTATCCAGCCGCCTCCCTCAGCCACACT

-1654 CTGAGACTCACACCAGAG**ACCTGCTCAAAGGGCATGC**CCACTGGCCCACAAATATTATGGGAGCAACTGACCACTTTTTGGGGGTGTATTTAAGGTCCAC
HNF4α

-1554 TTCATGAAATGGGACCCATCC**CTGACACTGC**TAAAATGCCTGAGAACCTGAAACTAGATAGAGCAAGGGC**TCTAGGGGAAAGCTCACTC**CAGTTATTCTA
Nrf2-RE (N-1527 to N-1516) HNF4α

-1454 AGGGCACAGGGTTATGACGCCTAATGACATATCGCTGGCTACATCCCTTGGCCAGCACATCACTGAAACCTCACCAGAGGAGCTTCTTGGAGTAGAAGGT

-1354 GATTAACAGAACTGTCCGTGACTGGATGACTTGCAGAGAGTGAGAGACTTGGG**AGCACTCAGACTTCAA**TGGGATGCTTGTATCTCACCCCTCTGCTAAA
Smad2/3

-1254 GATGCAGGTTCTAT**ACAGGAGGTGCAAAGATTGTAA**GAGTCAGAGTTGGAGGTTGTCTTCAGGAAAGCAGA**GTTTTGCAGACACAAC**AAAGCTGATGAAA
HNF4α Smad2/3

-1154 ATCTG**AACTCACAGACAGTGA**TAGC**ATGCACAAGACAG**ATATCTGAAC**TCACAGACCGTGA**TAACATGGCACAAAGACCTGCACAAAGTTCGAACCAAAT
Smad2/3 Smad2/3 Smad2/3

-1054 AAAATCTGAGCATGGAAAATGAGGTGTGGGCACAAAGTCCCACTCCTAAGTAAGAAACTACTTGCAGTTGACAGCTACTAGGAGAGAGAAATGGGTTTTC

-954 TTC**AATGGAGAGACAC**TGGGTGTATCAACTGCACCCCAAGGCAGGCCACACGCTCAGGAAGAGTTGGCCAACACAAAACACACTCCGTGTTTGGTTTTCT
Smad2/3

-854 GTTTGCTTGGGTTTGTTTTTGTGCTTTTATTCTTCCTTCCTTTCTTTCTTTGTTTCTTTGTTTCTCTCTCTCTTTCTCTCTTTTTCTCTCTCCCTCCTCC

-754 TCTTCTTCTTTCTTCTTTTTCTTCTTCTTCTTCTTCTTCTTCTTTTTCTTCTTCTTCTTCTTCTTTTTCTTCTTCTTCTTCTTCTTCTTCTTCTT

-654 TCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTGATCAGAGAGGGGAGGGGATCTGGGAAAAGTTTGGGGAGAGGAAAGAATTTGCCC

-554 AAAATATATTGCATAAAAACTTTTTAAAAATAAATTTAAAACAATTTTTAGGATAGAGCAAAGAGAAAGTAGAAAATATTTGGGTTGGGAAGGGCAGGAG

-454 AATGAGGGAAATGTGATTTTTTTCTCCCTAGGTTTTGTATGGCAGAAGTC**AGGGCATGGC**ATGCGTGACAGTCAGGCTGAGGAACATGTATGTCCTGCTG
SP1

-354 ACTGTC**AGGGGGTGCT**ATGGAGGACTTGTGCGGAGGACACTGTCAGAGTTGGATTCGGACCTTCCTAATCAAAGTTAGAGGGTGTATTTTCAGAGAACGC
SP1

-254 AGGAAGGAACTTTGCTTGGAACACTGGGTATAGGATGGATCCTAAACCCAGGAAGGAGTGCTCTTGAATTCCAAATGGTCCAGCGCCCCAGGACCAGCCT

-154 TCGGCCTTGATAGATCCTGATTCAGATAAATAAAGCTGGAGAAG**GAGGCTGAGA**CCTGGGGGACTTGTCGGGTCAGTGCTC**CTGAGGTAACCATTAATCC**
SP1 HNF1α

