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Diabète maternel et/ou hypertension et dommages rénaux induits par le système rénine-angiotensine intrarénal : rôle de Nrf2

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Résumé

L'expression 'programmation périnatale' est employée pour décrire les effets à long terme d'un environnement gestationel néfaste observés chez la progéniture. Ce concept est aujourd'hui bien reconnu. Notre laboratoire a déjà démontré l'impact de l'hyperglycémie maternelle sur le développement rénal des embryons à l'aide des souris HoxB7-GFP transgéniques (Tg) et qui se traduit par une augmentation des espèces réactives de l'oxygène (ROS) et une néphrogenèse perturbée. Les rejetons affectés présentent ainsi des reins plus petits et possédant un nombre inférieur de néphrons à la naissance, et développent une hypertension et des dommages rénaux à l'âge adulte (20 semaines).

Dans la première étude, nous avons tenté de réduire la production excessive de ROS dans les reins en développement par la surexpression de la catalase (CAT). Pour ce faire, nous avons croisé les souris CAT-Tg qui surexpriment la CAT dans les cellules des tubules proximaux rénaux (RPTCs) aux souris HoxB7-GFP-Tg afin de générer les souris HoxB7/CAT-GFP-Tg. Nous espérons observer la normalisation du nombre de néphrons et la prévention de l'hypertension et des dommages rénaux observés chez la progéniture issue d'un environnement gestational hyperglycémique.

Nous avons observé que la surexpression de CAT dans les RPTCs permet de normaliser la dysmorphogenèse rénale présente chez les embryons de mères diabétiques. À l'âge adulte, la surexpression de CAT dans les RPTCs permet également de réduire la génération des ROS et l'hypertension, tout en améliorant la morphologie et la fonction rénale. Afin de définir les mécanismes impliqués dans ce processus, nous avons étudié le rôle potentiel de Nrf2 ('nuclear factor-erythroid 2p45 (NF-E2) related factor-2'; un facteur de transcription des gènes antioxidants) et HO-1 (hème oxygénase-1'; une enzyme antioxidante). À la fois Nrf2 et HO-1 sont de forts antioxidants et ont été rapportés comme protecteurs pour le rein. Nous avons observé une surexpression des gènes et protéines Nrf2 et HO-1, en plus d'une translocation nucléaire accrue de Nrf2, dans les RPTCs de la progéniture des mères diabétiques, indiquant

que chez les souris surexprimant CAT, Nrf2 et HO-1 sont tous deux bien activés et fonctionnels.

En conclusion, nos études suggèrent que la surexpression de CAT dans les RPTCs permet de prévenir la programmation de l'hypertension et les dommages rénaux observés à l'âge adulte chez la progéniture issue de mères diabétiques, en partie suite à l'activation du système de défense Nrf2-HO-1 dans leurs reins.

Il a déjà été démontré que l'activation du système rénine-angiotensine (RAS) intrarénal induit l'hypertension en augmentant la constriction des artérioles et la réabsorption du sodium par les tubules rénaux. Une activation du récepteur AT1R et de ses voies de signalisation induit également les dommages rénaux observés dans plusieurs pathologies. Dans le cadre de mon second article, nous avons identifié un nouveau mécanisme par lequel l'angiotensine (Agt) intrarénale induit l'hypertension et des dommages rénaux en réduisant l'expression de l'aquaporine 1 (AQP1, le canal pour l'eau le plus important dans les RPTCs).

Des souris transgéniques surexprimant l'Agt de rat (rAgt-Tg) dans leurs RPTCs et des clones stables de cellules immortalisées de tubule proximal de rein de rat (IRPTCs) surexprimant le rAgt (pRSV/rAgt-IRPTC) ont été étudiés. Lorsque comparés aux souris non-transgéniques, les souris rAgt-Tg développent de l'hypertension et des dommages rénaux. Ces changements sont atténués par le traitement avec une double inhibition du RAS (losartan et perindopril). L'expression des protéines AQP1 et HO-1 est réduite dans les RPTCs, tandis que Nrf2 et le transporteur sodique NHE3 sont augmentés, à la fois in vivo et in vitro. Ces changements sont renversés par la double inhibition du RAS chez les animaux expérimentaux. Même si les niveaux de Nrf2 sont élevés, une accumulation cytosolique causée par une augmentation de l'export nucléaire induit par GSK3β se produit et ne parvient donc pas à induire l'expression des gènes en aval comme HO-1, ni à réduire l'expression de l'AQP1.

En conclusion, nos résultats suggèrent qu'une déficience en Nrf2 nucléaire mène à une diminution de l'expression de HO-1 et une régulation négative de l'AQP1, jouant un role dans l'hypertension et les dommages rénaux induits par l'Agt intrarénal.

L'hypertension et les dommages rénaux sont des maladies très hétérogènes et multifactorielles qui impliquent l'interaction de diverses molécules et voies de signalisations, et sont influencées par plusieurs facteurs environnementaux tels la diète ou la programmation périnatale. Tous ces différents facteurs contribuent à la progression de l'hypertension et des dommages rénaux, rendant les stratégies de traitement d'autant plus complexes. Dans notre étude, nous avons évalué le développement de l'hypertension dans deux circonstances : l'hypertension de la progéniture programmée par le diabète maternel et l'hypertension induite par l'activation du RAS intrarénal. Nous avons démontré que la génération des ROS dans les reins constitue un facteur majeur commun dans nos deux modèles d'hypertension chez la souris. De plus, le gène/facteur de transcription antioxydant Nrf2, sensible aux ROS, joue un rôle important dans le processus. Grâce à une meilleure compréhension des diverses voies qui mènent à la progression de l'hypertension, nous espérons qu'il sera possible de développer de meilleurs traitements pour faire face à l'hypertension.

Mots clés: Aquaporine 1, catalase, HO-1, hypertension, système rénine-angiotensine intrarénal, diabète maternel, Nrf2, progéniture, programmation périnatale, espèces réactives de l'oxygène

Abstract

The term 'perinatal programming' is used to describe the phenomenon that maternal adverse environment during pregnancies which have profound influences to their offspring later in life. And this concept is well accepted. Previously, we successfully created an *in vivo* murine model and demonstrated that maternal diabetes constitutes an adverse *in utero* environment that may fundamentally impair nephrogenesis and subsequently program of the offspring to develop hypertension and kidney injury in adulthood. It appears that enhanced reactive oxygen species (ROS) generation, activation of the nuclear factor-kappa B (NF-kB), intrarenal renin-angiotensin system (RAS) and p53 pathways were involved in the underlying mechanisms.

In our first study, we investigated whether overexpression of catalase (CAT) in renal proximal tubular cells (RPTCs) could prevent the perinatal programming of hypertension and kidney injury in male offspring of diabetic dams and examined the potential underlying mechanisms both *in vivo* and *in vitro*. Our data demonstrate that CAT overexpression in RPTCs exert a direct effect on nephrogenesis *in utero* and ameliorate maternal diabetes- induced dysnephrogenesis. And further consequently, CAT overexpression in RPTCs preventing maternal diabetes-induced perinatal programming, mediated at least in part, via the nuclear factor-erythroid 2p45 (NF-E2) related factor-2 (Nrf2)- heme oxygenase (HO)- 1 defense system.

Intrarenal RAS activation has attracted more attention in recent years due to studies have been reported that activation of the intrarenal RAS can elicit hypertension and kidney injury independently from the systemic RAS. Previously, we established a murine model (Agt-Tg) that specifically overexpress rat angiotensinogen (Agt) in their RPTCs and develops hypertension and nephropathy. Aquaporin 1 (AQP1) is the major water channel within renal RPTCs, but whether it has a regulatory role in the development of hypertension and nephropathy remains elusive. Our second study aimed to examine the regulation of AQP1 expression in an intrarenal RAS-induced hypertension and kidney injury, focusing on underlying molecular mechanisms. We believe that both our *in vivo* and *in vitro* studies

identified a novel mechanism(s) in which Agt overexpression in RPTCs enhances cytosolic accumulation of Nrf2 via the phosphorylation of pGSK3β Y216. Consequently, less intranuclear Nrf2 is available to trigger HO-1 expression as a defense mechanism and subsequently diminishes AQP1 expression in RPTCs. In conclusion, our data suggest that Agt mediated-downregulation of AQP1 and Nrf2 signaling may play an important role in intrarenal RAS-induced hypertension and kidney injury.

Hypertension and kidney injury is a heterogeneous and multifactorial disease that involves the interaction of various molecules/pathways and the influence of environmental factors, for instance, diet and perinatal programming. Such diverse causes contribute to the progression of hypertension and kidney disease, making the strategy of treatment even more complex. In our present study, we evaluated the development of hypertension under two circumstances: maternal diabetes-programmed hypertension in offspring and intrarenal RAS activation-induced hypertension. We found that ROS generation in the kidneys is a major and common factor in both hypertensive mice model. Also, the ROS-sensitive antioxidant gene/transcription factor – Nrf2, plays an important role in the process. By understanding the pathways that lead to hypertension progression, we can hopefully develop more effective treatments to cope with the disease.

Key words: Aquaporin 1, catalase, HO-1, hypertension, intrarenal renin-angiotensin system, maternal diabetes, Nrf2, offspring, perinatal programming, reactive oxygen species

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List of Abbreviations

ACE: Angiotensin converting enzyme
ACE2: Angiotensin converting enzyme 2
ADH: Antidiuretic hormone
Agt: Angiotensinogen
Agt KO: Agt knockout
Ang I: Angiotensin I
Ang II: Angiotensin II
AQP1: Aquaporin 1
AQP1KO: AQP1 knockout
AQPs: Aquaporins
ARE: Antioxidant response element
AT1b: Type 1 angiotensin II receptor subtype b
AT1R: Ang II type 1 receptor
AT2R: Ang II type 2 receptor
ATP: Adenosine triphosphate
AVP: Arginine vasopressin
BMI: Body mass index
bZIP motif: Basic leucine zipper motif
CAT: Catalase
CDA: Canadian Diabetes Association
CNC family: Cap-n-collar family
CO: Carbon monoxide
Cul-E3: Cullin 3-based ubiquitin E3
DAB: 3,3'-Diaminobenzidine
DOHaD: Developmental origins of health and disease
ECM: Extracellular matrix
eGFR: Estimated glomerular filtration rate
EMT: Epithelia –mesenchymal transition
GCK: Glucokinase
GFR: Glomerular filtration rate
GPX: Glutathione peroxidase
GSH: Glutathione
H_2O_2: Hydrogen peroxide
HAPO study: Hyperglycemia and adverse pregnancy outcomes study
HbA1c: Glycated hemoglobin
HBW: High birth weight
HFD: High fat diet
HNF1A: Hepatocyte nuclear factor-1alpha
HO-1: Heme oxygenase-1
Hoxb7-GFP-Tg mice: Hoxb7-green fluorescence protein-transgenic mice
IDF: International Diabetes Federation
IL-6: Interleukin-6
IRS1 mice: Insulin receptor substrate-1 haploinsufficient mice
IUGR: Intrauterine growth restriction

JGA: Juxtaglomerular apparatus
KAP: Kidney androgen- regulator protein
KCNJ11: Potassium inwardly rectifying channel subfamily J, member 11
Keap1: Kelch-like ECH-associated protein 1
LBW: Low birth weight
MAPK: Mitogen-activated protein kinases
MCP-1: Monocyte chemoattractant protein-1
MET: Mesenchymal–epithelial transition
MM: Metanephric mesenchyme
mTOR: Mammalian target of rapamycin
NAFLD: Non-alcoholic fatty liver disease
NCC: Sodium chloride cotransporter
NCC: Na+/Cl cotransporter
ND: Normal diet
NFkB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NHE3: Na+/H+ exchanger-3
NKCC2: Na-K-Cl cotransporter 2
NOS: Nitric oxide synthases
NOXs: NADPH oxidase
NQO1: NAD(P)H:quinone oxidoreductase1
Nrf2: Nuclear factor (erythroid-derived 2)-like 2
OGTT: Oral glucose tolerance test
O_2 .-: Superoxide anion
.OH: Hydroxyl radical
OR: Odds ratio
PAI-1: Plasminogen activator inhibitor-1
PGDM: Pre-gestational Diabetes Mellitus
PKC: Protein kinase C
RAS: Renin-Angiotensin System
RNS: Reactive nitrogen species
ROS: Reactive oxygen species
RPTCs: Renal proximal tubule cells
SBP: Systolic blood pressure
SOD: Superoxide dismutase
STZ: Streptozocin
T1DM: Type 1 diabetes mellitus
T2DM: Type 2 diabetes mellitus
TCF7L2: Transcription factor 7-like 2
TG: Triacylglycerol
TGF-β1: Transforming growth factor TGF-β1
TNF-α: Tumor necrosis factor-α
UB: Ureteric bud
VEGF: Vascular endothelial growth factor
WHO: World Health Organization

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CHAPTER 1: INTRODUCTION

1.1 Perinatal programming

1.1.1 Definition

Perinatal programming refers to the well accepted concept that the intrauterine environment profoundly affects the developing fetus, and if it is abnormal, this can lead to health problems in the adult [1]. This phenomenon is also called the "developmental origins of health and disease" (DOHaD) [2]. The concept of 'perinatal programming' is substantially supported by many studies that show how an adverse environmental stimulus experienced during a critically sensitive period of development *in utero* can induce short- and/or long-term structural and functional effects resulting in health alterations later in life [2, 3].

My thesis deals with how maternal diabetes influences fetus' kidney development in the intrauterine high glucose environment during pregnancy, by using streptozocin (STZ)-induced maternal diabetes mouse model in Hoxb7-GFP-Tg male mice. The reduced nephron number and higher risks of developing hypertension and kidney injuries for offspring from diabetic mother were ameliorated in Hoxb7/Cat-GFP-Tg male offspring which overexpress catalase (CAT) in their proximal tubules. The work described below has been published in Diabetes 61(10): 2565-2574, 2012, entitled "*Catalase Prevents Maternal Diabetes-Induced Perinatal Programming via the Nrf2-HO-1 (Nuclear factor (erythroid-derived 2)-like 2-Heme oxygensae-1) Defense System*".

1.1.2 The impact of an adverse intrauterine environment on fetal development

In 1989, Hales and Barker conducted a study in England that established a link between insufficient nutrition in pregnancies and the offspring with low birth weight (LBW; defined as less than 2,500 g, [4]) and increased risk of developing cardiac disease, metabolic syndrome and type 2 diabetes mellitus (T2DM) in adulthood [5]. This study was the first to describe the concept of perinatal programming that the authors termed "*the thrifty phenotype hypothesis.*" [6]. Further studies supported the theory that perinatal programming is an adaptive response to an adverse intrauterine nutritional environment, resulting in phenotype changes that begin in the fetus and affect the adult later in life [7].
The term "intrauterine growth restriction" (IUGR) describes a fetus which fails to achieve

full growth potential. IUGR is related to placental insufficiency, which is due to a combination of factors, such as maternal, fetal, placental and environmental factor. Placental insufficiency can be caused by dysfunction of fetal-placental perfusion, caused by maternal pre-eclampsia or hypertension, where hypoxia and ischemia affect both the maternal and fetal circulations [8, 9]. Abnormal placentation, including abnormal angiogenesis and vasculogenesis, also contributes to placental insufficiency. Poor maternal nutrition is the major contributing factor to IUGR. Maternal undernutrition influences the availability of nutrients for the fetus. During maternal starvation, low maternal food intake results in reduced nutrients available to the fetus, causing restriction in fetal growth [10].

In contrast to maternal undernutrition, maternal overnutrion leads to fetal overnutrition, which also constitutes a significant health hazard for both mother and fetus. Maternal overnutrition leads to neonatal macrosomia (also called Big Baby Syndrome), one of the most common pregnancy related health concerns in modern society. Macrosomia is characterized by babies with a birth weight over 4000 g, or greater than the 90^{th} percentile for gestational age, and is characterized by increased fetus adiposity and a number of metabolic and immune system changes [11, 12]. The adipose tissue in the macrosomic infant secretes inflammatory cytokines which lead to chronic inflammation and immune system activation, as well as increased insulin resistance [13]. Excess fat also induces hyperlipidemia and hyperinsulinemia, and leads to lipotoxicity and a build-up of lipids in non-adipose tissue, such as the pancreas, kidney, liver and heart [14].

Similar to maternal obesity, maternal diabetes is characterized by increased glucose level, and shares common factors, such as maternal hyperglycemia, hyperinsulinemia and insulin resistance [15]. The offspring born to a diabetic mother with poorly controlled glycaemia experiences a high risk of congenital malformations during gestation, and is predisposed to develop multiple chronic diseases, such as hypertension, T2D, cardiovascular and kidney diseases in adulthood [16].

A wide range of other perinatal insults and gestational events is known to alter the fetal developmental trajectory, includes environmental exposure to endocrine-disrupting chemicals, disease states, lifestyle (*e.g.* stress), substance abuse (*e.g.* smoking and alcohol

drinking) and medical interventions (*e.g.* androgens and glucocorticoids) during pregnancy. The maternal conditions and fetal outcomes are thoroughly discussed by Padmanabhan *et al* [1] (Figure 1-1).

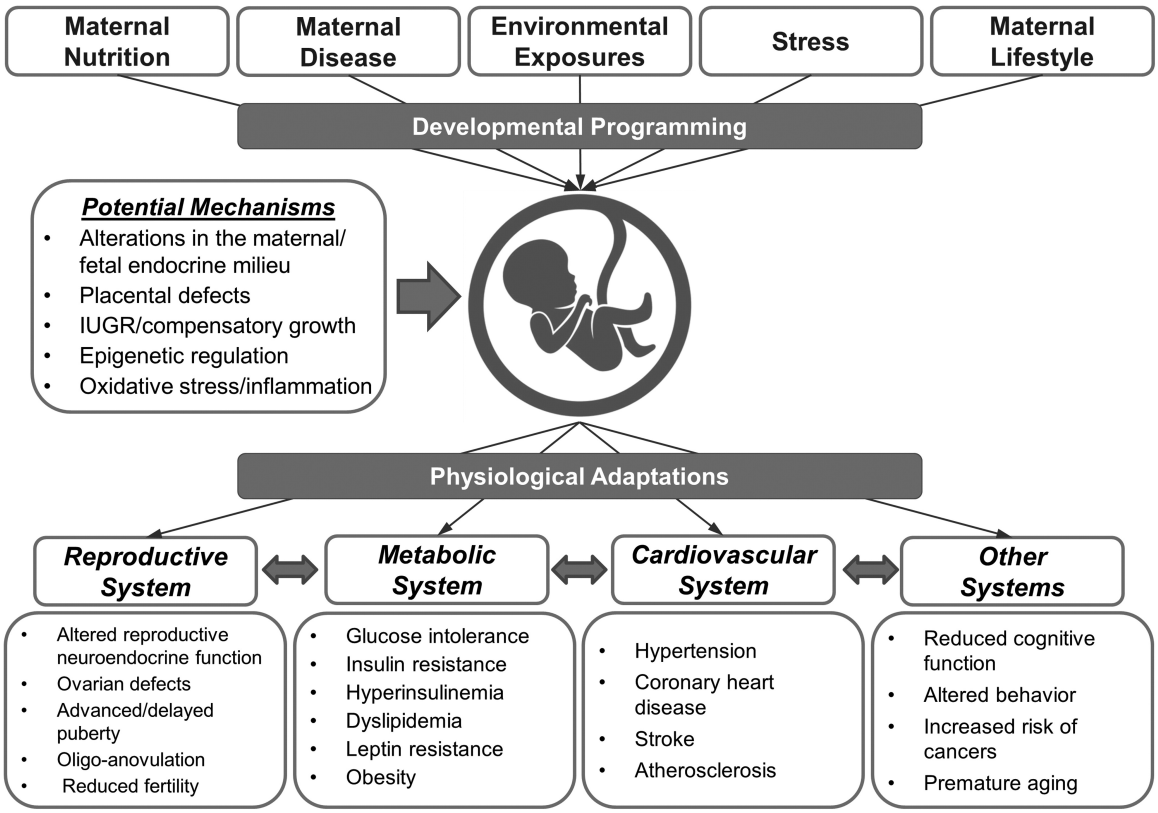

Figure 1-1. Impact of perinatal insults in programming adult pathologies in the offspring. Exposure of the fetus/offspring to different insults during critical periods of development may lead to adaptations that prove to be detrimental and associated with adult defects in several organ systems [1].

1.2 Perinatal programmed hypertension and chronic kidney disease (CKD)

1.2.1 Two important hypothesis

Two hypotheses brought to light the association between maternal environment, birth weight, nephron endowment and diseases later in life. As previously mentioned, in 1989, Barker DJ *et al* reported a correlation between maternal malnutrition, infant's LBW and

higher systolic blood pressure (SBP) later at 2 different population of age: 10 and 36-years-old, respectively [17] — Barker's hypothesis suggested that differences in the intrauterine environment predisposing to differences in blood pressure and cardiovascular mortality in the babies. At about the same time, Brenner BM *et al* hypothesized that a decrease nephron mass may result in a diminished glomerular filtration surface and increased nephron glomerular filtration rate (GFR) of the remaining nephrons [18]. Over time, overloading of the functional capacity of the remaining nephrons, leads to glomerular hyperfiltration, glomerular sclerosis and proteinuria, and eventually causes kidney injury [19] — Brenner's hypothesis postulated that a renal abnormality that contributes to essential hypertension in the general population is a reduced number of nephrons. These two studies shed light on how an adverse intrauterine environment can results in babies with LBW who are susceptible to subsequent chronic kidney disease and hypertension in adulthood.

1.2.2 Reduced nephron endowment is a major mechanism

Many studies were conducted to verify the correlation of birth weight with nephron number and the incidence of hypertension in adulthood [20]. A small case-controlled study conducted in Germany 2003 (n=20) examined the nephron numbers postmortem of adults who's age ranges from 35 to 59 years. This study found a 50% decrease in nephron number in hypertensive adults compared to age-matched controls [21]. A large population conducted in Norway (n=2 millions, 2008) indicated that children born with LBW had a relative risk of 1.7 (95% confidence interval 1.4 to 2.2; $P < 0.001$) for developing end-stage kidney disease later in life [22]. A study in 2008 in Netherland gave salt sensitivity test to 27 healthy male adults (average 37 years) and found a close correlation between LBW and salt-sensitive hypertension in these adults [23]. Similarly, a study in 2008 Switzerland assessed salt sensitivity on 50 children who aged 7 to 15 years and demonstrated that renal mass is reduced in children born with LBW and depends on the degree of growth retardation, which then determines lower GFR, increased salt sensitivity, and elevated blood pressure [24]. Consistent with the observations in humans, a vast of studies in rodent models conducted between 1999 to 2007 also confirmed that late-onset hypertension is associated with LBW and significantly reduced nephron numbers, and together with human data were reviewed by Brenner in 2010 [25].

A meta-analysis in humans have shown a correlation between LBW and renal dysfunction, as well as the effect of speeding up the progression of primary kidney disease [26]. Proteinuria and estimated glomerular filtration rate (eGFR) are important predictive biomarkers of progressive kidney injury [27]. A cohort study (1994 patients and 20,032 controls) in US of mixed races but a majority of white children under 21-year-old found a significant correlation between LBW and enhanced risk of chronic kidney disease (CKD) with adjusted odds ratio=2.88 [28]. Also, averaging the data from 18 human studies, the odds ratio for CKD associated with LBW, was found to be 1.73 [29]. These epidemiological studies suggested a strong association between LBW and late-onset kidney injury.

Studies both in human and in animal models have shown LBW has association with reduced nephron numbers [30-34]. Considering all the evidence, the concept that the maternal intrauterine environment can profoundly affect fetal nephrogenesis, reduce the nephron endowment in the fetus and thus predispose the offspring for developing hypertension later in life is well established [35, 36]. Nowadays, the research focus has been shifted to identify the underlying mechanisms involved in abnormal nephrogenesis, and to develop effective treatments.

Nephron endowment refers to the total number of nephron which a person has at birth, although this number may decline due to age or disease state later in life. In fact, even among healthy individuals, the total number of nephrons is highly variable. This is illustrated in a series of Australian study (from 2003 to 2010) of adult human nephron endowment, where nephron numbers were found to range from 0.2-2.7 millions in African Americans (n=176), and from 0.2-1.6 millions in Caucasians (n=132) [37]. The currently accepted range for a normal human nephron number is approximately 0.9-1 millions per kidney [37]. In mice, the average nephron number of both C3H/HeJ and C57BL/6J mice is 2,000 at birth and around 20,000 at adulthood (8 weeks old) while the kidney development is completed [38]. Currently, the technology available that is used to count nephron numbers accurately in humans relies on autopsy and dissociation of nephrons from kidney tissue by acid maceration [39, 40]. That technique creates a disadvantage when the kidney is destroyed and can not be used for other research purpose [39]. Less invasive methods,

such as ultrasound scanning, provide a second method to assess renal mass in humans, which is based on kidney size determination to attempt to estimate nephron endowment [41]. Direct microscopic observation and nephron counting is used in experimental animal studies, to analyze the detailed changes in nephrogenesis that occur in the fetus exposed to hazardous environmental conditions *in utero*, as well as the pathogenetic mechanisms that occur later in adulthood.

Nephrogenesis refers to the complex process of nephron formation during kidney development [42]. In humans, nephrogenesis starts at the fifth week after gestation and is completed at week 34-36. At birth, the kidneys are fully formed and functional, and the number of nephrons does not increase thereafter [43]. In contrast, nephrogenesis in rodents is not complete at birth, and continues up to 21 days after birth [38].

Nephrogenesis involves a complex process called "branching morphogenesis", which requires the coordinated interaction between the developing tubular epithelia and the renal vasculature. The development takes place in five stages: (1) ureteric bud (UB) development; (2) cap mesenchyme formation; (3) mesenchymal–epithelial transition (MET); (4) glomerulogenesis and tubulogenesis; (5) interstitial cell development [44]. In brief, the primary UB originates at the posterior end of the Wolffian duct as a solid aggregate of epithelial cells that proliferate, migrate, and progressively invade the surrounding metanephric mesenchyme (MM). UB branches in a highly reproducible manner, and the nephron formation is induced at each of its tips. While UB are branching into the MM, some MM cells, including self-renewing progenitor cells, condensate and aggregate around the UB tip of branches, transforming themselves into the cap mesenchymal cells. Cap mesenchyme progressively undergoes MET, which will form most of the epithelia of the nephron. Glomerulogenesis and tubulogenesis stages can be subdivided into four more steps: (1) renal vesicle formation; (2) transformation of the renal vesicles into comma-shaped bodies and then into S-shaped bodies; (3) development of the renal vascular system; (4) progressive development of the nephron and differentiation of the renal interstice [44]. Ultimately, MM differentiates into the main components of the renal corpuscles and the tubular segments, including proximal tubules, the loop of Henle, distal tubules, the juxtaglomerular complex, macula densa, mesangium, and part of the

afferent arterioles, whilst the branches form the collecting system, including collecting ducts, renal pelvis and ureter (Figure 1-2).

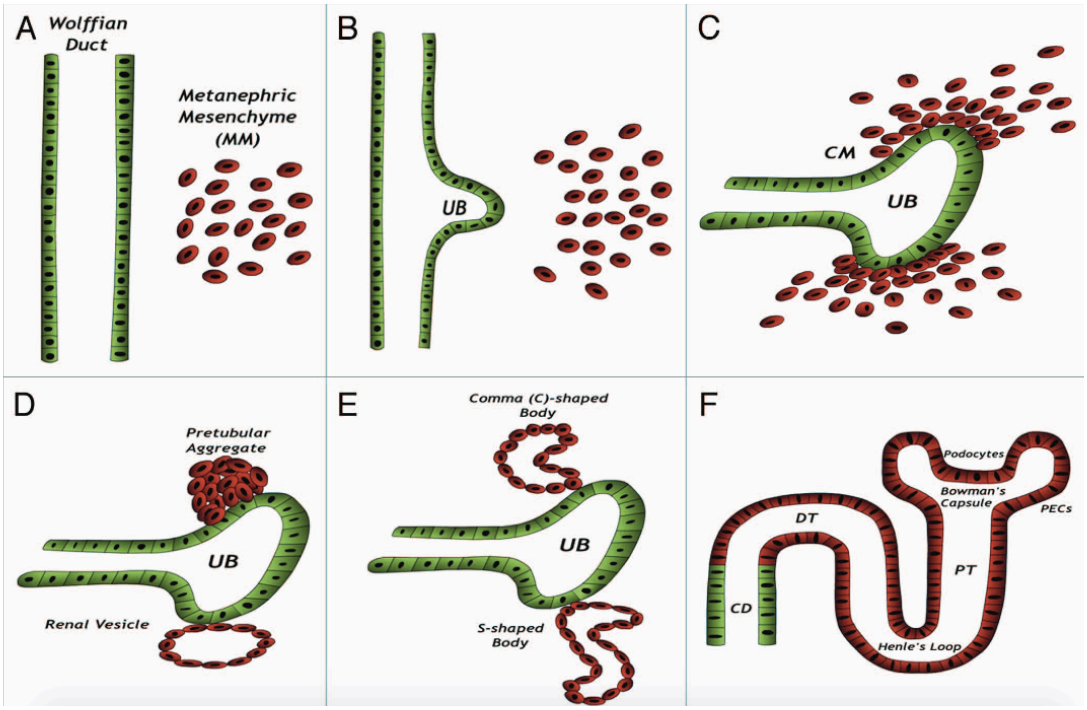

Figure 1-2. Kidney development: (A) The kidney is formed via reciprocal interactions between the Wolffian duct and the MM. (B) MM-derived signals induce the formation of the ureteric bud (UB) from the Wolffian duct. The UB then invades the MM and attracts MM cells. (C) MM cells condense around the tips of the branching UB, forming the condensed mesenchyme, or CM. In response to UB signals, CM cells are induced to undergo mesenchymal-to-epithelial transition (MET). (D–F) The induced cells acquire an epithelial phenotype concomitantly with shutting down of the major transcription factors described before. The cells sequentially form the pretubular aggregate, renal vesicle, C- and S-shaped bodies and finally the mature nephron. The cells derived from the CM form most of the nephron body (from glomerulus to distal tubule), whereas the UB-derived cells form the collecting duct [45].

Hundreds of signaling genes and factors participate in a specific spatial and temporal pattern to play a role in the UB origin, elongation, and branching [44, 46]. For example, multiple genes were reported to act either as inducers (c-Ret, ETv4, ETv5, GDNF, SOX8, SOX9, Wnt11, Angiotensin II, FGFR1, FGFR2, FGF8, p53, MMP-9, Cofilin1, Destrin, AT1R, AT2R, and PAX2), or inhibitors (Spry1, class 3 semaphorins, Robo2, Slit2, BMP4, FoxC1, and FoxC2) [46].

One human UB can branch on average 15 times during nephrogenesis and in murine, 10–11 UB branching events occur during kidney morphogenesis [47]. The developmental process is normally well synchronized, but it can become less well synchronized due to adverse environmental conditions *in utero* (e.g. high glucose), with the results that both kidney morphology and nephron number will become abnormal [48].

1.2.3 Other mechanisms involved in programming hypertension and kidney injury in the infant later in life

Evidences have shown that more other mechanisms participate in programming hypertension and kidney injury, in addition to reduced nephron number. An IUGR animal study done in a placental restriction induced by ligation of bilateral uterine vessel in Wistar Kyoto rat dam model (7-10 mothers per group and 5 pups per litter) proposed that the hypertension observed in the IUGR offspring could be ameliorated by cross-fostering with a normal control dam [49]. Furthermore, in a maternal glucocorticoid administration programmed hypertension rat model, hypertension was observed in offspring that had a reduction in glomeruli as well as in a group that did not have a reduction in glomerular number. The data suggested that a reduction in nephron number is not the only cause for the development of hypertension [50]. Taken together, it appears there is an existence of additional factors that contribute to perinatal programmed hypertension. More potential mechanisms that programmed hypertension in offspring exposed to maternal adverse environment are discussed below.

1.2.3.1 Impaired kidney sodium handling and programmed hypertension

Reduces sodium excretion either by decreasing GFR or by increasing tubular reabsorption of sodium can cause hypertension. The renal sympathetic nerve plays important role in

kidney function, include renin secretion and sodium reabsorption. Two studies which used the maternal placental insufficiency SD rat model [51] and maternal glucocorticoid administration SD rat models [52], observed that fetuses exposed to maternal adverse stimuli develop hypertension later in life, and the high blood pressure could be ameliorated by bilateral renal denervation, which altered sympathetic innervation and caused decreased sodium reabsorption. Aberrant sodium management is also associated with hypertension. Two studies in SD rat with maternal glucocorticoid exposure and maternal low protein diet found increased sodium channels, and increased Na+/H+ exchanger-3 (NHE3), Na-K-Cl cotransporter 2 (NKCC2), Na+/Cl cotransporter (NCC) in renal tubules of the offspring [53, 54].

1.2.3.2 Epigenetic changes and programmed hypertension – an implication of glycemic memories

In utero adverse environment modulates epigenetic modification, which is one of the mechanisms leading to perinatal programming [55]. Epigenetic changes refer to gene expression altered by several mechanisms, including DNA methylation, histone modification, and microRNA expression, without affecting the genetic code. Epigenetic changes influence mRNA transcription resulting in phenotype changes eventually [56, 57].

The term “glycemic memory” (also called “metabolic memory”) was coined in 1990 while the researchers found fibronectin and collagen were highly and persistently produced in endothelial cells of diabetic rats despite their glycaemia had been normalized for two weeks after having diabetes for two weeks [58]. Many large-scale clinical trials and experimental animal studies ensued and supported this concept, and were nicely reviewed [59]. These studies identified early exposure to hyperglycemia or poor glycemic control contribute to intense and prolonged diabetic complications development. The disease progression persists despite glycemic control is improved afterwards. This indicates a memory of glycemic insult and is due to epigenetically alteration of relevant genes [59, 60].

DNA methylation involves the covalent modification of cytosine residues that precede guanines-CpG dinucleotides, with the “p” referring to the phosphodiester bond between

the cytosine and guanine nucleotides [61]. The CpG dinucleotides are clustered in CpG-rich regions of the 5' end of genes where lies promoters, enhancers and suppressors. Methylation of CpGs recruits multiple factors to form a complex that is bound to the promoter and in turn prevents access of transcription factors and RNA polymerases to the DNA and results in the silencing of transcription [62, 63].

Epigenetic changes in the renin angiotensin system (RAS, a major system that controls blood pressure will be described in later section) have been observed in the adrenals of rat offspring of maternal low protein diet. The type 1 angiotensin II receptor subtype b (AT1b) promoter was hypomethylated compared to control, resulting in increased AT1b mRNA expression at 12 weeks of age [64]. This low protein diet model is known to produce hypertensive offspring identified as early as 4 weeks of age [65]. Another study examined the offspring's kidney following maternal IUGR in the rat and found a decrease in CpG methylation of the p53 promoter resulting in increased expression of p53 mRNA levels. Increased renal apoptosis and reduced glomeruli number were also observed in the affected offspring [66]. As reported by another group using the same maternal IUGR model, it was found that affected offspring develop hypertension at 22 weeks of age [67]. These findings emphasized the potential role of epigenetics in developmental programming. However, the exact effects of changes in gene methylation are not always easy to assess [68].

1.2.3.3 Oxidative stress and inflammation and programmed hypertension

Oxidative stress and inflammation are likely to be the common factors that are important in many pathological contexts, not to mention perinatal programmed hypertension [69]. In the rat maternal low protein diet model, both of these two factors were found to play a role in programming hypertension in the offspring [69]. However, the mechanism of how reactive oxygen species (ROS) regulates hypertension in offspring born to diabetic mothers is still unclear. Our laboratory has published a number of studies addressing the role of ROS generated by maternal high glucose intrauterine environment on the developing kidney of the fetus and on the adult-onset kidney disease and hypertension. Details will be discussed later in the following sections.

1.2.3.4 Mechanisms correlate LBW and late-onset kidney injury — the two hit model of kidney disease

The two hit hypothesis is a model which requires two hit of risks in order to generate the clinical phenotype and was originally used to describe the onset of other kidney diseases [70, 71]. In that model, the first hit is an early priming in a genetically predisposed individual and the second hit is a likely environmental insult. The dual hits increase vulnerability of the individual under adverse conditions. Later, other researchers expanded this concept and used it to propose that low nephron number renders the kidney more susceptible to kidney injury. According to this hypothesis, an insufficient nephron endowment is the "first hit," which then subsequently predisposes the person to more severe renal dysfunction if a "second hit" is added, which can be hypertension [55] (Figure 1-3). This hypothesis has been confirmed in IUGR perinatal programmed hypertension rat model [72]. The kidney with less nephron numbers was impaired as a first hit, and giving anti-Thy1 to induce glomerulonephritis as second hit could accelerate the progress of acute glomerulonephritis and lead to more sclerotic lesions [72, 73].

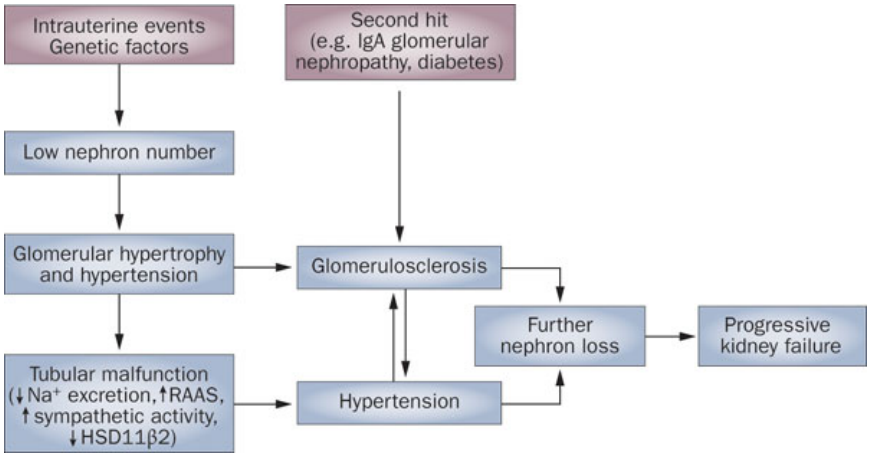

Figure 1-3. Theoretical model for how disturbed nephrogenesis contributes to progressive kidney disease. Prenatal programming causes low nephron numbers, which results in glomerular hypertrophy, tubular malfunction and hypertension, and can lead to glomerulosclerosis and progressive loss of renal function. The kidney is more easily damaged by superimposed kidney diseases; for example, IgA nephropathy and diabetic nephropathy, respectively, tend to have a more severe course and more rapid loss of renal function in individuals with a history of LBW caused by disturbed intrauterine development. Abbreviations: HSD11β2, corticosteroid 11-β-dehydrogenase isozyme 2; Na^+, sodium ion; RAAS, renin–angiotensin–aldosterone system [55].

1.3 Maternal diabetes and adverse outcome

1.3.1 Maternal diabetes

Maternal diabetes refers to either pre-existing diabetes in a pregnant woman (diabetes mellitus type 1 or type 2; T1/T2DM), or the development of insulin resistance and subsequent high blood glucose that is first diagnosed during pregnancy (gestational diabetes mellitus; GDM). According to the latest report of the International Diabetes Federation (IDF), 17% (21millions) of live births in 2013 had hyperglycemia in pregnancy (disregard of stillbirths). Hyperglycemia is the leading cause of perinatal complications to the fetus. Uncontrolled maternal hyperglycemia during pregnancy can result in birth complications, which affect both mother and child, and these are described further below [74].

1.3.2 Maternal diabetes and the maternal complications

Preexisting diabetes in a pregnant woman is known as pre-gestational diabetes mellitus (PGDM). According to the Canadian Diabetes Association (CDA), the diagnostic criteria for diabetes are a fasting plasma glucose level of ≥7.0 mmol/L; random plasma glucose ≥11.1 mmol/L; 2-hour plasma glucose value ≥11.1 mmol/L in a 75 g oral glucose tolerance test (OGTT) or glycated hemoglobin (HbA1C) ≥ 6.5%. In The Northern Diabetic Pregnancy Survey that with a majority of White British ethnicity showed the prevalence of PGDM is rising, reflecting that the prevalence of both T1DM and T2DM in women of childbearing age is increasing [75]. Diabetic patients are already at a higher risk of developing complications, such as retinopathy, nephropathy, and cardiovascular disease. For women with diabetes, pregnancy could worsen the progression of diseases and reduce their lifespan. Details are nicely reviewed by this publication of CDA, entitled *Diabetes and Pregnancy* [76].

Becoming diabetic during pregnancy can occur in women who have never had diabetes before conception. It usually manifests as glucose intolerance resulting in hyperglycemia of variable severity that is first diagnosed during pregnancy, and is called gestational diabetes mellitus (GDM) [77]. GDM generally occurs around the late second trimester of pregnancy and ends after delivery. For women with GDM, although blood glucose levels return to normal after delivery, mothers are at an increasing risk of developing T2DM in

the future (29.4% in a 45-month follow-up period) [78]. Also, a large-scale study included 47,909 women during a follow-up period of more than 10 years indicated that GDM is associated with long-term maternal cardiovascular morbidity (odds ratio=2.7) [79]. In Canada, CDA announced in 2016 that 3-20% of pregnant women develop GDM, depending on their risk factors [80]. Table 1-1. The incidence of aboriginal women is 2-3 times higher than non-aboriginal woman probably because of the genetic background and the poor involvement of public health services [81, 82].

Table1-1 Risk factors for GDM [80].

Being:
- 35 years of age or older
- From a high-risk group (Aboriginal, Hispanic, South Asian, Asian and African)
- Obese (BMI of 30kg/m2 or higher)
- Giving birth to a baby that weighed more than four kilograms (nine pounds)

Using:
- Corticosteroid medication

Having:
- Prediabetes
- Gestational diabetes in a previous pregnancy
- A parent, brother or sister with type 2 diabetes
- Polycystic ovary syndrome (PCOS) or acanthosis nigricans (darkened patches of skin)

Besides the risk factors listed in table 1-1, recent studies have identified several gene variations that are associated with an increased risk of GDM, includes the transcription factor 7-like 2 (TCF7L2), potassium inwardly rectifying channel subfamily J, member 11 (KCNJ11), Glucokinase (GCK), hepatocyte nuclear factor-1alpha (HNF1A), etc [83, 84]. These genes are related to glucose-stimulated insulin secretion, insulin synthesis, pancreatic β-cell proliferation and islet cell volume, and the two studies suggested that the metabolic imbalance observed during GDM pregnancy occurs in women who are genetically predisposed to it. Thus, it appears that GDM results from an interaction between certain genetic backgrounds and environmental factors.