-54 TTCCCCCAGGGGAATCCAGGGACTAGCCCCTTGAGGGACAGATGGTGGAGAGA**ATG**GAGCAACACGTAGAGGCAGGCTCTGAACTTGGGGAGCAGAAGGT
+1TSS

+54 CCTGATTGATAATCCTGCTGACATTCTGGTTATCGCTGCCTATTTCCTGCTGGTCATTGGTGTTGGCTTGTGGGTGAGACATTGAGGGGGGTTGGATAGG

+154 GAAATGCTTCTGGGGCTTGAGGGTAAAGATTTAGGGAGACCTCAGAGAGGAGTGGGAGAAAAGGGTGCTTGGATATAATGAGGGAGAAACCTAGATTTAG

+254 TAGGCAAGCCAATTTTAATTCTTTGTCTTCGTACCTTCTGGATTGTGCAAAAGAGACTGGGGGTATCAATAGGTTTTTTTTTAATTCAAGTGTTCTAACA

+354 AGTGCTCTAAGAGATGTATCAGTTCCCACGTCTGTATTATGGCTGAGCAGCAGCCTATATTTAAGGTCACCAGGCAAGTTAGGCTGAATCTAGGCATATC

+454 TAGGTTCCAGTAGTTGCGCTAGGATTAGGGCCTGGGTTGTTCTGAGTGTCGGGGAAGGTTGGGGGTAAGGAGGTGCAGTCTGGGGAGTCCAGGGCTGGTT

+544 AATCTTCAGCCTGAAACAAGGCTGAGGAATGTGTTGAGGAAGCTAAGGAAGTCCAAAGATGTGCCCCAATCCCAGTTTCCCCCCACTTCTGTTTCCCAGT

+684 CTATGTTCAGAACCAATAGAGGCACAGTTGGTG

5.3 Effect of Ang II on Sglt2 expression *in vivo* and *in vitro*

As discussed in 4.5, SGLT2 inhibitors have been considered as combination therapy with RAAS inhibitors. Currently, we are investigating the interaction of iRAS and SGLT2 in diabetic condition. It has been well-acknowledged that Ang II contributes to the development of DN and administration of angiotensin II receptor blocker (ARBs) is one of the major approaches in the treatment of DN. Losartan is one of ARBs that used to treat high blood pressure, cardiovascular disease and diabetic nephropathy [360]. In HK2 cell, losartan could attenuate the Ang II-induced necroptosis, inflammation and oxidative stress [361, 362]. As shown in Figure 29, our preliminary data showed that Ang II can stimulate SGLT2 expression from the concentration of 10^{-13} to 10^{-7} M dose-dependently and then markedly decrease at 10-5 M. The stimulatory effect of Ang II at 10^{-7} M was attenuated by 10^{-6} M of losartan, indicating the effect of upregulation of SGLT2 gene in HK2 cells is Ang II-dependent.