Diagnosis of GDM, according to the recommendations by the World Health Organization (WHO), should be determined by a 2 h, 75 g oral glucose tolerance test administered

anytime during pregnancy (Table 1-2) [78].

Table 1-2. 2013 World Health Organization (WHO) recommendation for the diagnosis of gestational diabetes [85].

Gestational diabetes mellitus should be diagnosed at any time in pregnancy if one or more of the following criteria are met	
Fasting plasma glucose	5.1-6.9 mmol/L (92-125 mg/dL)
1-h plasma glucose following a 75 g oral glucose load	≥ 10.0 mmol/L (180 mg/dL)
2-h plasma glucose following a 75 g oral glucose load	8.5-11.0 mmol/L (153-199 mg/dL)
If fasting plasma glucose ≥ 7.0 mmol/L (126 mg/dL), and/or 2-h plasma glucose ≥ 11.1 mol/ L (200 mg/dL) and/or random plasma glucose ≥ 11.1 mol/L (200 mg/dL) in the presence of diabetes symptoms overt diabetes is diagnosed.	

1.3.3 Maternal diabetes and its complications for the offspring

1.3.3.1 Short-term complications

1.3.3.1.1 Macrosomia

In humans, the risk of being macrosomic (big baby syndrome, also called high birth weight; HBW) for babies born to diabetic mothers is 3 times greater than normoglycemic mothers. According to a 2008 international population study (also called hyperglycemia and adverse pregnancy outcomes (HAPO) study) which included 25,505 pregnant women from 9 countries, maternal fasting blood glucose level higher than 6.9 mmole/L is significantly correlated with fetal birth weight above the 90th percentile (odds ratio=1.38) [86]. When maternal glycemia is high during pregnancy, the excess glucose is transported to the fetus through the placenta. In response to high level of circulating glucose, the fetus secretes insulin after entering the second trimester of gestation, when their pancreas development is mature. As a result, fat and protein uptake is increased, and growth is accelerated (Figure 1-4) [87].

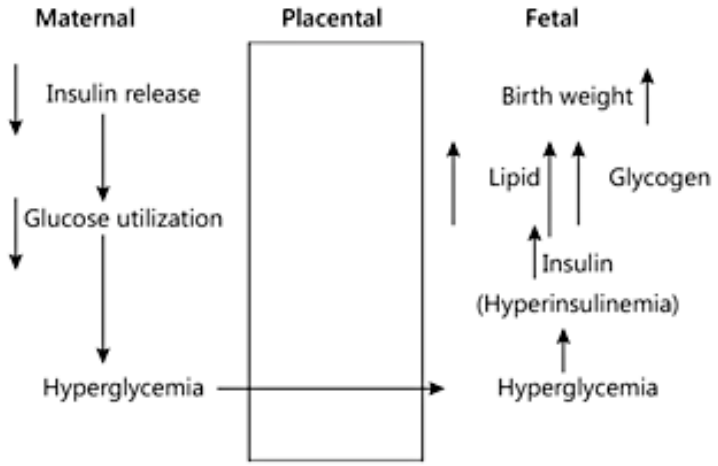

Figure 1-4. Fetal results of maternal hyperglycemia. Modified according to Pedersen's hypothesis [87].

A large fetus size can increase difficulty during vaginal delivery: the fetus may become stuck in the birth canal, requiring additional instruments and/or a C-section. The more difficult and prolonged delivery process could cause complications, such as fetus hypoxia, birth trauma, shoulder dystocia and cerebral palsy [88].

1.3.3.1.2 Congenital malformations

PGDM is associated with a higher incidence of congenital anomalies, compare to GDM. A meta-analysis review study in 2012 indicated that the risk for congenital malformations in PGDM is 1.9–10-fold higher than total population, while the risk is slightly increased in GDM compared to the general population (odds ratio=1.1-1.3) [89]. For PGDM, good glycemic control is the key to prevention of congenital anomalies. The level of risk for newborns is highest just before conception and during the first 5-11 weeks while the baby's organs are beginning to develop [90]. Congenital malformations occur when the development of the embryo is disregulated, such as arrested, delayed or misdirected development effects may involve multiple organs and systems, including respiratory, intestinal tract, cardiovascular, neural tube, genitourinary, musculoskeletal and is a leading cause of infant death [91]. Although with stringent maternal glycemia monitoring and control intervention, the risk of congenital abnormalities for the fetus of diabetic mothers is still 1.7- to 3-fold higher compared with the background population [92].

1.3.3.1.3 Stillbirth and perinatal mortality

Stillbirth is defined as fetal death at or later than 20 weeks of gestation or birth weight of 350 g or greater, while perinatal mortality is defined as the total number of stillbirths and neonatal deaths up to 28 days of life [93]. The perinatal mortality is mainly due to congenital anomalies or complication of prematurity. Both PGDM and GDM are associated with high rate of stillbirth. Patients with PGDM overall have a odds ratio of 3.8 to 6.3 of perinatal mortality compared to women with normal glycemia according to a literature review [93]. The risk of perinatal mortality in GDM is not as high as PGDM [16].

A US study in 2005 compared women who were diagnosed with GDM after 37 weeks (and left untreated till the end) with women who were treated for GDM and women without diabetes. The stillbirth rates in the three groups were 5.4, 3.6, and 1.8 per 1000 births, respectively [94].

1.3.3.1.4 Premature birth

Preterm delivery is defined as labor with gestation less than 37 weeks completed [95]. Preterm birth is responsible for 75% of neonatal mortality and 50% of long-term neurologic impairments in children [96]. Prematurity could be caused by premature rupture of membranes, or maternal hormones and cytokine disorders [97]. Newborns are under the risk of prematurity associated complications, such as infection, respiratory difficulties, and intensive cares are needed [95].

1.3.3.1.5 Hypoglycemia at birth

Immediately after delivery, the newborn's blood insulin level is still high due to the *in utero* high glucose environment, even though the glucose supply from the mother is interrupted while gluconeogenesis and ketogenesis in the newborns are still immature to produce glucose for their selves [98, 99]. Neonatal hypoglycemia is commonly observed in the first hours of life of newborns [99] and persists up to 72 hours and may even last up to 1 week [100], which can lead to cardiopulmonary, central nervous system damage and subsequent mental retardation and recurrent seizure activity [87]. The prevalence of hypoglycemia in newborns of diabetic mothers is as high as 40% compared to newborns

from non-diabetic mothers [98]. Immediate glucose testing and feeding or intravenous glucose injection to the infant is used to effectively treat this condition [101].

1.3.3.2 Long-term complications — perinatal programming

1.3.3.2.1 Obesity

Population of obesity is markedly increasing in all age groups and has become a global issue in the last two decades. A worldwide study in 2014 indicated that nearly 30% of the population including children and adults are either overweight or obese [102]. According to the WHO guideline on this subject, body mass index (BMI) is a useful index of obesity, and is defined as the individual's weight (in kilograms) divided by the square of his/her height (in meters). Individuals with a BMI of 25 or more are considered overweight, and those with a BMI of 30 or more are considered obese [103]. Obesity is associated with a number of metabolic disorders, and causes a great burden of medical care to societies worldwide [104], hence obesity is a major problem which urgently needs to be solved.

Although the individual's eating habits may be one of the causes conferring obesity, studies have shown a strong link between maternal diabetes, body weight at birth and obesity in adulthood [105]. The concept of the obesity growing into a world pandemic, due to a "vicious cycle," was first proposed by Pettitt in 1988, to explain how the maternal diabetic intrauterine environment is transmitted to future generations, by increasing the risk of obesity and T2DM in the offspring, thus contributing to a growing population of obese and diabetes [106] (Figure 1-5).

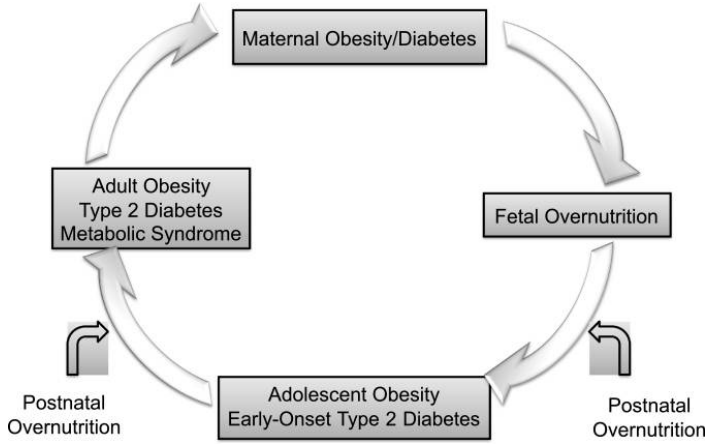

Figure 1-5. Vicious cycle of obesity and diabetes [106].

1.3.3.2.2 Type 2 diabetes mellitus (T2DM)

T2DM is a disease of insulin resistance combined with pancreatic β-cell dysfunction [107]. In the early stage of T2DM onset, both β-cell mass and function are increased to compensate for peripheral insulin resistance. β-cells become inadequate over time, followed by insulin deficiency [107]. Both epidemiological and animal studies indicate that offspring exposed to high glycemia *in utero* are at a higher risk of developing T2DM compared to controls.

A US population studies in 2008 analyzed youth of aged 10–22 years comprised 79 T2DM and 190 nondiabetic controls and found 47.2% (95% CI) of T2DM in youth could be attributed to intrauterine exposure to maternal diabetes and obesity [108]. In the same year, another study in Denmark (n=597) examined young adults aged 18–27 years and concluded that the odds ratio for offspring from GDM mothers to develop T2DM or impaired glucose tolerance is 7.76 while that from T1DM mothers the odds ratio is 4.02, all compared to offspring from control mothers [108]. A study pre-screened healthy participants without T1DM and measured insulin sensitivity and insulin secretion in these participants. Fifteen offspring were born to T1DM mothers whose fathers were healthy and 16 offspring were born to T1DM fathers whose mothers were healthy. They found 33% offspring from T1DM mothers developed glucose intolerance reduced insulin secretion while no offspring from healthy control mothers and T1DM fathers developed the impairment [74].

A rat study continuously infused dams during the last week of pregnancy to mimic mild hyperglycemia intrauterine environment and found the offspring from hyperglycemic dams started to show mild glucose intolerance and impairment of glucose-induced insulin secretion at 1 month of age. This situation persisted and eventually developed to constantly hyperglycemia and severe impairment of glucose tolerance and insulin secretion at 10 month of age [109]. It appears offspring born to mild hyperglycemic mothers (*i.e.* GDM or mother with T2DM) presented with islet hyperplasia, and increased pancreatic and plasma insulin concentrations [110]. In contrast, mouse offspring born to severely hyperglycemic mothers (*i.e.* with T1DM) displayed enhanced islet mass, with degranulated β-cells, suggesting overstimulation by hyperglycemia [111, 112]. This early exhaustion of the

pancreatic insulin secretory capacity may explain the low pancreatic insulin content and low plasma insulin levels in late intrauterus period. Immediately after birth, the mouse pup's islet mass was decreased, the granular content was normalized, and the secretory capacity was restored. However, later during adulthood, the offspring developed insulin resistance [110].

1.3.3.2.3 Cardiovascular disease (CVDs)

CVDs manifests heart and blood vessel dysfunctions and is the number 1 cause of death globally [103]. Coronary heart disease and stroke are the two leading factors causing death [103]. Atherosclerosis is a condition that develops when the arterial walls are repeatedly injured and results in plaque built-up in the walls. The artery walls become sclerotic and narrow over time, and can be broken or blocked, which can lead to heart attack or stroke [103]. Many circulation cell adhesion molecules, such as intercellular adhesion molecule 1 (ICAM1), vascular adhesion molecule 1 (VCAM 1) and E-selectin, are used as a marker to predict CVDs [113]. Although the correlation between obesity, smoking, hypercholesterolemia and cardiovascular disease in adults is well established, evidence from both human and animal studies showed that vascular dysfunction can be programmed as early as the perinatal stage by various adverse maternal environments [114].

A UK study examined children aged 5-11 years (n=61) and found offspring from T1DM mothers expressed higher markers of endothelial dysfunction compared with offspring of nondiabetic pregnancies [115]. A similar US study (n=91) looked at the same endothelial dysfunction markers in children (aged 6-13 years) of maternal GDM exposure and found these children had increased values compared with non-exposed children, independent of BMI [116]. The above data suggested that exposure to maternal diabetes during pregnancy confers risks for the development of CVDs later in life and is independent of other risk factors of the offspring, such as adiposity [117].

The endothelium also plays an important role in the development of the vasculature by secreting vasoactive substances, such as vasodilators (*e.g.* nitric oxide) and vasoconstrictors (*e.g.* angiotensin II), which act on the adjacent vascular smooth muscle cells to cause vasodilation or vasoconstriction [118]. Reduced endothelium-dependent

vasodilatory capacity of the artery is found in 12 month old rat offspring of dams that were induced diabetic with STZ 10 days before mating, compared to offspring of non-diabetic control mother [119]. Altered angiogenesis is an important element in predisposing the development of vascular dysfunction in infants of diabetic mothers. A rat study examined 19 day old offspring of dams which were induced diabetic by STZ on the 5th day of gestation and found that maternal diabetes led to marked alterations in blood vessel differentiation and cardiomyopathy [120]. More specifically, it was pointed out that *in utero* exposure to high glycemia affect angiogenesis via decreased proliferation of endothelial cells via decreased production of vascular endothelial growth factor (VEGF) and VEGF receptor, resulting in embryonic vasculopathy [121].

1.3.3.2.4. Hypertension

According to the WHO, normal adult blood pressure is defined as 120/80 mmHg (systolic/diastolic). When the SBP is ≧ 140 mmHg, and/or the diastolic blood pressure is ≥ 90 mmHg, the blood pressure is considered to be high. High blood pressure can lead to lethal complications, such as stroke, heart attack and kidney failure [122]. One in every four people globally is hypertensive in 2000, and it is anticipated that this population will increase from 972 million to 1.5 billion by 2025 [123]. Even though many treatments for hypertension are currently available, there is still a need to elucidate its cause and underlying mechanisms in order to take precautious intervention and hence halt its growing prevalence.

The causes of hypertension are multiple and complex [124]. Although there is clear evidence that an unhealthy lifestyle (little exercise and an unhealthy diet) contributes to the risk of developing hypertension, there is compelling data from epidemiological and experimental studies which show that maternal adverse environment can also increase the risk of their offspring developing hypertension later in life [125, 126], suggesting that adulthood hypertension might be determined before birth by altered fetal development.

In humans, a population study on Pima Indian (US first Nation, n=42, aged 7-11 year old) indicated that offspring of mothers with maternal diabetes had higher SBP (about 11 mmHg) than mothers with normal glycemia during pregnancy [127]. More recently a

meta-analysis study reviewed 15 publications on the association of diabetic pregnancy and offspring blood pressure in childhood. The review article indicated that SBP was higher in offspring of diabetic mothers (both PGDM and GDM) (mean difference 1.88 mmHg; p=0.009) [128].

A study of rat induced diabetes with STZ 10 days before mating has found that young offspring (3 month old) from diabetic mothers developed hypertension without affecting the nephron number. However, the nitric oxide (NO)-related vascular response is decreased which may contribute to hypertension [119]. In addition to vascular aspect, studies in our lab in the past have revealed that kidney also has an important role to play in the development of maternal diabetes-induced hypertension, and this will be discussed later.

1.3.3.2.5 Chronic kidney disease (CKD)

In 2015, 10% of the population worldwide is affected by CKD, and millions of people die every year because of unaffordable treatment for the patients [129]. Currently there is no cure to reverse CKD but only treatments to slow down the progression. Strategies for early stage of CKD are proper diet control and medication, while in the end stage of CKD hemodialysis or a kidney transplantation is needed. Hypertension and diabetes account for the major causes of CKD, however, it is gaining more and more attention that *in uterus* exposure to adverse maternal environment, such as IUGR, is a risk factor for offspring to develop late-onset CKD [130].

The population studies of the correlation between maternal hyperglycemia exposure and offspring predisposition to CKD later in life is relatively scarce. A study on Pima Indian (n=308, aged 20-61) in 1998 found that 64% of subjects from diabetic mother were of HBW, and the odds ratio to develop albuminuria in subjects with HBW was 3.2 compare to those with normal birth weight [131].

Also, there is an absence of animal studies that look at the influence of maternal diabetes to the offspring kidney function at adulthood. Most of studies focus on nephron number change during development, for example, a study induced rat dams diabetes on day 0 of

pregnancy and found nephron deficit compared to normal glycemic pregnancy by assessing offspring's kidney at 14 day of age [132].

1.4 Mechanism(s) mediating maternal diabetes programmed hypertension and kidney injury: objective of my study

There is a lack of human epidemiological studies correlating maternal diabetes with reduced nephron number in their children. Human epidemiological studies have shown a high correlation between maternal diabetes and their offspring macrosomia at birth [87] and with the development of hypertension and CKD later in life [127, 131]. The 1998 US Pima Indian study described above is a good example [131]. Together with the thrifty phenotype (LBW correlates with hypertension and kidney injuries), it appears that the relationship between birth weight and the risk of developing hypertension and kidney injury later in life is not linear, but instead is U-shaped [34, 131, 133] (Figure 1-6). Only a single French case-controlled clinical study (n=18-19, aged 18-41) measured GFR as an indirect measure of kidney functional reserve (nephron numbers), and concluded that the children of T1DM mothers have reduced nephron numbers and hypertension (Pearson correlation coefficient r=0.61, p=0.006) compared to children of T1DM fathers (r=−0.08, P=0.76) [134]. There is no human study, however, that examines the effect of maternal diabetes and HBW on nephron numbers in human neonates, children or adults.

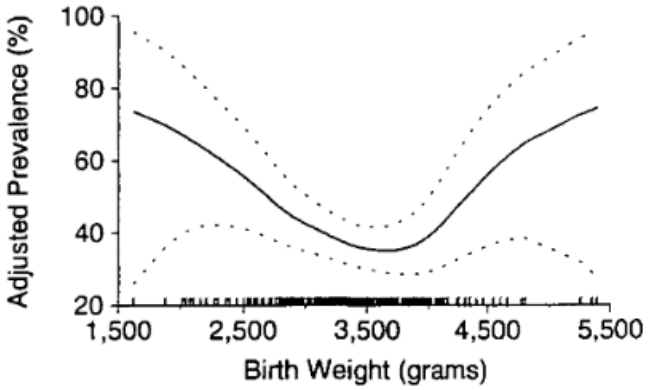

Figure 1-6. Prevalence of elevated urinary albumin excretion (albumin-to-creatinine ratio ≥30 mg/g), by birth weight, adjusted for age, sex, duration of diabetes, HbA1c and mean

arterial pressure in Pima Indians from Gila River Indian Community in Arizona, 1983-1996. Dashed lines represent twice the pointwise asymptotic standard errors of the estimate curve, and the vertical tics on the x-axis are a frequency plot of birth weights [131].

In experimental rodent studies, our research group has shown that pups born to diabetic mother had an increased risk of developing hypertension by 8 weeks of age after birth ($n=9-14$; $p<0.001$; about 50% of offspring are affected) [135]. We demonstrated that pups born to uncontrolled diabetic dams were about 27% smaller than babies born to non-diabetic normal at birth and exhibit approximately 40% nephron loss due to increased apoptosis, that occurred *in utero*. The diabetic dams were treated with insulin right after hyperglycemia was detected (two days after STZ injection), and this normalized the fetus body weight and nephron numbers [135]. We also detected activated renal renin angiotensin system (RAS) components (both protein and mRNA levels) in the hypertensive offspring [135]. Furthermore, we found that transforming growth factor TGF-β1 (TGF-β1) and plasminogen activator inhibitor-1 (PAI-1), which are associated with tubule-interstitial fibrosis, are overexpressed in the kidney of hypertensive offspring born to diabetic mothers [135].

Elucidation of the link between maternal diabetes and the increased risk of programmed hypertension and kidney injury seen in their adulthood offspring is needed in order to develop strategies for the prevention of these chronic disease (hypertension and kidney disease) worldwide. A number of animal studies have shown that gestational diabetes induced birth defects are associated with increased ROS or impaired antioxidant defense systems [136, 137], indicating the enormous influence of ROS on the developing fetus. Also according to our study, renal ROS appears to be a major mechanism involved in nephrogenesis [138]. However, studies that assessed the effect of treating women with gestational diabetes with antioxidants, on the outcomes for their offspring, have given contradictory results or found these ineffective [139, 140].

In my thesis study, I used murine models of maternal diabetes to study the underlying

mechanisms that control the development of late-onset hypertension and kidney injury in the offspring, and focused particularly on the renal ROS pathways. I also tested a novel therapeutic approach aimed at enhancing the oxidative stress defense system in the offspring kidneys, by overexpressing CAT specifically in mouse renal proximal tubule cells (RPTCs).

1.5 Reactive Oxygen species (ROS)

ROS is found to be a critical mediator in many diseases progression, such as and diabetic nephropathy [141]. ROS refers to molecules of oxygen that contain an unpaired electron and thus are very unstable and active [142]. These oxygen molecules are generated constantly within cells because they are a byproduct of aerobic respiration. Cells have developed an antioxidant system to cope with this ROS generation under normal condition. Once the intracellular ROS begin to be produced in an unregulated manner or the defense system is impaired, the excess reactive molecules begin to attack cellular structures, activate redox-sensitive signal transduction pathways and this eventually results in processes that all lead to cellular pathological changes (*e.g.* necrosis, apoptosis, inflammation, fibrosis). This adverse condition is called oxidative stress [141].

There is an emerging class of reactive signaling molecules other than ROS which have also been well studied [142]. These include molecules contain reactive nitrogen species (RNS) and carbon monoxide (CO), which have share similar properties with ROS, but differ in other aspects [143]. In fact, the ROS plays an important role as a signal transduction factor/second messenger, and initiates cellular protective responses, such as activation of cell survival and DNA repair pathways [144, 145].

1.5.1 Origin of ROS

The source of ROS could be endogenous (cellular) or exogenous (xenobiotic). Exogenous ROS concerns drug metabolism, expose to toxic substances, ionizing radiation or infections [144], and is beyond the scope of my thesis. The major source of intracellular ROS includes: 1) NADPH oxidase (NOXs) complexes that are located on cellular membranes, which have seven isoforms that are expressed in different cell types [146]; 2)

mitochondrial respiration chain molecules involved in energy production (*i.e.* adenosine triphosphate, ATP) [147]; 3) the flavoenzyme ERO1 located in the endoplasmic reticulum; 4) lipoxygenases; 5) cyclooxygenases; 6) cytochrome P450s; 7) oxidases for polyamines and amino acids; and, 8) nitric oxide synthases (NOS) [148]. The major forms of ROS are superoxide anion ($O_2^{\cdot -}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2).

Mitochondria are the major site of $O_2^{\cdot -}$ production among them [149]. Briefly, oxygen is converted to water by 4-electron reduction by hydrogen ($O_2+4H\rightarrow 2H_2O$), in one step, which is the so-called electron transport chain. Approximately 1- 3% of electrons leak from this reaction and produce superoxides (e.g. $O_2^{\cdot -}$ and H_2O_2) (Figure 1-6).

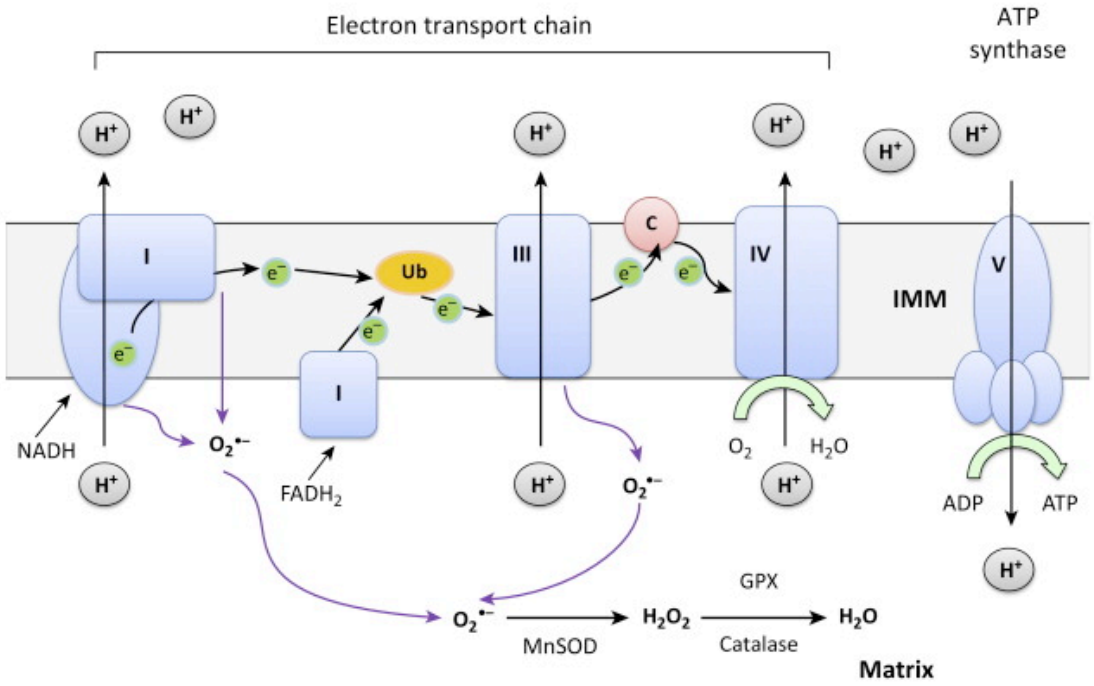

Figure1-6. Mitochondrial respiratory chain. The electron transport chain receives electrons (e^-) from NADH and FADH2 and mediates electron transfer from complex I to complex IV, via ubiquinone (Ub) and cytochrome c (C). At complex IV, electrons reduce molecular oxygen to form water. As a byproduct of the respiratory chain, reactive oxygen species (ROS) are generated. $O_2^{\cdot -}$ is formed at complexes I and III and is dismutated to H_2O_2 by matrix manganese superoxide dismutase (MnSOD). H_2O_2 can then be safely reduced to water by catalase or glutathione peroxidase (GPX) [150].

Besides mitochondria, a variety of other intracellular sources contribute minor amounts of $O_2^{\cdot -}$ and H_2O_2 [151] (Figure 1-7).

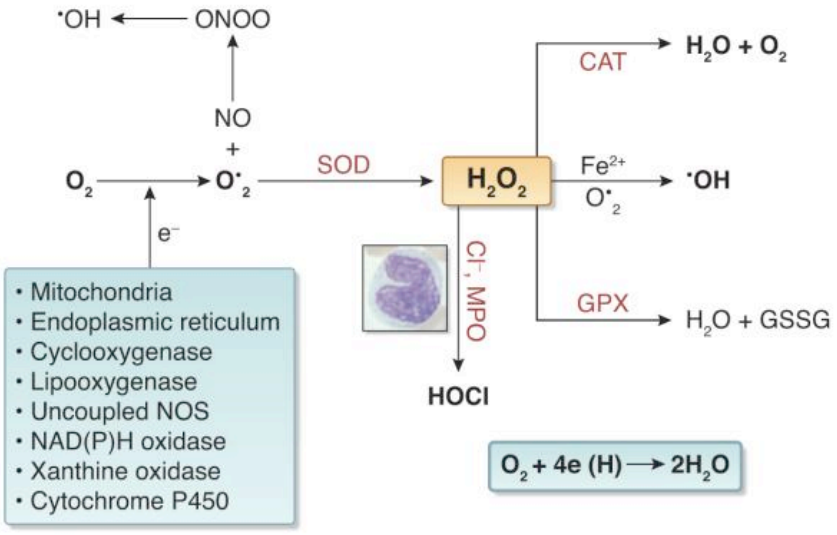

Figure1-7. Production and metabolism of ROS [141]. The primary ROS produced in the body is $O_2^{\cdot -}$, which is formed from single electron reduction of molecular oxygen. The primary sources of $O_2^{\cdot -}$ include the mitochondria, endoplasmic reticulum, cyclooxygenase, lipoxygenase, uncoupled nitric oxide synthase (NOS), NAD(P)H oxidase, xanthine oxidase, and cytochrome P450. Antioxidants then act on ROS to generate less reactive species. For example, superoxide dismutase (SOD) converts superoxide into H_2O_2, which is then reduced by catalase (CAT) into water and oxygen and by glutathione peroxidase (GPx) into water and oxidized glutathione. However in pathological states H_2O_2 serves as the substrate for formation of highly reactive and cytotoxic oxidants such as hydroxyl radical by catalytically-active iron (Fe^{2+}) and hypochlorous acid by myeloperoxidase. An increase in ROS generation or decrease in antioxidant availability leads to oxidative stress and induction of the pro-inflammatory response, which contribute to disease pathogenesis.

1.5.2 The antioxidant defense system: antioxidant genes

The antioxidant defense system includes two categories of compounds: enzymatic and nonenzymatic [149]. Nonenzymatic antioxidants (e.g. vitamin C, β-carotene, glutathione

(GSH) serve as electron donors or react with/scavenge free radicals [152, 153]. Antioxidant enzymes (*e.g.* SOD, CAT, GPx), catalyze the breakdown or removal of free radicals, and hence convert dangerous oxidative molecules to H_2O_2 and eventually to water, through several steps [149]. Antioxidant enzymes include a broad spectrum of enzymes that have been well studied. In my project, I focus on the roles of two antioxidant enzyme genes: catalase (**CAT**) and heme oxygenase (**HO)-1**, as well as one of key antioxidant regulated transcription factors, called **Nrf2** (nuclear factor erythroid 2-related factor 2). These are described further below.

1.5.2.1 CAT

In mammalian cells, CAT is primarily located in intracellular peroxisomes [154, 155] and can also be detected in the cytoplasm [156, 157]. It is widely expressed in almost all organs, and at high levels in liver, kidney, and erythrocytes [158]. CAT is a tetrameric enzyme consisting of four identical 62.5 kDa monomers [159]. It is considered a housekeeping gene due to its lack of TATA box and initiator element sequence, and high promotor GC content [160].

The major function of CAT is to catalyze the dismutation of H_2O_2 and defend cell from injury by oxidative stress [161]. As mentioned above, $O_2.^-$ is produced from a variety of sources, and is first converted to H_2O_2 by SOD, then CAT reduces H_2O_2 to water and oxygen [162].

In the kidney, CAT is highly expressed in the proximal tubules of the juxtamedullary cortex, less in that of the superficial cortex, and not detectable in the glomerulus and other segments of the tubular, such as tubules of Henle's loops, distal tubules and collecting ducts [163]. Altered CAT activity is associated with several kidney injury animal models suggesting that kidney injury impairs CAT function, which can exacerbate oxidative stress and cause renal dysfunction [164, 165], Nrf2 and FoxO1 transcription factors known to upregulate CAT, are found decreased in several kidney disease condition, a putative cause of enhanced oxidative stress-induced cell damage [166, 167].

1.5.2.2 HO-1

There are two forms of HO: HO-1, the inducible form; and, HO-2, the constitutive form. HO-1 and HO-2 are encoded by two different genes, share 40% protein homology and have the same catabolic ability to degrade heme [168]. Briefly, HO catalyzes heme cleavage in the presence of oxygen and NADPH, and as a result, biliverdin is produced along with iron and CO. Biliverdin is further rapidly converted to bilirubin by the enzyme bilirubin reductase [169]. All three degradation products (iron, bilirubin, CO) have beneficial regulatory functions in cells. Iron is an essential component of hemoglobin and ferritin; CO is a messenger and signaling molecule that promotes vasodilation, anti-inflammation and anti-apoptosis [170]; bilirubin acts as a potent cellular antioxidant and anti-inflammatory agent [171]. These functions make HO as an antioxidant gene. However, HO-1 (but not HO-2) is responsive to oxidative stress and to numerous drugs and chemicals, including statins, aspirin, niacin, specific prostaglandins [172]. This makes HO-1 the preferred research target [172].

The basal expression of HO-1 in the kidney is abundant. In rodents, it can be detected by immunostaining in both proximal and distal tubules, as well as in the medullar collecting tubules and loops of Henle [173]. Responding to insult such as hyperglycemia, HO-1 is also expressed in glomeruli [174]. Moreover, HO-1 expression is detected in human proximal tubules under disease condition, such as ischemia/reperfusion acute kidney injury and diabetic nephropathy [175]. The upregulation of HO-1 is likely via transcription factors including Nrf2, nuclear factor-κB (NFκB), PI3K/Akt, p38 mitogen-activated protein kinases (MAPK)) [176, 177]. Overexpression of HO-1 specifically in the kidney, both by chemical induction (e.g. CoPP) or genetic engineering (e.g. HO-1 transgenic/knockout), in renal disease animal models, demonstrates that HO-1 has a cytoprotective function [175, 178, 179].

1.5.2.3 Nrf2

Nrf2, belongs to the cap-n-collar (CNC) family of transcription factors which contain the basic leucine zipper (bZIP) motif, is known to be an activator of almost all the phase II antioxidant genes [180, 181]. Under normal condition, Nrf2 associates with Keap1 (Kelch-like ECH-associated protein 1) and remains in the cytosol. Keap1 is a

redox/electrophile stress sensor, Nrf2-binding protein, and also an adapter protein between Nrf2 and the Cullin 3-based ubiquitin E3 (Cul-E3) ligase complex. Once Keap-1 binds to Nrf2, Keap1 promotes Nrf2 degradation by presenting ubiquitinated Nrf2 to proteasomes [182, 183]. When Keap1 senses oxidative stress, it undergoes conformational change and releases Nrf2. Nrf2 then translocates into the nucleus and targets genes which possess an antioxidant response element (ARE) in their promoter region [184]. Nrf2 is not only passively modulated by Keap1, it can also be induced by phosphorylation of certain serine or threonine residues of Nrf2 by upstream kinases (*e.g.* protein kinase C (PKC), MAPK) [185].

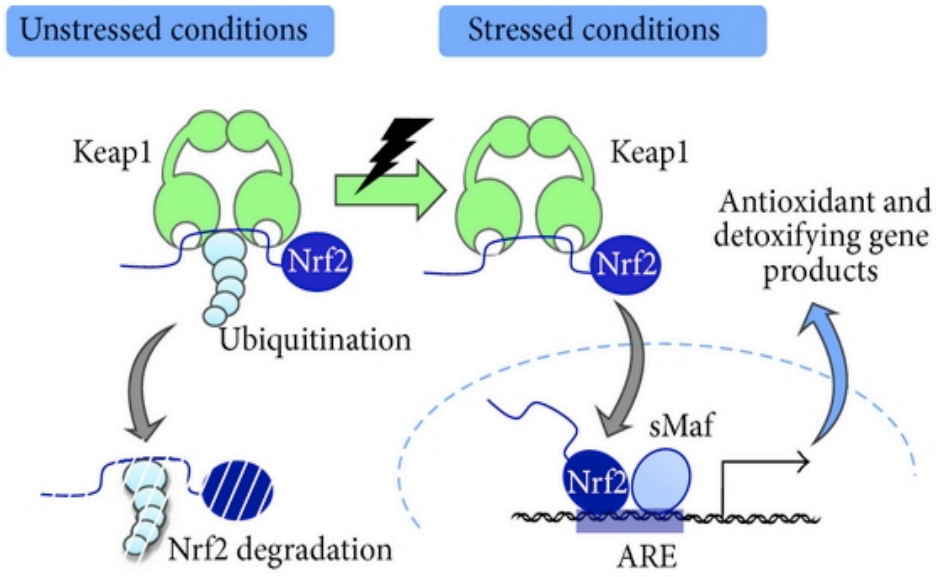

Figure 1-8. Keap1-Nrf2 stress response system. Stress-sensing system of Keap1 and Nrf2. Environmental stresses, including ROS and electrophiles, inactivate Keap1 and stall the ubiquitination and degradation of Nrf2. Nrf2 accumulates in the nucleus and forms a heterodimer with the sMaf protein. The binding of the Nrf2-sMaf heterodimer to the EpRE/ARE motif leads to the transactivation of Nrf2 target genes, which include a battery of antioxidant and detoxifying genes required for cellular protection [186] .

In the nucleus, Nrf2 binds to the ARE region of its target genes, to form a heterodimer with another transcription factor (*i.e.* Maf protein), thus activating transcription of the gene [187]. A large numbers of genes of antioxidant and phase II detoxifying enzymes are known to be regulated by Nrf2 (*e.g.* CAT, SOD, HO-1, NAD(P)H:quinone oxidoreductase1 (NQO1)) (Figure 1-8) [180, 181].

1.6 Renin-Angiotensin System (RAS)

Hypertension and kidney injury is a heterogeneous and multifactorial disease that involves the interaction of various molecules/pathways and the influence of environmental factors, for instance, diet and perinatal programming. In my second study, in addition to maternal diabetes-induced hypertension and kidney injury in offspring, I also investigate intrarenal activated RAS-induced hypertension and kidney injury. Under physiological conditions, RAS plays important roles in the regulation of renal functions and blood pressure and in the maintenance of homeostasis of electrolyte balance and body fluid composition. Once intrarenal RAS is dysregulated and activated, it can lead to development of hypertension and kidney injury.

In my second project, using a transgenic animal model, I identified a novel pathway that overexpress angiotensinogen (Agt) in renal proximal tubule cells (RPTCs) could affect one of key water channels, aquaporin 1 (AQP1), via Nrf2/GSK3β/β-catenin pathways. This article is entitled: *Overexpression of Angiotensinogen Downregulates Aquaporin 1 Expression via Modulation of Nrf2-HO-1 Pathway in Renal Proximal Tubular Cells of Transgenic Mice,* is published in Journal of the Renin-Angiotensin Aldosterone System 2016 Sep 15;17(3).

1.6.1 The systemic RAS

The systemic (also called circulating) RAS is a very important regulatory system that controls blood pressure through arterial modulation and mediates several cellular signaling pathways [188]. Not properly balanced/counterbalanced RAS or activated downstream signaling promote reactions that are harmful (*e.g.* inflammation, cytokine production, cell proliferation, fibrosis [189]) in many disease context (*e.g.* cancer [190] and high glucose

[191].) In this system, it majorly comprises: the precursor angiotensinogen (Agt) and its effective product Ang II; the enzymes renin, angiotensin converting enzyme (ACE) and ACE2; and Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R), two Ang II receptors. Ang II is generated through a sequential cleavage cascade from Agt and is the most important active peptide in this system.

Briefly, Agt is synthesized in liver hepatocytes, and then released into the circulation. Renin, an aspartyl protease generated in the juxtaglomerular (JG) apparatus of the kidney, cleaves 10 amino acids from the Agt N-terminus, to form angiotensin I (Ang I). This is the rate-limiting step in the RAS system, which controls the activity of the entire system. Angiotensin converting enzyme (ACE) is a dicarboxypeptidase that catalyzes the conversion from Ang I to Ang II, by removing two amino acids from the c-terminus of Ang I. ACE is a membrane-bound protein which is abundantly expressed in the vascular endothelium, the lung, renal proximal tubular epithelium and ciliated intestinal epithelium [192]. Ang II is a key regulator of RAS system, and executes its function by binding to its G-protein-coupled receptor. There are two types of Ang II receptor AT1R and AT2R. AT1R is located in arterioles and causes vascular smooth muscle cells to contract, which reduces the arterial lumen and increases resistance to blood flow, ultimately increasing blood pressure. AT2R also located in arterioles has shown to counteract AT1R effects, which is vasodilation. In addition to blood pressure regulation, AT1R also plays roles in cellular proliferation, inducing inflammation and fibrosis [193]. AT2R is found to exert antagonistic effects against effects of AT1R, such as anti-inflammation and anti-fibrosis [194, 195]. Animals lacking AT2R develop hypertension [196] positions AT2R an important counterbalancing element in the RAS system.

The ACE2/Ang-(1-7)/MasR pathway counterbalances the ACE/Ang II/AT1R axis. The ACE/Ang II/AT1R axis has long been recognized as the classic RAS system. In year 2000, an ACE protein homolog, ACE2, was discovered. ACE and ACE2 share more than 40% identity in the catalytic domain [197, 198], but have different function. ACE2 is generated in testis, lung, intestines and brain, and highly expressed in heart and kidney, where it was shown to be bound to the apical membrane of polarized cells and face externally [199]. The major function of ACE2 is to cleave Ang II into Ang-(1-7) peptide [200, 201].

Ang-(1-7) is a ligand for the Mas receptor (MasR), which mediates effects that oppose the AT1R effects, including vasodilation, anti-cell proliferation, and vascular protection [202-204]. ACE2 hydrolyzes Ang I to Ang-(1-9) and prevents the formation of Ang II by ACE [197]. This ACE2/Ang-(1-7)/MasR pathway counteracts the action of the classical RAS pathway and plays a protective role in many settings (Figure 1-9).

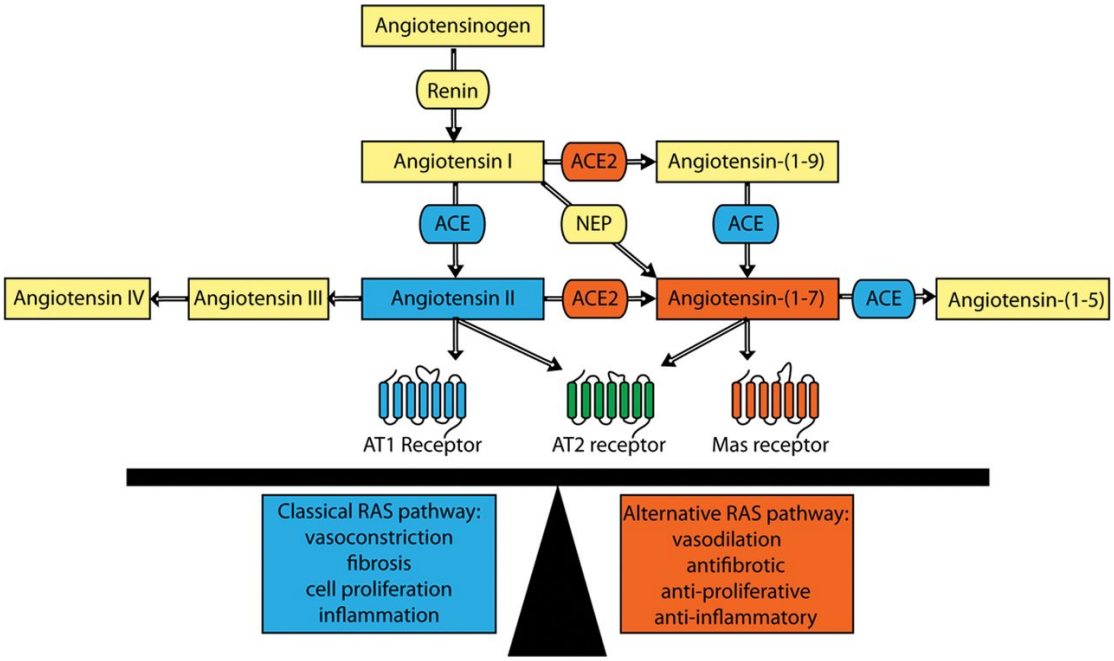

Figure1-9. Overview of the RAS [193].