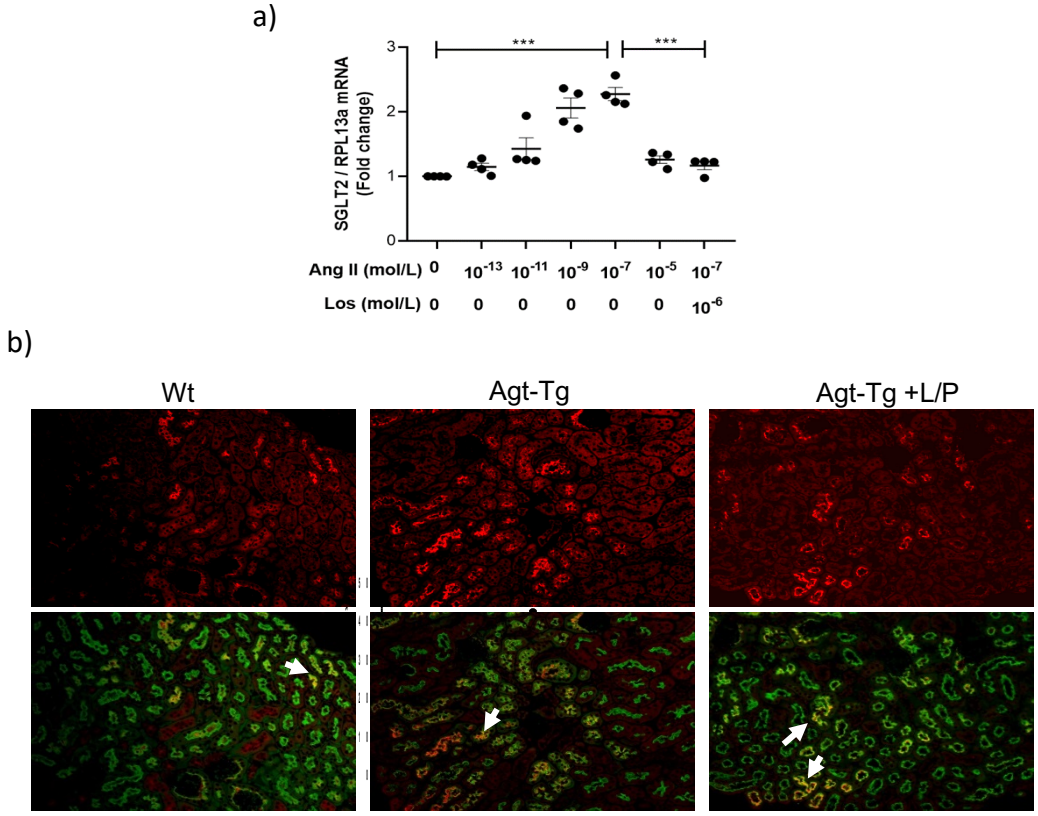

Figure 29. – Ang II stimulates SGLT2 expression. a) SGLT2 mRNA expression when exposed to Ang II 24 hrs in HK2 cells; b & c) SGLT2 protein expression and quantification by IF staining in Wt, Agt-Tg and Agt-Tg + L/P (L/P: losartan 30 mg· kg^{-1}·day^{-1} plus perindopril mg·kg^{-1}·day^{-1}).

Red: SGLT2; green: LTL; yellow: combined SGLT2 and LTL; Arrows indicate merged staining in each group. Statistical analysis was done by one-way ANOVA (***$p < 0.001$).

Furthermore, we previously demonstrated that in Agt transgenic (Agt-Tg) mice, specifically overexpression of rAgt in RPTC leads to hypertension, albuminuria and renal injury, which was accompanied with increased level of Ang II [363, 364]. As shown in Figure 29b, we observed elevated SGLT2 expression in Agt-Tg mice and treatment of RAS blockers attenuated the enhancement of SGLT2. We are currently administrating Ang II in mice investigate the effect of Ang II on SGLT2 *in vivo*.

5.4 Generation of Pax8-Nrf2 KO mice

To further study the role of tubular Nrf2 in DN, we are generating Pax8 Nrf2 KO mice line. Pax8 is a transcriptional factor that expresses in brain, thyroid gland and kidney [365]. Pax8-Cre mice were crossbred with Nrf2 lox/lox to generate the Pax8 Nrf2 KO mice based on the Cre-loxP recombination system (Figure 30a). As shown in Figure 30b, Nrf2 expression was much decreased in renal tubule cells but not in glomeruli. Compared to Ctrl, Nrf2 mRNA level was dramatically decreased in the isolated proximal tubule cells of Pax8 Nrf2 KO mice but not in brain and thyroid gland, indicating Nrf2 is successfully and mainly disrupted in renal tubule cells in this mice model.