1.6.2 The local RAS

In addition to the systemic RAS, a local RAS in different organ and tissue expressing all the components of RAS members plays an important role in response to disease or injury condition [193, 205, 206]. Some local RAS function independently in one organ only (e.g. testis and adipose tissue), while other RAS (e.g. heart, kidney) interact with the systemic RAS.

1.6.3 The intrarenal RAS system

The functional role of intrarenal RAS is to regulate water transport and hemodynamics, which are associated with sodium balance and blood pressure homeostasis. A dysregulated or abnormally activated intrarenal RAS can cause hypertension and kidney injury [207]. The local intrarenal RAS contains all the components of RAS. Agt is predominantly

produced in the S3 segment of kidney proximal tubules [208, 209]; renin is expressed in kidney JG cells and macula densa of collecting ducts; ACE is generated in kidney proximal tubules, endothelial cells and distal nephron segments [210, 211]; AT1R and AT2R are wildly distribute in all the glomerular and tubular tissue. Agt derived from proximal tubules is release into the kidney tubule lumen, reaches the distal tubules and eventually contributes urinary Ang II. To confirm this concept, Ding *et al* conducted a study of overexpression human Agt specifically in mouse proximal tubule cells demonstrated that human Agt was evident in the urine of transgenic mouse and no detectable human angiotensinogen protein in plasma [212, 213].

Intrarenal RAS regulate blood pressure via several ways. In glomeruli, Ang II controls the vascular tone of the afferent and efferent arterioles via AT1R. In renal tubules, Ang II regulates several transporters (e.g. NHE3 and $Na+/HCO3^-$ cotransporter) located in the proximal tubules, and sodium chloride cotransporter (NCC) located in the kidney distal tubules. In addition, Ang II is one of the mediators of the tubuloglomerular feedback mechanism, that modulates the interaction between glomeruli and the renal tubules [214]. When the body is in depleted of sodium, water, or in conditions of hypertension, the intrarenal RAS is increased or activated to balance to normal physiology.

The intrarenal RAS is formed independently from the systemic RAS and it is thought to be the major system modulating body fluid homeostasis; as supported by the following evidence. Firstly, the plasma Ang II concentration in rats is in the picomolar range (50-100 pM) [207], whereas Ang II concentration in the proximal tubule fluid of rats is in the nanomolar range (30−40 nM) [215], which is about 1000 folds higher, providing evidence that the regulation of the substantial intrarenal RAS system is autonomous from the systemic RAS. Secondly, it is debatable whether liver derived Agt is also an important source of renal Agt. Two organ-specific Agt knockout mice (liver Agt KO, kidney Agt KO and dual KO) were studied by Matsusaka *et al*, and found that the Ang II level present in whole kidney tissue remains the same in kidney of kidney Agt KO mice in comparison of WT controls. However, in liver Agt KO mice, the level of whole kidney Ang II is only 13% of the value in control mice. The data suggested that renal Agt II originates mainly from the liver, while a minor proportion is produced locally in the kidney [216]. Other

researchers challenge this conclusion and argued that this study is based on normal dietary conditions [217]. Intra proximal tubular Ang II is still the most potent stimulant to influence blood pressure, in terms of dynamic dietary salt intake. A study evaluated two mouse strains: the sodium-sensitive inbred C57BL/6 and the sodium-resistant CD1 outbred. Under high sodium and low sodium diet, C57BL/6 mice had elevated urinary total Agt levels compared CD1, while plasma Agt of both strains remained unaltered levels, reflecting tubular RAS response to challenges of sodium homeostasis instead of systemic RAS [218]. Another study overexpressed Agt specifically in RPTCs of mice observed salt-sensitive hypertension, without recruitment of the circulating RAS [219]. Also a study on rat demonstrated that high salt intake increased proximal tubule luminal Ang II concentrations while decreased plasma and total kidney Ang II concentration [220]. Thirdly, in response to increasing serum Ang II concentration, AT1R on JG cells sense and signal to inhibit renin secretion. Unlike the negative feedback of the systemic RAS, AT1R located in the kidney collecting ducts stimulate renin secretion upon receiving excess Ang II, resulting in further increasing Ang II in the kidney [221]. This emphasizes that while renin formation from kidney JG cells is shut down, intrarenal Ang II formation in the collecting ducts can still continue independently. Taken together, intrarenal RAS system plays more important role regarding water and salt homeostasis.

1.6.4 Intrarenal RAS and hypertension

Studies in both experimental animal models and human patients established a correlation between hypertension and an augmented intrarenal RAS [207, 222]. Ang II causes hypertension by inducing renal vasoconstriction and increasing proximal tubule sodium reabsorption, thus expanding blood volume through AT1R activation. The augmented intrarenal Ang II level is contributed from two sources: 1) AT1R activates the ligand-receptor complex of internalization, leading to Ang II uptake from the blood stream into the intrarenal compartment [223, 224]; 2) AT1R stimulates Agt mRNA synthesis and protein production in proximal tubules [225-227].

Inhibition of Ang II level or Ang II signaling by AT1R are important strategies currently used for treating hypertension, including ACE inhibitor (ACEI) and angiotensin receptor blockade (ARB) [228]. Other antihypertensive strategies, such as diuretics, beta-adrenergic

blockers and calcium antagonists, when tested in large clinical trials, provided no additional advantages for improving diabetic nephropathy [229] or hypertensive kidney disease [230]. RAS blockade by ACEI delayed the onset and prevented kidney complication progression, as well as reduced blood pressure [230]. Furthermore, the combination treatment of ACEI plus ARB showed greater effectiveness for reducing proteinuria in non-diabetic patients with persistent proteinuria, compare to single blockade or doubling the dose [231].

1.6.5 Intrarenal RAS and kidney injury

Interstitial fibrosis is the common final outcome of progressive renal disease, due to the fact that tubular interstitial damage, especially proximal tubules, is highly correlated with the decline of kidney function [232]. Ang II plays a pivotal role in promoting renal fibrogenesis [233]. TGF-β1 and oxidative stress are the major mediators of Ang II-induced kidney fibrosis progression, by causing apoptosis and EMT of the renal tubular epithelium. In proximal tubule cells, Ang II stimulates TGF-β1 expression [234] and triggers downstream production of PAI-1, and extracellular matrix (ECM) protein synthesis and deposition in the interstitial space [235, 236]. Induced by Ang II, oxidative stress contributes to EMT by activating Src kinase that phosphorylates caveolin-1 and its downstream adaptor proteins, eventually leading to EMT [237]. Furthermore, Ang II can activate cell signaling pathways, such as mammalian target of rapamycin (mTOR), nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), which causes extracellular matrix remodeling [238, 239]. Ang II also acts as a proinflammatory factor that promotes inflammation in many tissue and organs, activating the expression of numerous inflammatory cytokines, such as interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) in the kidney [240, 241].

Since the consequences of RAS activation are well documented, pharmacological blockade of RAS is the most potent strategy to prevent hypertension and kidney injury elicited by RAS [228]. Indeed, RAS blockade, by ACEI and ARB, are extensively used in treating hypertension both in human and experimental animals. Also, these treatments reduce blood pressure, inflammation, oxidative stress, and prevent chronic kidney disease progression [242].

1.7 The kidneys and proximal tubules

The primary function of kidneys is maintaining a constant composition and volume of the extracellular fluid by controlling ion (Na^+, K^+, Cl^-) and water transport. Since the adult has variable daily intake, to keep virtually no change in the volume and composition of the extracellular fluid volume is an enormous task that the kidney is facing. [243]. The kidney consists of an outer region (cortex) and an inner region (medulla). In the cortex and medulla, nephrons, blood vessels and lymphatics interact with each other (Figure 1-10).

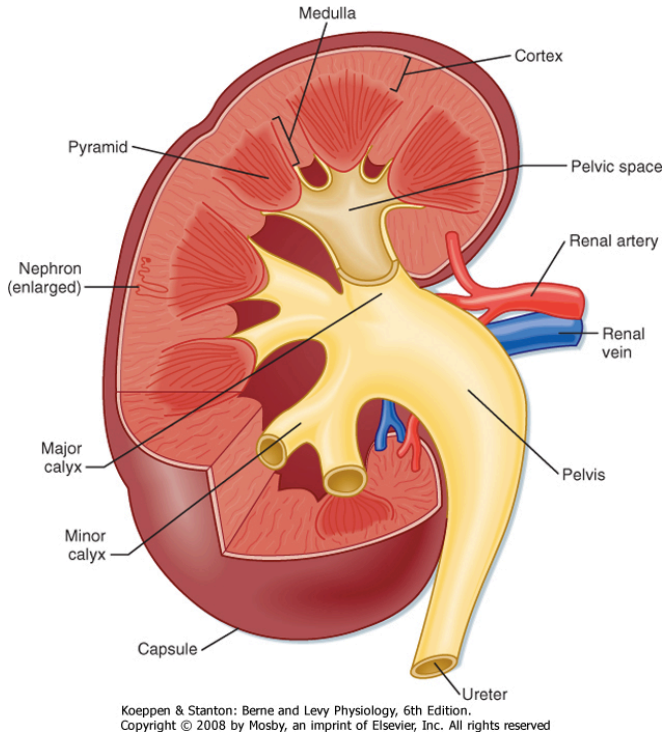

Figure 1-10. Structure of a human kidney, cut open to show the internal structures [243].

Nephron is the smallest functional unit of the kidney. The nephron is a long, tubular structure, consisting of a single monolayer of cells, and is divided into several segments: renal corpuscle, proximal tubule, loop of Henle, distal tubule and collecting duct (Figure 1-11). Inside the renal corpuscle lies a cluster of blood capillaries that is surrounded by

Bowman's capsule, called a glomerulus. Each part of the kidney plays a distinct yet pivotal role in urine formation.

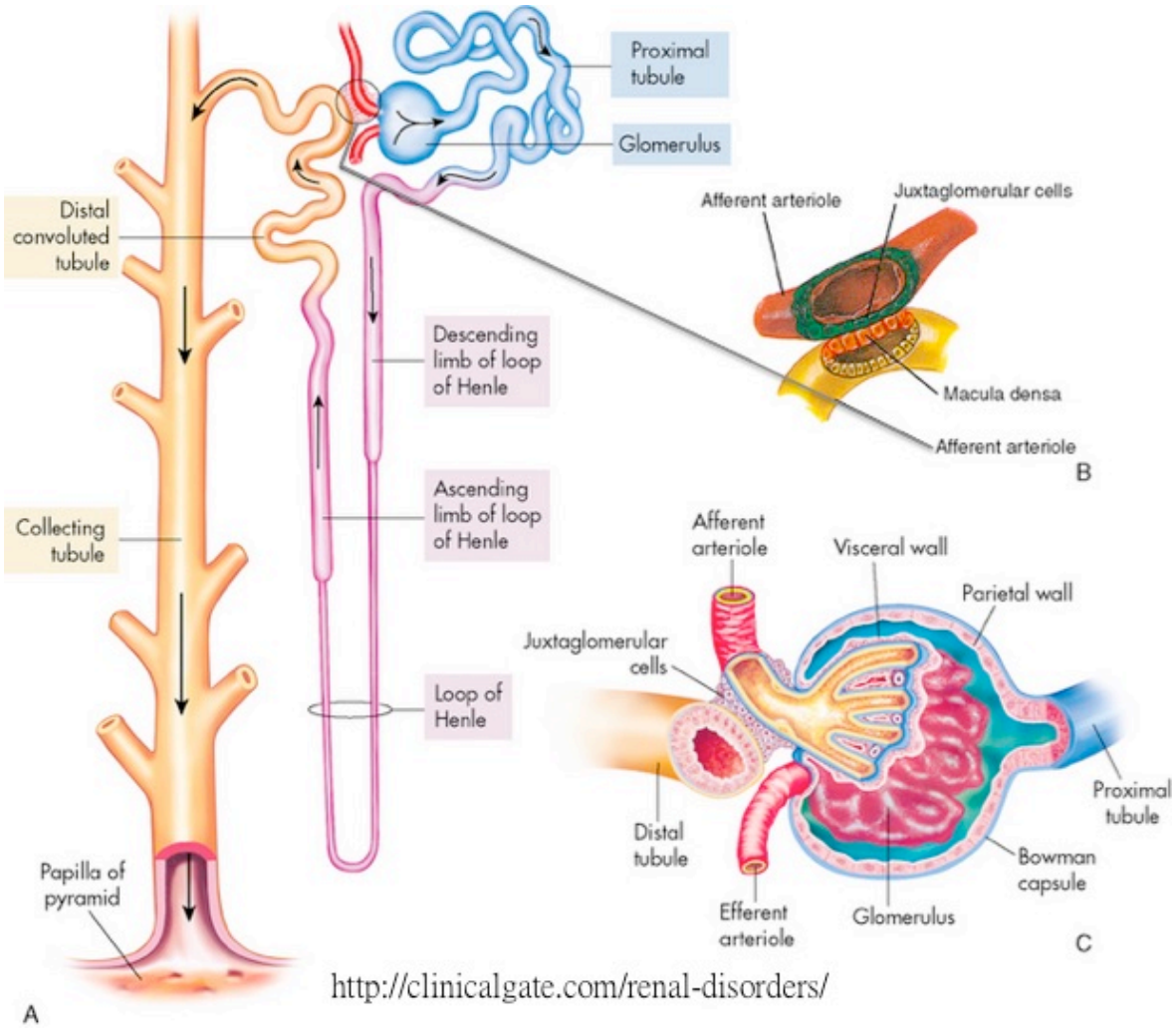

Figure 1-11. Components of the nephron. *A*: one nephron. *B*: Cells of juxtaglomerular apparatus. *C*: Glomerulous and juxtaglomerular apparatus [244].

1.7.1 Glomerulus

All the components in blood stream travel to the blood capillaries in the glomerulus, who's main function is to filter plasma to produce glomerular filtrate. Unique, highly differentiated epithelial cells (podocytes) cover the outside of the capillaries and function as a sieve. The capillary endothelial cells, glomerular basement membrane and foot of the

podocytes, the three major components together form the filtration barrier [245].

The glomerular filtration barrier is freely permeable to water, small and midsized solutes in plasma (e.g. urea), while larger molecules and negatively charged proteins retain in plasma. The ultrafiltration fluids flow into Bowman's space [243].

1.7.2 Proximal tubules

The renal proximal tubule, comprising the proximal convoluted tubule (S1 and S2 segments) and the proximal straight tubule (S3 segment), is responsible for 65–70% of sodium and water reabsorption under normal conditions [246]. A RPTC is a cuboidal epithelium cell, which has a specific orientation. The RPTC's apical membrane faces toward the lumen of the tubule, which contains the urine, and its unique feature is its extensively amplified membrane surface (brush border). The RPTC's basolateral membrane faces the blood side of the tubule and is highly invaginated. RPTCs express numerous kinds of channels and reabsorb most of the sodium and water, and all of the glucose as well as many other substances from the ultrafiltration fluid that comes from Bowman's space. The reabsorbed substances then flow back into the bloodstream from this location [243].

1.7.3 Juxtaglomerular apparatus (JGA)

The JGA is located between the afferent arteriole and the returning distal convoluted tubule of the same nephron. It is responsible for regulating both intrarenal RAS and extrarenal (systemic RAS) mechanisms necessary to maintain both renal and entire body volume status. The JGA is composed by three components: 1) the juxtaglomerular (JG) cells of the afferent arteriole, synthesize and store renin. 2) The macula densa, a region of the distal convoluted tubule but these tubular epithelial cells are more densely packed than other tubular epithelial cells. The macula densa senses decreased NaCl and determines whether to release renin. 3) mesangial cells, which connects afferent and efferent arterioles and determine vasoconstriction or vasodilation via mesangial cell contraction [243].

1.7.4 Other compartments of renal tubular

Loop of Henle: This is composed of the thin descending limb, the thin ascending limb and

the thick ascending limb. The thin descending limb is very permeable to water and this helps to concentrate the ultrafiltrate. The thin ascending limb and the thick ascending limb are not permeable to water, and instead reabsorb ions. This creates an osmotic gradient, which increases water reabsorption and maximizes conservation of water. The macula densa is a group of cells that has distinct properties and functions that are different from those of tubule cells [243].

The distal tubule: This is composed of a simple cuboidal epithelium, as the distal tubule cells have no brush border. The permeability of water is regulated by arginine vasopressin (AVP) and helps to concentrate urine further in the distal tubule. Being immediately downstream of the macula densa, the distal tubule is responsible for a variety of homeostatic processes, including sodium chloride reabsorption and potassium secretion, similar to the other sections of the nephron [243].

Collecting duct: This is composed of two cell types: principal cells and intercalated cells. The former plays an important role in reabsorption of sodium chloride and secretion of potassium ion, while the latter plays an important role in regulating pH [243].

1.8 Water homeostasis in kidney and blood pressure regulation

For all cells to survive and function properly in the body, the tonicity balance of extracellular fluid and intracellular composition is extremely important [243]. In mammalians, the serum sodium concentration must be maintained in a very narrow range despite dynamic food and liquid intake. The kidney, especially the renal tubule, is the primary organ involved in regulation and maintenance of the body's tonicity and water homeostasis.

Under normal conditions, 65-70% of the glomerular ultrafiltrate is reabsorbed in the kidney proximal tubules, including both water and electrolytes. The reabsorption of sodium into the proximal tubular interstitium creates a major driving force and is energy consuming. Water reabsorption is passive, driven by osmolality between the tubule lumen and the renal interstitium, through water channels. The remaining 30% of the ultrafiltrate

flows to Henle's loop and generates a medullary interstitial osmolality gradient, which is an essential step before the ultrafiltrate entering the distal tubule. A decreasing amount of filtrate coming from proximal tubule can perturbs water homeostasis by interfering with the tonicity gradient. Finally, the ultrafiltrate is delivered to the distal tubule and collecting duct, and undergoes additional water reabsorption. At this point, the concentration of the urine is controlled by AVP, a hormone that is synthesized in the hypothalamus and released into the bloodstream upon sensing a change in plasma osmolality. AVP binds to its receptor located on the collecting duct, and this signals activation of water channel expression on the apical side of the cell membrane of distal tubules. Due to its anti-diuretic function, AVP is also called antidiuretic hormone (ADH).

In humans, every day 180 liter of plasma is filtered through the kidney glomeruli and less than 1% becomes urine and is excreted, thus 99% of this water is reabsorbed by renal tubules. Approximately 70% of the reabsorption takes place in the proximal tubule and 15% in the descending thin limb of Henle, and these membrane structures rely on the AQPs to greatly enhanced water permeability, although water also can cross cell membranes by diffusion.

1.9 Aquaporin1 (AQP1) and its role in kidney pathological conditions

Aquaporins (AQPs) are a family of integral membrane proteins that predominantly serve as semi-permeable water channels. Thirteen AQPs have been identified in mammalian cells, namely AQP0-12. AQPs are widely express in tissues that deal with fluid homeostasis maintenance, such as lungs, eyes, liver, brain and kidneys [247]. AQP is a small protein, consisting of six membrane-spanning alpha-helical domains and a center pore [248]. It can further be divided into three sub-families according to their permeability to small neutral solutes other than water, such as glycerol and urea [249].

Eight AQPs are expressed in different segments of the nephron of human kidneys (Figure 1-13). The major water channels responsible for water reabsorption in proximal tubule is AQP1 and, in collecting duct is AQP2. Table 1-3 provides further details regarding renal AQP expression.

Table 1-3. AQPs in the kidney [250].

Aquaporin	Localization	Subcellular distribution	Extrarenal localization	Phenotype of mice with mutations or gene knockout	Phenotype of humans with mutations
AQP1	Proximal tubules, descending thin limbs of Henle, outer medullary descending vasa recta	Apical and basolateral plasma membrane	Erythrocytes, ciliary and lens epithelium choroid plexus, pulmonary vascular endothelium	Urinary concentrating defects, impaired pain sensation	Mild urinary concentrating defects
AQP2	Principal cells of the collecting duct	Apical plasma membrane and subapical vesicles	Epididymis	Diabetes insipidus	Diabetes insipidus
AQP3	Principal cells of the collecting duct	Basolateral plasma membrane	Conjunctiva, pulmonary airway epithelia, colonic epithelia, keratinocytes, erythrocytes	Urinary concentrating defect, reduced skin hydration, impaired wound healing, resistance to the formation of skin tumors	No obvious abnormality
AQP4	Principal cells of the collecting duct	Basolateral plasma membrane	Astroglia, ependyma, retinal glia, muscle fiber cells, keratinocytes pulmonary airway epithelia, stomach parietal cells	Mild urinary concentrating defect, impaired vision, hearing and olfaction	No abnormalities reported
AQP6	Intercalated cells of the collecting duct	Intracellular vesicles	Cerebellum, synaptic vesicles	No report	No abnormalities reported
AQP7	S3 segment of proximal tubules	Apical plasma membrane	Adipose tissue, testis, skeletal muscle, heart, brain, intestine	Glyceroluria, defective glycerol metabolism, obesity, smaller islet cells	Defective glycerol metabolism
AQP11	Proximal tubules	Endoplasmic reticulum	Testis, thymus, intestine, liver	Polycystic kidney disease (fatal)	No abnormalities reported

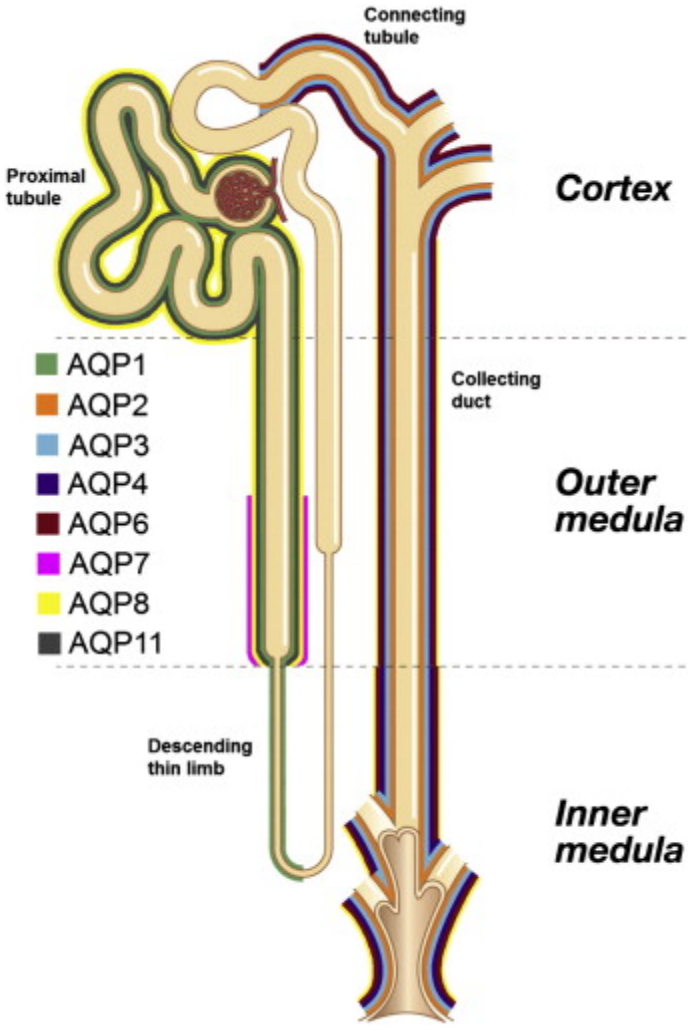

Figure1-12. Expression of renal AQPs along the nephron. Blood is filtered at the glomerulus, and the filtrate is modified as it travels through the nephron to make the final urine. Most of the glomerular filtrate is reabsorbed through AQP1 in the proximal tubule and descending thin limbs of Henle, although AQP7 is also expressed in the S3 segment of the proximal tubule. AQP1 is also expressed in the descending vasa recta, facilitating the removal of water. In the connecting tubule and collecting duct, AQP2 is mainly expressed at the apical membrane and intracellular vesicles of principal cells, while AQP3 and AQP4 are present at the basolateral membrane of the principal cells, representing exit pathways for water reabsorbed via AQP2. In contrast to these AQPs, AQP6, AQP8 and AQP11 are localized in intracellular membranes only. AQP6 is localized to intercalated cells of the collecting duct and connecting tubule, AQP8 is expressed in proximal tubules and weakly in collecting ducts, while AQP11 is localized to proximal tubules [251].

AQP1 is abundant in the proximal tubule (both convoluted and straight proximal tubules), descending thin limb and the outer medullary descending vasa recta endothelia, express in both apical and basolateral plasma membranes [252, 253] (Figure 1-14). Normally the expression of AQP1 is constitutively high, and not responsive to the regulation of AVP, the classical antidiuretic hormone [254]. An *in vitro* study showed that AQP1 also exists in the cytoplasm. Upon hypotonic stimulation, AQP1 rapidly translocates to the cell membrane. Microtubules, PKC and calcium are involved in this process [255, 256]. Because the tubular fluid in the proximal tubule is nearly always isosmotic with the cortical interstitial fluid, so the osmotic equilibration across renal tubules that is created by the sodium gradient is thought to be the driving force of water flow [257, 258].

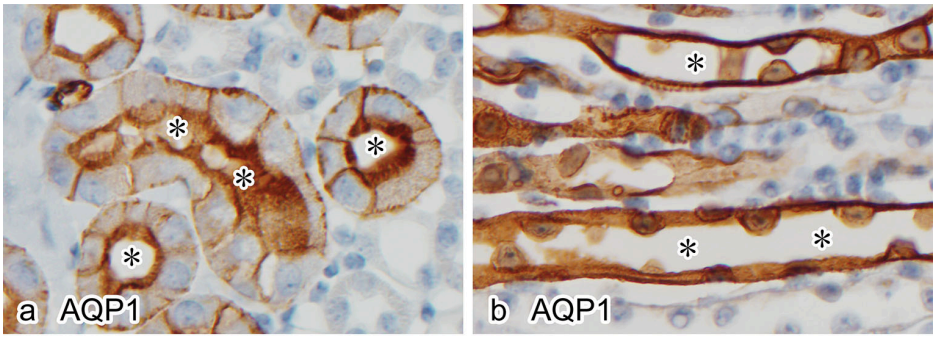

Figure 1-13. Immunohistochemical stainnig of AQP1 in the paraffin sections of mouse kidney. AQP1 at both the apical and basolateral membranes of the proximal tubule S3 segment cells in the outer medulla (a) and descending thin limbs of the loop of Henle (b) [259].

A study on AQP1 knockout (AQP1KO) mice specifically in RPTCs found these transgenic mice were grossly normal in terms of survival, physical appearance, and organ morphology. The body and kidney weights of AQP1KO mice were slightly lower than age-matched controls, but weight ratio was not different. The mean arterial blood pressure was significantly lower in AQP1KO compared to WT (88 ± 3 mm Hg vs. 102 ± 4 mm Hg, P = 0.019). The AQP1KO mice were polydipsic (excessive thirst) and polyuric, consuming about 3 times more fluid per day then WT mice when given free access to water [260].

However, under water deprivation condition for 36 hours, the knockout mice became severely dehydrated and lethargic, with body weight decreased by 35±2%, serum osmolality increased to >500 mOsm, and urinary osmolality (657±59 mOsm) did not change. In contrast, both WT and heterozygous mice remained active, body weight decreased by 20–22%, serum osmolality remained normal (310–330 mOsm), and urine osmolality rose to >2500 mOsm. The results suggested AQP1 is required for the formation of a concentrated urine by the kidney [261].

1.10 Glycogen synthase kinase 3β (GSK3β) and its signaling pathway

Glycogen synthase kinase 3 (GSK3) is a serine-threonine kinase that expresses ubiquitously. GSK3 was originally identified in rat skeletal muscle as a negative regulator, able to phosphorylate and inhibit glycogen synthase, which is a key regulator of glycogen synthesis [262, 263]. Initially GSK3 was considered as a key enzyme in metabolism. Recently, research indicate that GSK3 has central roles in a number of intracellular signaling cascades, including Wnt pathway, hedgehog signaling pathway, growth factor, cytokine, and G protein-coupled receptor cascades; regulates a wide range of cellular events, such as gene transcription, differentiation, cell growth and apoptosis [264]. When GSK3 is not regulated well, it leads to the progression of many human diseases, such as bipolar disorder, Alzheimer's disease, noninsulin-dependent diabetes mellitus (NIDDM) and cancer [264].

In mammals, GSK3 exists in two isoforms: GSK3α and GSK3β, encoded by different genes on separated chromosomes. Both isoforms are widely expressed and share 98% homology in their kinase domains [265]. Most studies focused on GSK3β but not GSK3α because global GSK3β knockout is lethal while GSK3α knockout is not [266]. Under resting or unstimulated conditions, GSK3β is active, and it can be inhibited by phosphorylation of serine 9 (S9) [267].

1.10.1 GSK3β expression and function in the kidneys

The role of GSK3β in the kidneys was unveiled 10 years ago [268], while it has been observed a long time ago by clinicians that lithium treatment of bipolar disorder can cause

polyuria in 40% of patients [269]. Lithium, a GSK3 inhibitor, was used as mental health therapy for over 60 years. However, a substantial number of long-term lithium therapy recipients develop nephrogenic diabetes insipidus (NDI). These patients with NDI had significantly reduced ability to concentrate urine and showed no response to AVP [270]. In animal studies, lithium caused a marked AQP2 protein decrease in the collecting duct [271]. Further animal study using renal collecting duct-specific GSK3β knockout mice model indicated that the mice were not overtly polyuric under basal conditions. However, their urine concentrating capacity in response to water deprivation was diminished, along with great reduction in AQP2 mRNA and protein levels, and AQP2 membrane trafficking ability [272]. These data suggest that ablation of GSK3β in collecting duct reduces it's response to the hydro-osmotic effects of vasopressin.

As to the impact of GSK3β on blood pressure regulation, a rat model of Li-induced NDI was used to assess whether the marked decreases in urine output could result in blood pressure change [273]. The mean arterial pressure did not differ between control group and the group that fed standard rat chow plus Li for 4 week [273].

1.10.2 GSK3β regulates Nrf2 signaling

In addition to the well-accepted Keap1-Nrf2 pathway, a novel mechanism of Nrf2 regulation in response to oxidative stress was discovered [274]. It was reported by the same group previously that a tyrosine kinase, Fyn, can mediate Nrf2 nuclear export by phosphorylating Nrf2 on tyrosine 568 [275]. After that, this research team used a series of *in vitro* studies to demonstrate that GSK3β acts upstream of Fyn in control of Nrf2 nuclear export [274]. Inhibition of GSK3β by chemicals or siRNA leads to Nrf2 nuclear accumulation and transcriptional activation of its target gene, whereas H_2O_2 can stimulate phosphorylation of tyrosine 216 (Y216) on GSK3β, and results in activation of GSK3β. The activated GSK3β phosphorylates Fyn at threonine residue(s). Phosphorylated Fyn accumulates in the nucleus and phosphorylates Nrf2 at tyrosine 568. This leads to nuclear export and degradation of Nrf2 by proteasome [274]. Notice that H_2O_2 is a major form of ROS and is known to induce Nrf2 activation. In this study they found H_2O_2 caused Nrf2 signaling activation in a short-term manner (*i.e.* 1 hour); but caused Nrf2 nuclear export and Nrf2 signaling inactivation in a long-term manner (*i.e.* 4 hours). The author presumed

that H_2O_2 induced Nrf2 signaling activation might be regulated by protein kinase C (PKC), which both inactivate GSK3β and activate Nrf2 in a synergistic fashion in response to early oxidative stress. However, the signaling events between H_2O_2 and pGSK3β(Y216) could be a delayed response.

1.11 β-catenin and it's signaling

1.11.1 The canonical Wnt/β-catenin signaling pathway

The Wnt/β-catenin signal pathway is very important in growth and development during embryogenesis. This signaling pathway is finely tuned and found dysregulated in disease condition, such as cancer [276]. Briefly, in the absence of Wnt, which serves as a ligand, Frizzled/low-density-lipoprotein receptor related protein (Fz/LRP) receptors are not activated. Casein kinase 1 (CK1) and GSK3 sequentially phosphorylate Axin-bound β-catenin at S45, S41, S37 and S33. Phosphorylated β-catenin then forms a destruction complex with β-transducin repeat containing (β-TrCP) in cytosol, which is part of an E3 ubiquitin ligase complex. As a result, β-catenin is ubiquitinated and targeted for rapid destruction by the proteasome and this prevents transcriptional activation of β-catenin target genes. With the presence of Wnt, the Wnt signaling pathway is on and phosphorylation of β-catenin by CK1 and GSK3 is suppressed. The destruction complex falls apart, and β-catenin is free to form a complex with other transcription factors in nucleus, and subsequently promote expression of its target genes (figure 1-14) [276].

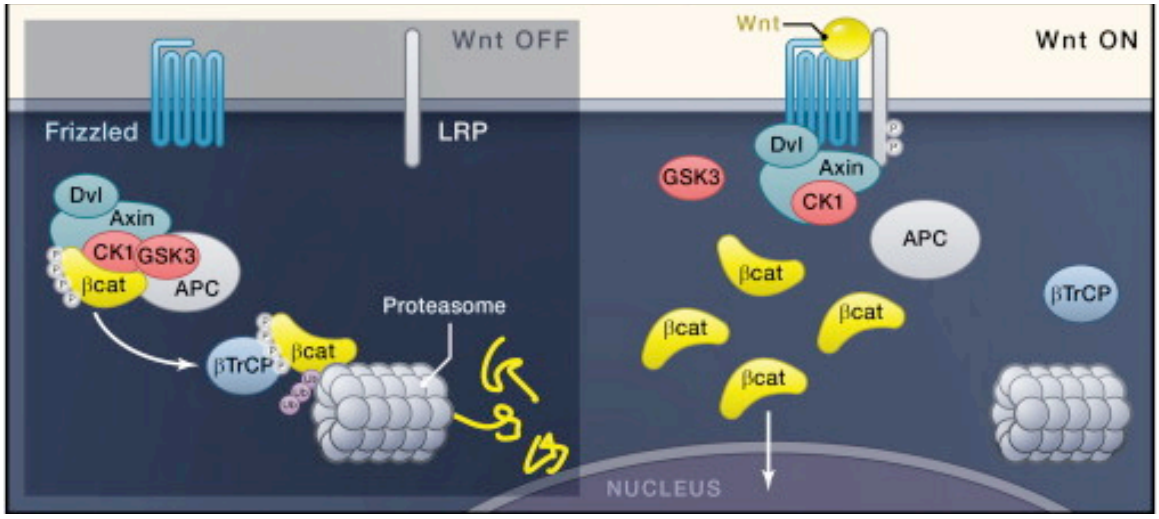

Figure 1-14. The current Wnt signaling model. In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds and phosphorylates β-catenin. The latter then leaves the complex to be ubiquitinated by β-TrCP (which binds to the phosphorylated "degron" motif in β-catenin) and is then degraded by the proteasome. Wnt induces the association of Axin with phosphorylated LRP. The destruction complex falls apart, and β-catenin is stabilized [276].

1.11.2 β-catenin and kidney disease

It was recently recognized that dysregulation of Wnt signaling pathway in adults is linked with progressive kidney injury. Progressive kidney injury is associated with glomerulosclerosis and interstitial fibrosis. In an Adriamycin-induced podocyte injury and proteinuria mouse model, Wnt/β-catenin pathway was activated. Further activation of Wnt signaling by intravenously injection of Wnt activator can activate glomerular β-catenin in glomeruli and aggravated albuminuria, whereas administration blockade of Wnt signaling can ameliorate podocyte lesions [277]. Also in the same study, podocyte-specific knockout of β-catenin can protect mice from developing albuminuria after injury [277]. These data suggest that Wnt/β-catenin signaling plays an important role in podocyte injury and proteinuria. In human with kidney diseases complicated by proteinuria, such as diabetic nephropathy and focal segmental glomerulosclerosis, upregulation of Wnt and active β-catenin in podocytes were observed.

Progressive renal injury also features increasing interstitial fibrosis of the kidney. In response to insults, kidney tubular epithelial cells undergo a phenotypic conversion and give rise to the matrix-producing fibroblasts and myofibroblasts. The process is termed epithelial-mesenchymal transition (EMT) [278]. An *in vitro* study using rabbit primary RPTCs demonstrated that administration of β-catenin inhibitor can reverse high glucose-induced EMT, suggested tie role of Wnt/β-catenin signaling activation in the process of EMT [279]. Another experimental study using both T1D and T2D mouse model indicated that Wnt/β-catenin signaling is activated in diabetic mice kidney. Treatment of insulin can attenuate activated Wnt/β-catenin signaling in the kidney. Moreover, inhibition of WNT signalling by using monoclonal antibody to block LRP receptor can ameliorate renal inflammation and fibrosis, as well as reduce proteinuria in diabetic mice [280].

1.11.3 β-catenin and its interaction with AQP1

Interestingly, lately a study reported that AQP1 could interact with the transcription factor β-catenin and have protective effect in an autosomal dominant polycystic kidney disease (PKD) mouse model [281]. The study induced cysts formation by forskolin in immortalized canine kidney epithelial cells, and overexpression AQP1 can inhibit cyst development as well as decrease β-catenin expression and downregulate Wnt signaling. *In vivo*, cyst number is significantly greater in AQP1KO PKD mice, following decreased β-catenin phosphorylation and increased β-catenin expression compared to PKD mice with normal AQP1 [281]. The result implicated that AQP1 may be involved in the inhibition of Wnt signaling by recruiting and forming a complex. The author hypothesized that AQP1 may interact and therefore increase the stability of the "destruction complex". In that complex, β-catenin is phosphorylated and subsequently degraded by proteasome. Without AQP1 in the cells, the stability of the "destruction complex" is decreased and β-catenin is released from the complex, resulting in β-catenin translocate into the nucleus and promote transcription of Wnt target gene. This study provided us an insight of how AQP1 could contribute to kidney injury.

1.12 Animal models used in present study

1.12.1 Maternal diabetic murine model

The etiology of GDM is complex. It is considered to be a complex disease with several risk factors. Thus creation of appropriate animal models that are perfect replicas of this disease is rather difficult. Hence the choice of animal model depends whether the study focuses on the health consequences for the offspring of mothers with maternal diabetes, or on the physiology of the diabetic mothers.

Rat and mouse are the most commonly used animal model for the study of maternal diabetes, although other vertebrates (pig, sheep, dog and non-human primates) are also options. There are various strategies to induce diabetes at gestation (Table 1-4). In our study, we chose the STZ-induced diabetic mouse model, and treated the dams with STZ starting on embryonic day 13 for 48 hours, as described [282]. This model mimics the elevated glycemia at the end of the second trimester of gestation observed in human mothers with GDM. STZ specifically destroys β-cells by transporting STZ through the glucose transporter 2 (GLUT2) [283], causing β-cell apoptosis, leading to decreased insulin secretion and hyperglycemia [284]. Though STZ is able to cross into the placenta, its half-life is less than five minutes, and therefore does not significantly affect the developing embryo [285]. It is a well-accepted animal model to investigate offspring of diabetic dams.

Table 1-4. Strategies in generating animal models of GDM. Currently available models for the study of GDM include surgery, chemical induction, nutritional manipulation, or genetic manipulation and are viable options for a wide variety of model organisms [286].

Strategy	Methods	Examples of Species Used	Major Advantages	Major Disadvantages
Surgery	Pancreatectomy	Dogs Rats	• Plausible strategy in animals where other options are not feasible	• Requires highly trained personnel • High mortality rate • Not accurate pathogenesis of GDM
Chemically Induced	Streptozotocin Alloxan	Mice Rats Rabbits Pigs Sheep Nonhuman primates	• Affordable • Proven technique in many different species	• Potential nonspecific consequences • More severe hyperglycemia • Not accurate pathogenesis of GDM
Nutritional Manipulation	High-fat diet Glucose infusion	Mice Rats Dogs Sheep	• Affordable • Plausible strategy for larger animals	• Ignores genetic contribution to disease • Does not reflect cases of GDM not due to diet
Genetic Manipulation	Gene knockouts Transgenic overexpression	Mice	• Spontaneous development of GDM • Glucose intolerance specific to pregnancy	• Not an option for many animals • Overly simplistic representation for most cases of GDM

1.12.2 Hoxb7/catalase-GFP-Tg mouse

This double transgenic mouse model was generated by crossbreeding Hoxb7-GFP-Tg mice along with Cat-Tg mice as description in detail below:

1.12.2.1 Hoxb7-GFP-Tg mouse

Nephron number loss is a marker for impaired nephrogenesis in the offspring of diabetic mouse dams [287]. To visualize kidney development, Hoxb7-Green Fluorescence Protein-transgenic (Hoxb7-GFP-Tg) mice were utilized in this study. During kidney development, a UB, the precursor of the renal collecting duct, keeps branching and inducing MM, which are a group of nephron progenitor cells, to form an entire nephron [288]. The number of UB counted during the embryonic stage is a surrogate marker of the

number of nephrons in adulthood. These transgenic mice express GFP in UB under the control of the Hoxb7 promoter, which allows monitoring of UB growth and branching even when the kidneys are cultured *ex vivo* for several days [289].

1.10.2.2 CAT-Tg mouse

A Cat-Tg mouse that overexpresses rat CAT specifically in mouse RPTCs, driven by the human kidney androgen- regulator protein 2 (KAP2) promoter, was kindly provided by Dr. John S.D. Chan [290]. The activity, function and tissue expression of the overexpressed rat catalase were characterized by Dr. Chan's group [290].

Briefly, to generate CAT-Tg mice specifically in RPTCs, KAP2-rCAT construct that driven by androgen was introduced. As a result, only male mice were studied in the fallowing experiments. To select a transgenic line that specifically expresses transgene in RPTCs but not other tissues, mRNA and protein expression was tested in various organs.

After the line has been determinate, next is to demonstrate whether transgenic CAT protein is functional. RPTCs extract were used to perform CAT activity assay in the presence or absence of H_2O_2, and H_2O_2 was consumed effectively in CAT-Tg RPTCs extract compare to WT. Also, RPTCs were isolated, *ex vivo* incubated and ROS generation was induced with high glucose medium (25 mM D-glucose) compare to normal glucose medium (5 mM D-glucose plus 20 mM D-mannitol). ROS level was augmented in RPTCs of WT mice in response to high glucose, but no apparent changes in RPTCs of CAT-Tg mice. Diabetes was induced by STZ injection to both WT and CAT-Tg adult mice. WT diabetic mice developed albuminuria, kidney hypertrophy and kidney structural damage, while all these pathological changes were attenuated in diabetic CAT-Tg mice kidneys. All *in vivo* and *in vitro* data suggested that transgenic CAT in RPTCs can cleanse ROS generated in the kidney and is a good model to investigate maternal diabetes perinatal programmed hypertension and kidney injury, focusing on the role of intrarenal ROS.