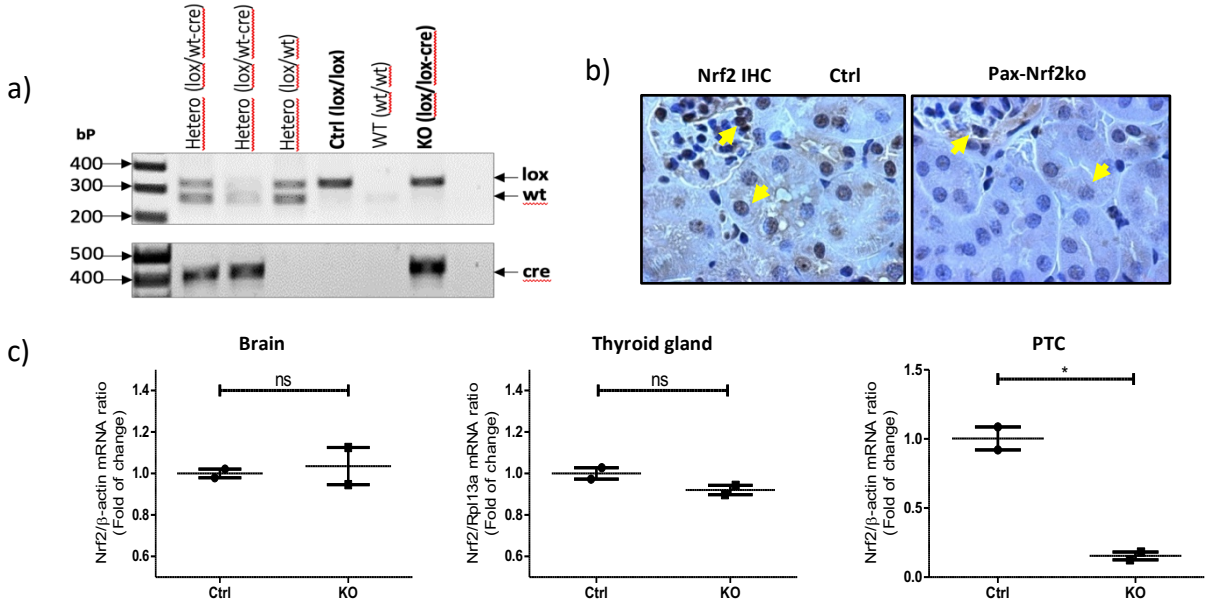

Figure 30. – Establishment of Pax8-Nrf2 KO mice line. a) Genotyping of the Ctrl (control: lox/lox) and KO (Pax-Nrf2 KO: lox/lox-cre); b) Nrf2 IHC staining of Ctrl and KO, 600X; Yellow arrows show the Nrf2 positively/negative-stained cells in control and KO mice. c) Nrf2 mRNA expression in brain, thyroid gland and PTC (proximal tubule cell). Statistical analysis was done by unpaired *t-test*.

5.5 Future experiments

5.5.1 Generation of Akita Pax8 Nrf2 KO mice

We are currently crossbreeding Pax8 Nrf2 KO mice with Akita to generate Akita Pax8-Nrf2 KO mice. We will follow the mice from 12 to 20 weeks of age. General appearance, body weight, glycaemia and blood pressure would be measured every two weeks. Before euthanizing, GFR would be measured. 24 hours urine, food and water consumption, the kidneys and heart would be collected for following studies such as urinary ACR measurement, mRNA and western blotting. We anticipated that compared to Akita lox/lox, Akita Pax8-Nrf2 KO would exhibit decreased hyperglycemia, hypertension and kidney injury. Moreover, we will measure urine parameters, such as urine volume, sodium and potassium level. Furthermore, gene expression related to phenotypes would be studied. We believe that his study would provide more detailed perspectives of Nrf2 in renal tubules in diabetic condition.

5.5.2 Generation of db/db $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg Mice

To study the impact of overexpression of Nrf2 in type 2 diabetes (db/db model), we will generate the db/db $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg mice on BKS background. $Nrf2^{-/-}$/^{RPTC} Tg mice (on C57BLK/6 background) would be back crossed for 8-9 generations with db/m $Nrf2^{-/-}$ (on BKS strain background) to obtain db/db $Nrf2^{-/-}$/$Nrf2^{RPTC}$ Tg mice on BKS background. Both male and female mice would be followed from week 12 to week 16. Body weight, blood glucose and blood pressure would be measured every two weeks. Similar to previous studies, GFR would be measured at week 16 and urine, kidneys and heart would be collected for following studies. We anticipated that increase of Nrf2 in RPTCs would increase SGLT2 and Agt expression and lead to higher blood pressure, hyperglycemia and kidney injury. This work would provide further evidence for Nrf2's role in type 2 diabetes induced DN.

5.6 Other publications

- Kana N. Miyata, Chao-sheng Lo, Shuiling Zhao, et al; Kana N. Miyata, Chao-sheng Lo, Shuiling Zhao et al; Angiotensin II Upregulates Sodium-Glucose Co-Transporter2 (SGLT2) Expression and SGLT2 Inhibitor Attenuates Ang II-Induced Hypertensive Renal Injury in Mice. (In revision, Clinical Science CS-2021-0094, **2020**).
- Kana N. Miyata, **Shuiling Zhao**, et al; Comparison of the effects of Insulin and SGLT2 inhibitor on the Renal Renin-angiotensin System in Type 1 diabetes mice. Diabetes Res Clin Pract. **2020**; 162:108107.
- Anindya Ghosh, **Shuiling Zhao**, et al; Heterogeneous Nuclear Ribonucleoprotein F Mediates Insulin Inhibition of Bcl2-Modifying Factor Expression and Tubulopathy inDiabetic Kidney. Sci Rep. 2019; 9(1):6687.
- Chao-sheng Lo, Kana N. Miyata, **Shuiling Zhao**, et al; Tubular Deficiency of Heterogeneous Nuclear Ribonucleoprotein F Elevates Systolic Blood Pressure and Induces Glycosuria in Mice. Sci Rep. 2019; 9(1):15765
- Ghosh Anindya, Abdo Shaaban, **Shuiling Zhao**, et al; Insulin Inhibits Nrf2 Gene Expression via Heterogeneous Nuclear Ribonucleoprotein F/K in Diabetic Mice. Endocrinology 2017; 158(4):903-919.

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