1.12.3 rAgt-Tg mouse

The Agt-Tg mouse line was kindly provided by Dr. John S.D. Chan and was well characterized [291]. The strategy used to generate transgenic rat Agt-Tg mice is similar to

that used for Cat-Tg. Briefly, to generate Agt-Tg mice that overexpress Agt specifically in RPTCs, KAP2-rAgt construct that driven by androgen was introduced. It was confirmed that the transgene only express in RPTCs but not any other tissue by mRNA RT-PCR. Transgenic Agt protein expression was found to be increased in RPTCs of male Agt-Tg mice, and their SBP was 15 mmHg higher compared to male non-Tg littermates [292]. SBP of male Agt-Tg mice can be further enhanced by administration of testosterone by averages 20-30mmHg compared to the non-induced controls. The male transgenic mice displayed markedly increased albuminuria and kidney injury compared to non-transgenic littermates, and the levels of urinary albumin can be normalized by treatment with losartan or perindopril [291]. Taken together, this mice model is a perfect model for us to study intrarenal RAS activation-induced hypertension and kidney injury, discovering the role of AQP1 and underlying mechanism.

CHAPTER 2: PUBLISHED ARTICLE

Catalase Prevents Maternal Diabetes-Induced Perinatal Programming via the Nrf2-HO-1 Defense System

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Catalase Prevents Maternal Diabetes-Induced Perinatal Programming via the Nrf2-HO-1 Defense System

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Key words: Catalase, Maternal Diabetes, Offspring, Hypertension, Kidney Injury

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2.1 Abstract

We investigated whether overexpression of catalase (CAT) in renal proximal tubular cells (RPTCs) could prevent the programming of hypertension and kidney disease in the offspring of dams with maternal diabetes. Male offspring of non-diabetic and diabetic dams from two Tg lines (Hoxb7- GFP-Tg (controls) and Hoxb7/CAT-GFP-Tg, which overexpress CAT in RPTCs), were studied from the prenatal period into adulthood. Nephrogenesis, systolic blood pressure (SBP), renal hyperfiltration and kidney injury as well as reactive oxygen species (ROS) generation were assessed. Gene expression of TGF-β1, nuclear factor-erythroid 2p45 (NF-E2) related factor-2 (Nrf2), and heme oxygenase-1 (HO-1), were tested in both *in vitro* and *in vivo* studies. Renal dysmorphogenesis was observed in offspring of Hoxb7-GFP-Tg dams with severe maternal diabetes; the affected male offspring displayed higher renal ROS generation, developed hypertension and renal hyperfiltration as well as renal injury with increased TGF-β1 expression in adulthood. These changes were ameliorated in male offspring of diabetic Hoxb7/Cat-GFP-Tg dams via the Nrf2-HO-1 defense system. CAT promoted Nrf2 gene nuclear translocation and HO-1 gene expression, seen both in *in vitro* and *in vivo* studies. In conclusion, CAT overexpression in the RPTCs ameliorated maternal diabetes-induced perinatal programming, mediated, at least in part, by triggering the Nrf2-HO-1 defense system.

2.2 Introduction

Gestational diabetes occurs in 3-14% of pregnancies world wide (www.diabetes.com), conferring substantial risk to the offspring. Infants of diabetic mothers are thus prone to develop a variety of disease later in life, such as metabolic syndrome, hypertension and

chronic kidney disease (CKD) (1;2). This phenomenon, in which intrauterine events are linked with later changes, is termed "perinatal programming," but the mechanisms by which it occurs are incompletely delineated (3;4).

The broad spectrum of birth defects seen in offspring of women with gestational diabetes and in animal models is thought to be associated either with increased reactive oxygen species (ROS) or diminished antioxidant defense systems (both enzymatic and non-enzymatic defense systems), leading to increased susceptibility to ROS- induced injury in multiple tissues, including the kidney (5-7). Studies to determine whether antioxidant supplementation and/or the provision of non-enzymatic antioxidants prevent these abnormalities are needed. To date, reports on the efficacy of antioxidant supplementation to pregnant women with or without diabetes are preliminary and controversial (8;9), as is the case in experimental models (7). Hence, the present study focuses on antioxidant enzymatic pathways, specifically the catalase (CAT)- nuclear factor-erythroid 2p45 (NF-E2) related factor-2 (Nrf2) - heme oxygenase-1 (HO-1) pathway.

The key initial step in the formation of all ROS is the conversion of oxygen to superoxide anion ($O_2^{\bullet -}$). $O_2^{\bullet -}$ has a very short half-life and is rapidly converted to less reactive hydrogen peroxide (H_2O_2) by superoxide dismutases (SODs) and then reduced to H_2O by CAT and glutathione peroxidase (GPx) (10;11). In the kidneys, CAT is localized to the renal proximal tubular cells (RPTCs) (12-14). CAT has been postulated to be a key enzyme regulating H_2O_2 levels since cells overexpressing CAT are more resistant to H_2O_2 toxicity and oxidant-mediated injury (15;16), whereas, overexpression of GPx alone is not protective against renal injury in diabetic mice (17).

Nrf2 is a transcriptional factor that acts as a key regulator of cellular antioxidant enzymes including CAT, HO-1, SODs, glutathione S-transferease, peroxidase, NAD(P)H quinone oxidoreductase and thioredoxin, etc. (18) via its binding to the antioxidant-response element to protect against oxidative stress (18;19). Under basal conditions, Nrf2 is bound within the cytoplasm to protein kelch-like ECH-associated protein 1 (Keap1, an oxidative stress sensor) and then undergoes rapid ubiquitination, with subsequent proteasome-dependent degradation. Upon exposure of cells to oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it subsequently guides expression of antioxidant stress genes to trigger the cellular anti-oxidant defense response (18;19).

Nrf2 is highly expressed in the kidney (19), and it is thought that the Nrf2-HO-1 defense system is renoprotective and that its induction might even improve kidney function in renal fibrosis (20), diabetic nephropathy (21) and acute ischemic kidney injury (22), as well as in the progression of focal glomerulosclerosis (23;24). Moreover, HO-1 induction has been considered as a useful target for the development of antihypertensive drugs, since HO-1 or its metabolites can attenuate the development of hypertension and lower blood pressure in models of established hypertension (25).

Previously, we reported that a high-glucose milieu *ex vivo* or severe maternal diabetes *in utero* (defined as maternal blood glucose concentration ~30 mM) induces ROS generation, which impairs nephrogenesis, resulting in offspring with relatively smaller kidneys and nascent nephron deficiency due to excessive apoptosis [via activation of the nuclear factor-kappa B (NF-kB) and p53 pathways] (26-28). Moreover, we have shown that severe maternal diabetes is linked to low birth weight in offspring (mean decrease 20%), which later manifests hypertension, glucose intolerance and kidney injury in adulthood along with heightened ROS

generation (26;29). Taken together, our prior data suggest that an imbalance between ROS production and anti-oxidative capacity can lead to a state of "oxidative stress" that is intimately associated with perinatal programming of hypertension and kidney disease.

In the present studies, we investigated whether overexpression of CAT in renal proximal tubular cells (RPTCs) could prevent the perinatal programming of hypertension and kidney injury in male offspring of diabetic dams and examined the potential underlying mechanisms both *in vivo* and *in vitro*.

2.3 Materials and methods

Animal models

We used both Hoxb7- green fluorescent protein (GFP) –transgenic (Tg) (Hoxb7-GFP-Tg) and Hoxb7/Catalase-GFP-Tg (Hoxb7/Cat-GFP-Tg) murine lines (both in C57/BL6 background); both lines are fertile with a normal phenotype at birth and during adult life. Hoxb7-GFP-Tg mice (GFP expression specifically in ureteric bud (UB) driven by Hoxb7 promoter) provided by Dr. Frank Costantini (Columbia University Medical Center, New York, NY, USA) (30;31) were engineered to allow UB branching morphogenesis to be visualized in real time *in vivo* as reported previously (28). Cat-Tg mice (e.g., rat CAT gene overexpressing specifically in RPTCs driven by kidney-specific androgen-regulated protein (KAP2) promoter) were obtained from Dr. John S.D. Chan (CRCHUM-Hôtel-Dieu Hospital, Université de Montréal) (32-34). High levels of androgens have been reported in the fetal and maternal circulation in both humans (35-38) and mice (39), rendering the KAP2 promoter a feasible way to direct CAT transgene expression during nephrogenesis (which occurs in mice both in the prenatal and postnatal periods). We thus created hybrid Hoxb7/Cat-GFP-Tg mice by cross-

breeding Cat-Tg mice with Hoxb7-GFP-Tg mice; the resultant hybrid mice permit the visualization of CAT impact on nephrogenesis--e.g., UB branching morphogenesis, which we examined both in the presence and absence of maternal diabetes *in vivo*.

Induction of Maternal Diabetes

We have successfully employed a single intraperitoneal injection of 150 mg/kg body weight (BW) of streptozotocin (STZ, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) at embryonic day 13 (E13) to create an *in vivo* murine model of maternal diabetes (26-29). We studied the male offspring from non-diabetic (control) and diabetic dams of both Hoxb7-GFP-Tg and Hoxb7/Cat-GFP-Tg.

Animal Care

Animal care and the procedures utilized were approved by the Institutional Animal Care Committee of the CRCHUM. Mice were housed under standard humidity and lighting conditions (12-h light-dark cycles) with free access to standard mouse chow and water.

Isolation of Metanephroi and Counting of UB Tips

E15-embryos were dissected aseptically from both timed-pregnant Hoxb7-GFP-Tg and Hoxb7/Cat-GFP-Tg mice with or without diabetic mellitus (DM). The E15-metanephroi were isolated under sterile conditions, and quantitative assessment of the number of UB tips in each group was performed as reported previously (28).

Physiological Studies

Blood glucose levels were measured with a Side-Kick Glucose Analyzer (Model 1500, Interscience, ON, Canada) in the morning after a 4-hour fast, as reported previously (26;27;29). Mean systolic blood pressure (SBP) was monitored by the tail-cuff method with the Visitech BP-2000 Blood Pressure Analysis System for mice (Visitech System Inc., Apex, NC, USA), as reported elsewhere (29;33;34). The animals were acclimated to BP measurement (2-week period of pre-training starting at 6 weeks of age, followed by actual measurement of SBP thrice weekly from 8 weeks until 18 weeks of age). Although the technique of tail-cuff measurement is generally considered less sensitive than telemetry, we judged that our SBP data is valid, based on the substantial numbers of animals used and the fact that the animals were well-acclimated and used to the measurement in our longitudinal studies, thus minimizing stress.

Urine samples, collected from mice individually housed in metabolic cages, were assayed for albumin and creatinine (ELISA, Albuwell and Creatinine Companion, Exocell Inc., Philadelphia, PA, USA) as reported previously (29;33;34). All animals were euthanized at 20 weeks of age under CO_2, and the kidneys removed immediately. Body weight (BW) and kidney weight (KW) were rapidly recorded. The left kidney was utilized for renal morphology and immunohistochemistry (IHC) (29;33;34). The right kidney cortex was reserved for ROS generation and gene expression experiments as previously reported (29;33;34).

Measurement of Glomerular Filtration Rate (GFR)

As reported previously (40), we estimated the GFR in 20 week-old male animals by the fluorescein isothiocyanate-inulin (FITC-inulin) method, as described by Qi et al. (41) and recommended by the Diabetic Complications Consortium (DCC) (www.diacomp.org).

Renal Morphology, Mean Glomerular Volume and Nephron Number

Kidney morphology was assessed with hematoxylin and eosin (H & E) and periodic-acid schiff (PAS) staining. As in previous reports (26;27;29), mean glomerular volume (Vg) was determined using PAS–stained images with the aid of an image analysis software system (Motics Images Plus 2.0, Motic, Richmond, BC, Canada) (42); and quantification of neonatal nephron number was adapted from Bertram's method, using serial sections (43).

ROS Generation

Freshly isolated renal cortex was used immediately for ROS measurement by the lucigenin method, as described elsewhere (26;27;32;33). ROS production was normalized by protein concentration and expressed as relative light units (RLU) per μg protein.

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

Total RNA extracted from freshly isolated renal cortex was assayed for gene expression by real time quantitative PCR (RT-qPCR), as reported previously (27;29;40). The Fast SYBR® green mastermix kit and the 7500 Fast real-time PCR system (Applied Biosystems, Life Technologies, Foster City, CA, USA)] were employed for this purpose (26;27;32;33).

Immunohistochemistry

Immunohistochemistry (IHC) was performed by the standard avidin-biotin-peroxidase complex method (ABC Staining System, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), as described elsewhere (26;27;29). Polyclonal anti-CAT antibody was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada); polyclonal anti-Nrf2 antibody was purchased from Abcam (Cambridge, MA, USA); transforming growth factor-beta 1 (TGF-β1) and HO-1 antibodies, were purchased from Santa Cruz Biotechnologies.

Immortalized Renal Proximal Tubular Cells (IRPTCs)

The IRPTC cell line reported previously (44) was employed for our studies *in vitro*. This *in vitro* setting is useful for studies of the effect of high glucose (25mM D-Glucose) on both Nrf2 and HO-1 gene expression as well as Nrf2 nuclear translocation with or without the administration of CAT (250 unit (U)). The cells incubated in low glucose (5mM D-Glucose) medium with 20mM mannitol to reproduce the same osmolality as high glucose served as the control.

Nuclear protein (N.P.) and cytosolic protein (C.P.) extracts were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Thermo scientific, Burlington, Ontario, Canada) as reported previously (26;27). Anti-Histone H3 (3H1) rabbit mAb was purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Western blot (WB) and immunofluorescence (IF) staining on both Nrf2 and HO-1 genes in IRPTCs were performed as reported previously (45).

Statistical Analysis

Statistical significance between the experimental groups was analyzed by 1-way ANOVA, followed by the Bonferroni test using Graphpad Software, Prism 5.0 (http://www.graphpad.com/prism/Prism.htm). A probability level of $P \leq 0.05$ was considered to be statistically significant (26;27;29).

2.4 RESULTS

Hybrid Hoxb7Cat-Tg Mice Generation

The success of generating hybrid Hoxb7 /Cat-Tg mice was confirmed by PCR genotyping (Figure 1A) as well as GFP live image in E15-metanephroi (Figure 1B). Once these animals were obtained, we compared UB branching morphogenesis in E15-metanephroi after 2 days of STZ administration [e.g., maternal blood glucose concentration (mM): non-diabetic dams (Hoxb7-GFP-Tg: 9.45 ±1.69; Hoxb7/Cat-GFP-Tg: 9.68 ± 1.01) vs. diabetic dams (Hoxb7-GFP-Tg: 28.6 ± 2.14; Hoxb7/Cat-GFP-Tg: 27.9 ± 2.0)]. As compared to the E15-metanephroi isolated from non-diabetic Hoxb7-GFP-Tg animals, the E15-metanephroi from diabetic Hoxb7-GFP-Tg mice displayed smaller size (Figure 1B) with less number of UB tips (Figure 1C), and those UB branching impairments appear to be ameliorated by diabetic Hoxb7/Cat-GFP-Tg mice (Figure 1B and 1C).

Neonatal Kidney Outcomes in Offspring

Neonatal renal morphology was reviewed by H & E staining and CAT-IHC. CAT-IHC revealed that CAT is highly expressed in RPTCs in the neonatal kidneys of Hoxb7/Cat-GFP-Tg compared to Hoxb7-GFP-Tg mice (Figure 2A). As compared to the offspring of non-

diabetic Hoxb7-GFP-Tg dams, the neonates of diabetic Hoxb7-GFP-Tg dams had smaller kidneys with small glomeruli (Figure 2A), as well as fewer nephrons (Figure 2B). This dysnephrogenesis appeared to be attenuated in the neonatal offspring of Hoxb7/Cat-GFP-Tg diabetic dams (Figure 2A and 2B). Also, there is no significant difference in litter size and sex distribution among the four groups of animals (see the supplemental data).

Physical and Biochemical Measurements in the Male Offspring in Adulthood

Figure 3 displays the physical and biochemical findings in the male offspring at 20 weeks of age. The offspring of Hoxb7-GFP-Tg diabetic dams were significantly smaller and lighter as compared to the offspring of non-diabetic Hoxb7-GFP-Tg dams [Body weight (BW, g) in Hoxb7-GFP-Tg offspring: non-diabetic (Hoxb7-Con: 27.98 ± 1.095, N=16) vs. diabetic (Hoxb7-DM: 22.84 ± 1.506, N=12), $P \leq 0.05$]. In contrast, there were no significant differences between the BWs of the offspring from non-diabetic and diabetic Hoxb7/Cat-GFP-Tg dams [BW (g) in Hoxb7/Cat-GFP-Tg offspring: non-diabetic (Hoxb7/Cat-Con: 31.845 ± 1.35, N=13) vs. diabetic (Hoxb7/Cat-DM: 33.92 ± 1.82, N=14)] (Figure 3A). Although the 20 week-old male Hoxb7/Cat-GFP-Tg offspring had significantly bigger kidneys (kidney weight (KW, mg), as compared to those of Hoxb7-GFP-Tg mice (Figure 3B) [KW (mg): Hoxb7-Con (314.6 ± 37.96, N=15); Hoxb7-DM (298 ± 41.31, N=10); Hoxb7/Cat-Con (394 ± 26.08, N=11); and Hoxb7/Cat-DM (380 ± 5.07, N=12)], the KW to BW ratio among all groups of 20 week-animals, however, did not differ significantly (Figure 3C) as well as the fasting blood glucose levels (mM) (Figure 3D).

Mean Systolic Blood Pressure (SBP) in Adulthood

SBP as monitored by tail cuff is shown in Figure 4 A and B from age 8 to 20 weeks. Longitudinal studies (Figure 4A) revealed that the male offspring of diabetic Hoxb7-GFP-Tg dams have significantly higher SBP over the follow-up period, as compared to the control offspring. CAT overexpression in RPTCs seems to prevent maternal diabetes-induced perinatal programming of hypertension. Figure 4B summarized the SBP in the male offspring at 20 weeks (Hoxb7-Con: 108.74 ± 3.21 mmHg, N=23; Hoxb7-DM: 125.47 ± 2.08 mmHg, N=21; Hoxb7/Cat-Con: 113.35 ± 1.40 mmHg, N=22; Hoxb7/Cat-DM: 116.65 ± 1.52 mmHg, N=23).

ROS Generation and Renal Function Assay in Adulthood

The offspring of diabetic Hoxb7-GFP-Tg dams at 20 weeks of age have significantly augmented ROS generation in their freshly isolated renal cortex as compared to the offspring of non-diabetic Hoxb7-GFP-Tg dams (Figure 4C); The offspring of diabetic dams exhibited significantly increased urinary albumin/creatinine ratio (ACR) (Figure 4D) [ACR: Hoxb7-Con (0.026 ± 0.024, N=12) vs. Hoxb7-DM (0.38 ± 0.34, N=15), $P \leq 0.001$] and glomerular filtration rate (GFRs) (Figure 4E) [GFRs: Hoxb7-Con (21.50 ± 2.0, N=6) vs. Hoxb7-DM (36.1 ± 5.2, N=6), $P \leq 0.01$].

In contrast, offspring with overexpression of CAT did not have an increase in renal ROS (Figure 4C), ACR (Figure 4D) and GFR (Figure 4E), irrespective of whether the dams were non-diabetic or diabetic Hoxb7/Cat-GFP-Tg [ACR: Hoxb7/Cat-Con (0.031 ± 0.004, N=9) vs. Hoxb7/Cat-DM (0.087 ± 0.11, N=8); and GFR: Hoxb7/Cat-Con (22.22 ± 2.39, N=7) vs. Hoxb7/Cat-DM (22.35 ± 3.23, N=6)].

Renal Morphology and TGF-β1 Gene Expression in Adulthood

Enhanced extracellular matrix (ECM) protein expression and accumulation in glomeruli is a marker of glomerular injury. PAS staining of kidney sections revealed that ECM accumulation in the glomeruli (Figures 5A) and higher mean Vg (Figure 5B) were more pronounced in hypertensive offspring of diabetic Hoxb7-GFP-Tg dams; this finding was attenuated in offspring of both diabetic and non-diabetic Hoxb7/Cat-GFP-Tg dams (Figure 5A-B).

TGF-β1 is a ROS-inducible gene that is overexpressed in diabetes; it is directly associated with increases in ECM accumulation and tubulointerstitial fibrosis (10;11;44). Increments of TGF-β1 gene expression (Figure 5C), predominantly localized to glomeruli and the tubulointerstitium, were observed in kidneys of hypertensive Hoxb7-GFP-Tg offspring (Figure 5D). Further, the heightened TGF-β1 expression was attenuated in kidneys of offspring of diabetic Hoxb7/Cat-GFP-Tg dams (Figure 5C-D), indicating that suppressing ROS generation ameliorated glomerular and tubulointerstitial fibrosis.

Nrf2-HO-1 Gene Expression in Adulthood

We assessed Nrf2 and HO-1 gene expression in the renal cortex using RT-qPCR (Figure 6A-B). Compared to offspring of non-diabetic Hoxb7-GFP-Tg dams, both Nrf2 and HO-1 levels were significantly increased in hypertensive offspring of diabetic Hoxb7-GFP-Tg dams. Overexpression of CAT in RPTCs further enhanced Nrf2 and HO-1 gene expression in the affected male offspring of both non-diabetic or diabetic Hoxb7/Cat-GFP-Tg dams.

Consistent with the RT-qPCR data (Figure 6A-B), our IHC studies (Figure 6C) in paraffin-embedded renal sections showed that Nrf2 protein expression was not only detected

in glomeruli as reported as others (21;22), but also in RPTCs as well, whereas HO-1 appeared limited to the RPTCs. Most interestingly, the elevation of Nrf2 protein expression was strikingly increased in RPTCs, accompanied by nuclear translocation in offspring of Hoxb7/Cat-GFP-Tg dams, both diabetic and non-diabetic, indicating overexpression of CAT in RPTCs activates the Nrf2-HO-1 defense system to ameliorate maternal diabetes-induced perinatal programming of kidney injury in offspring.

Nrf2-HO-1 Gene Expression in IRPTCs in vitro

In order to confirm the effect of CAT on Nrf2 gene translocation, we performed additional *in vitro* studies with cultured IRPTCs (44). In this *in vitro* system, as in vivo, high glucose (WB, Figure 7A-B; IF staining, Figure 7C-D) significantly upregulates Nrf2 gene expression, as well as its translocation from the cytosol to nucleus, which targets the downstream HO-1 gene, resulting in significant up regulation, suggesting the Nrf2-antioxidative machinery on the operation. Meanwhile, CAT itself could trigger Nrf2 translocation and further upregulate HO-1 expression in IRPTCs, indicating that Nrf2-HO-1 anti-oxidative action could be mediated in a CAT-dependent manner.

2.5 Discussion

The present work demonstrates that intrarenal ROS generation induced by maternal diabetes can exert a direct effect on nephrogenesis *in utero*; and consequently trigger the perinatal programming of hypertension and renal injury in the offspring of diabetic dams when they reach adulthood. CAT overexpression in RPTCs appears to prevent this phenomenon, mediated, at least in part, via the Nrf2-HO-1 defense system.

In women with gestational diabetes, there is evidence of increased oxidative stress and impairment of antioxidant defense mechanisms, as seen in maternal plasma and cord blood as well as in placental tissue (46). It appears that ROS can directly impair nephrogenesis and elicit growth retardation and congenital kidney anomalies (5-7), and may also lead to adverse perinatal programming (26;29). In the current study, we created a unique murine model, hybrid Hoxb7/Cat-GFP-Tg mice, allowing us to directly study the functional role of CAT on the impact of maternal diabetes on nephrogenesis during prenatal period and on the development of perinatal programming of hypertension and kidney injury in adulthood in the exposed male offspring.

First, we characterized our hybrid Hoxb7/Cat-GFP-Tg mice and documented that the offspring of those Hoxb7/Cat-GFP-Tg mice rendered diabetic during pregnancy overexpress CAT in their RPTCs in isolated embryonic or newborn kidneys, which could prevent maternal diabetes-induced renal dysmorphogenesis (small kidneys with decreased nascent nephron number as well as less UB branching morphogenesis). One possible explanation is that since common progenitors in the S-shaped body migrate spontaneously and differentiate to form tubules (both proximal and distal) and glomeruli (glomerular tufts), tubulogenesis and glomerulogenesis directly influence each other(47). Thus, if CAT overexpression in RPTCs eliminates the impairment of maternal diabetes-induced ROS in tubulogenesis, glomerulogenesis is improved as well. Nevertheless, our data suggest that maternal diabetes-induced renal ROS may exert a direct effect on nephrogenesis *in utero*, and that the impaired nephrogenesis induced by maternal diabetes could be ameliorated by CAT overexpression in RPTCs.

We previously reported that CAT overexpression in RPTCs of *db/db* mice, which spontaneously develop diabetes, effectively attenuates hypertension, albuminuria, interstitial fibrosis, tubular apoptosis, and pro-apoptotic gene expression (32;33), suggesting that CAT overexpression in the RPTC might provide a novel approach to obviating or reversing the pathophysiological manifestations of maternal diabetes-induced perinatal programming of hypertension and kidney injury. Hence, we hypothesized that the protective role of CAT in RPTCs programmed for hypertension and kidney injury by maternal diabetes might be mediated via the Nrf2-HO-1 defense system.

To test this hypothesis, we followed male offspring of non-diabetic and diabetic dams until adulthood (20 weeks of age). We observed that the adult male offspring of diabetic Hoxb7-GFP-Tg dams displayed higher renal ROS generation and developed hypertension and renal injury-- features such as microalbuminuria, renal hyperfiltration (increased GFR and mean Vg), apparent glomerular injury (ECM accumulation and tubulointerstitial fibrosis with heightened TGF-β1 expression as compared to male offspring of diabetic Hoxb7-CAT-GFP-Tg dams. Thus, CAT overexpression in RPTCs of the male offspring of diabetic Hoxb7/CAT-GFP-Tg dams appear to normalize these abnormalities with upregulation of Nrf2 and HO-1 gene expression in the kidney.

It has recently been reported that the Nrf2-HO-1 defense system is renoprotective (20-24); further, a causal link between Nrf2 anti-oxidative pathways and oxidative stressors--e.g., ROS, angiotensin II (Ang II), TGF-β1 and NF-kB etc., has been established (20-24;48). Thus, it seems likely that Nrf2 mediated anti-oxidative capacity could act to counterbalance the stress induced by increased ROS production. When Nrf2 signals are impaired, either by reduction of Nrf2 pathway activation (20-24;48) or by disruption of Nrf2 gene expression

(Nrf2 knock-out mice) (21;49;50), renal damage may worsen, suggesting Nrf2-dependent regulation. Our present data indicate that overexpression of CAT in RPTCs promotes Nrf2 gene expression, and then decreases TGF-β1 related glomerular ECM accumulation, which confirms the findings of Jiang et al., who reported that knockdown of Nrf2 by siRNA enhanced TGF-β1 transcription and fibronectin production in cultured human mesangial cells (21). Moreover, previously, we established that there is a functional relationship between intrarenal ROS generation, the activation of the intrarenal renin-angiotensin system (RAS) and the NF-kB signaling pathway in our maternal diabetes murine model of perinatal programming (26;27;29). Taken together, our data suggest that CAT is capable of triggering Nrf2 translocation, and then targeting downstream genes, such as the HO-1 gene, which then interact with the intrarenal RAS and NF-kB signalling, improving renal outcome (27;29;34).

Finally, we observed that the augmented upregulation of Nrf2 with nuclear translocation was most evident in the RPTCs, rather than in glomeruli, as reported by others (21;22), whereas heightened HO-1-IHC expression seems to be only localized in the RPTCs, in agreement with other reports (20;22). Given this specific localization of Nrf2 expression in RPTCs, we further validated our *in vivo* data by employing IRPTCs *in vitro* (44). In addition to showing that CAT eliminates ROS-generation produced either by high glucose milieu, we confirmed that CAT itself could trigger Nrf2 translocation and further upregulate HO-1 expression in IRPTCs, indicating that the anti-oxidative action of Nrf2-HO-1 occurs in a CAT-dependent manner.

In conclusion, we demonstrated that CAT overexpression in RPTCs could exert a direct effect on nephrogenesis *in utero* and ameliorate maternal diabetes-induced

dysnephrogenesis consequently, preventing maternal diabetes-induced perinatal programming, mediated at least in part, via the Nrf2-HO-1 defense system.

Abbreviations

Ang II, angiotensin II; ACR, albumin/creatinine ratio; BW, body weight; CAT, catalase; CKD, chronic kidney disease; DM, diabetic mellitus; ECM, extracellular matrix; FITC, Fluorescein Isothiocyanate; GFP, green fluorescence protein; GFR, glomerular filtration rate; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; H&E, hematoxylin & eosin; HO-1, heme oxygenase-1; IF, immunofluorescence; IHC, immunohistochemistry; KAP, kidney-specific androgen-regulated protein; Keap1, kelch-like ECH-associated protein 1; KW, kidney weight; NF-kB, nuclear factor-kappa B; Nrf2, nuclear factor-erythroid 2p45 (NF-E2) related factor-2; $O_2^{\bullet-}$, superoxide anion; PAS, periodic acid schiff; RAS, renin-angiotensin system; RLU, relative light units; ROS, reactive oxygen species; RPTC, renal proximal tubular cells; RT-qPCR, real time-quantitative polymerase chain reaction; SBP, systolic blood pressure; SODs, superoxide dismutases; STZ, streptozotocin; Tg, transgenic; TGF-β1, transforming growth factor-beta 1; UB, ureteric bud; Vg, mean glomerular volume; WB, western blot.

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S-Y. C., Y-W. C., X-P. Z., and I.C., researched data and contributed to discussion; S.T., and A.S., researched data; J.R.I., contributed discussion and reviewed/edited manuscript; S-L.Z., researched data and contributed discussion as well as wrote/reviewed/edited manuscript. Dr. Shao-Ling Zhang is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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2.7 Legends and Figures

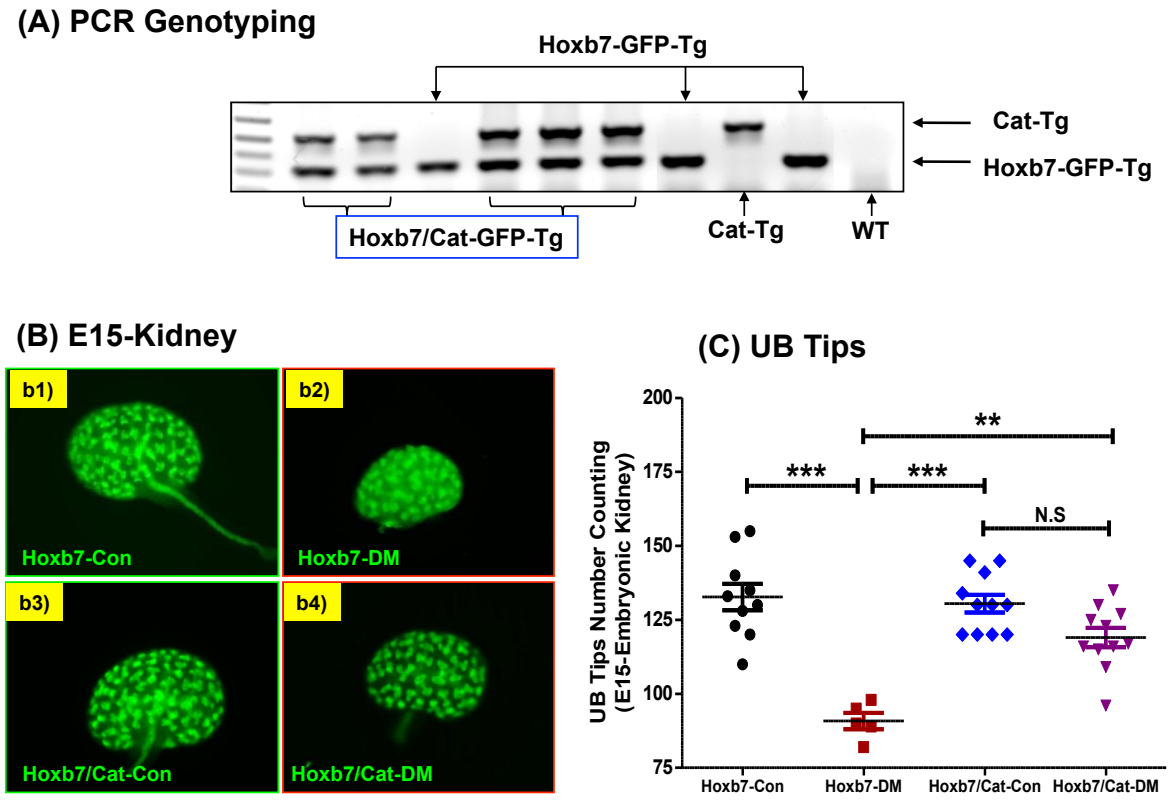

Figure 2-1: Characterization of Hoxb7-GFP-Tg and Hoxb7/Cat-GFP-Tg mice. (A) PCR Genotyping. (B) E15-metanephroi isolation either from Hoxb7-GFP-Tg dam [(b1) non-diabetic, Hoxb7-Con; (b2) diabetic, Hoxb7-DM] or Hoxb7/Cat-GFP-Tg dam [(b3) non-diabetic, Hoxb7/Cat-Con; (b4) diabetic, Hoxb7/Cat-DM], Magnification: 4X; (C) The number of E15-metanephroi UB tips. (●) Hoxb7-Con; (■) Hoxb7-DM; (♦) Hoxb7/Cat-Con; (▼) Hoxb7/Cat-DM; **, $P \leq 0.01$; ***, $P \leq 0.001$; N.S., non-significant.

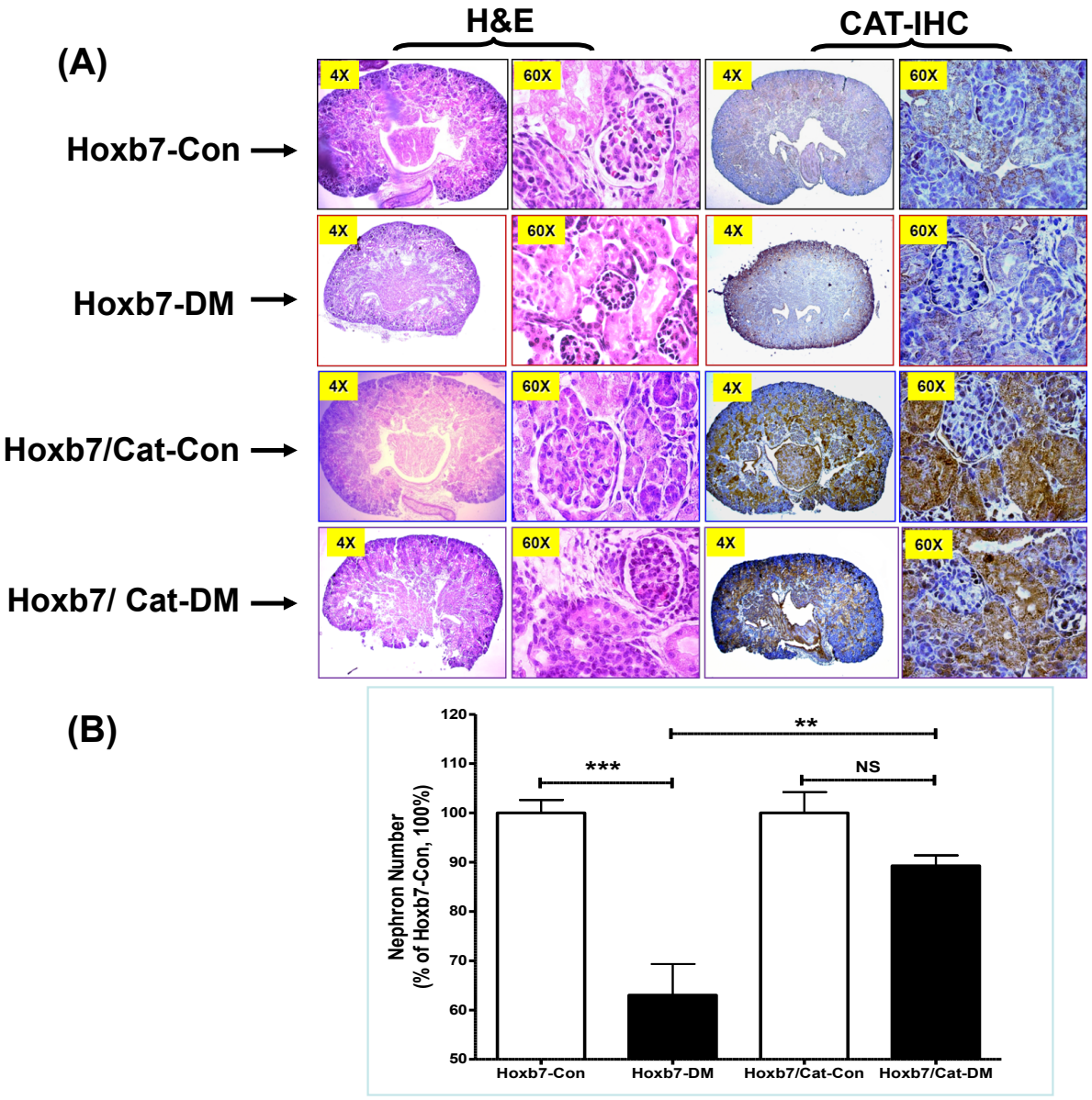

Figure 2-2: (A) Neonatal renal morphology reviewed by H&E staining and CAT expression (CAT-IHC). Neonatal offspring [Non-diabetic: Hoxb7-Con (black frame); Hoxb7/Cat-Con (blue frame) vs. Diabetic: Hoxb7-DM (red frame); Hoxb7/Cat-DM (purple frame)]; Magnification: 4X and 60X. (B) Quantification of neonatal nephron number. □, non-diabetic offspring (Hoxb7-Con, N=9; Hoxb7/Cat-Con, N=8); ■, diabetic offspring (Hoxb7-DM, N=7; Hoxb7/Cat-DM, N=9). The y axis shows the percentage of nephron number compared with Hoxb7-GFP-Tg control animal (Hoxb7-Con, 100%), **, $P \leq 0.01$; ***, $P \leq 0.001$; N.S., non-significant.

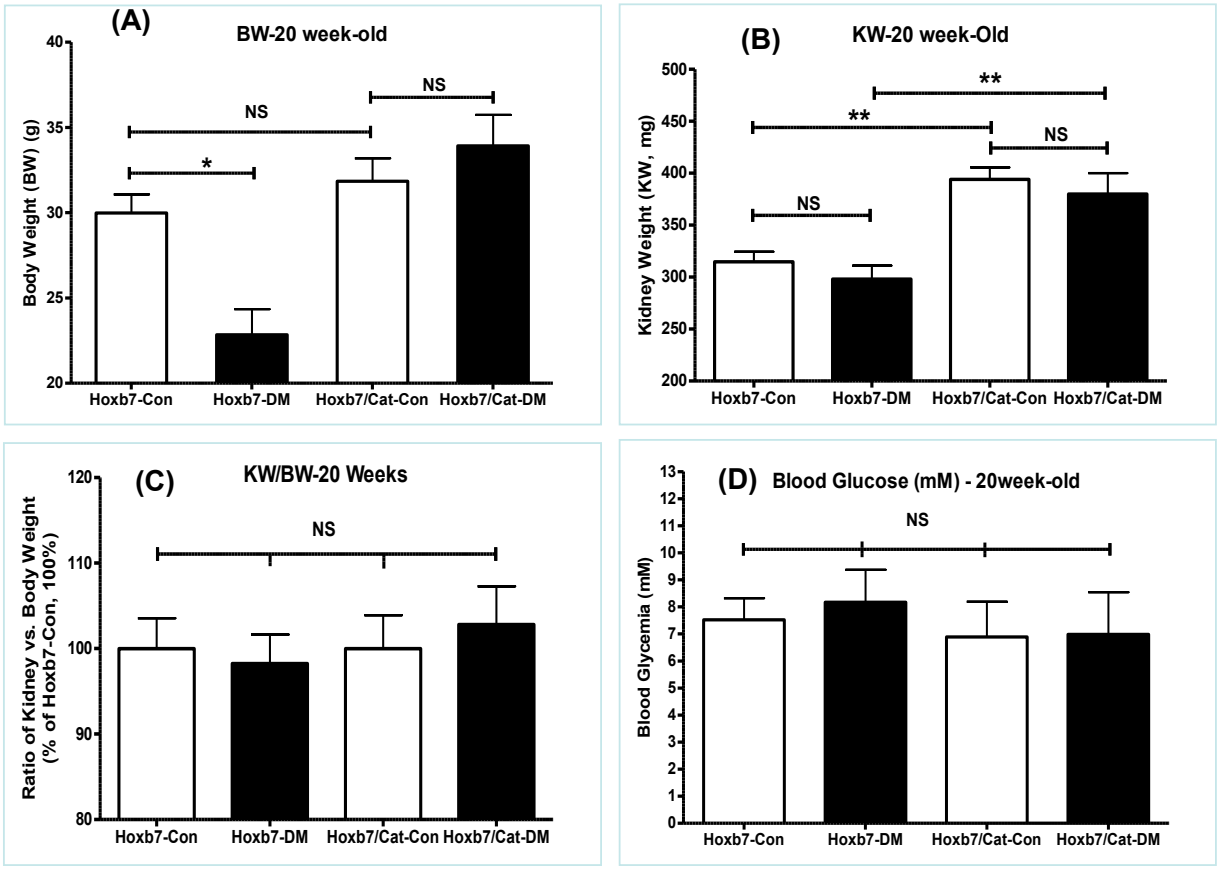

Figure 2-3: Physical parameters in the male offspring at 20 week-old. (A) Body weight (BW, g); (B) Kidney weight (KW, mg); (C) Ratio of KW vs. BW; (D) Fasting blood glucose concentration (mM). The y axis shows the percentage of value compared with Hoxb7-Con (100%). □, non-diabetic offspring (Hoxb7-Con; Hoxb7/Cat-Con); ■, diabetic offspring (Hoxb7-DM; Hoxb7/Cat-DM); *, $P \leq 0.05$; **, $P \leq 0.01$; N.S., non-significant.

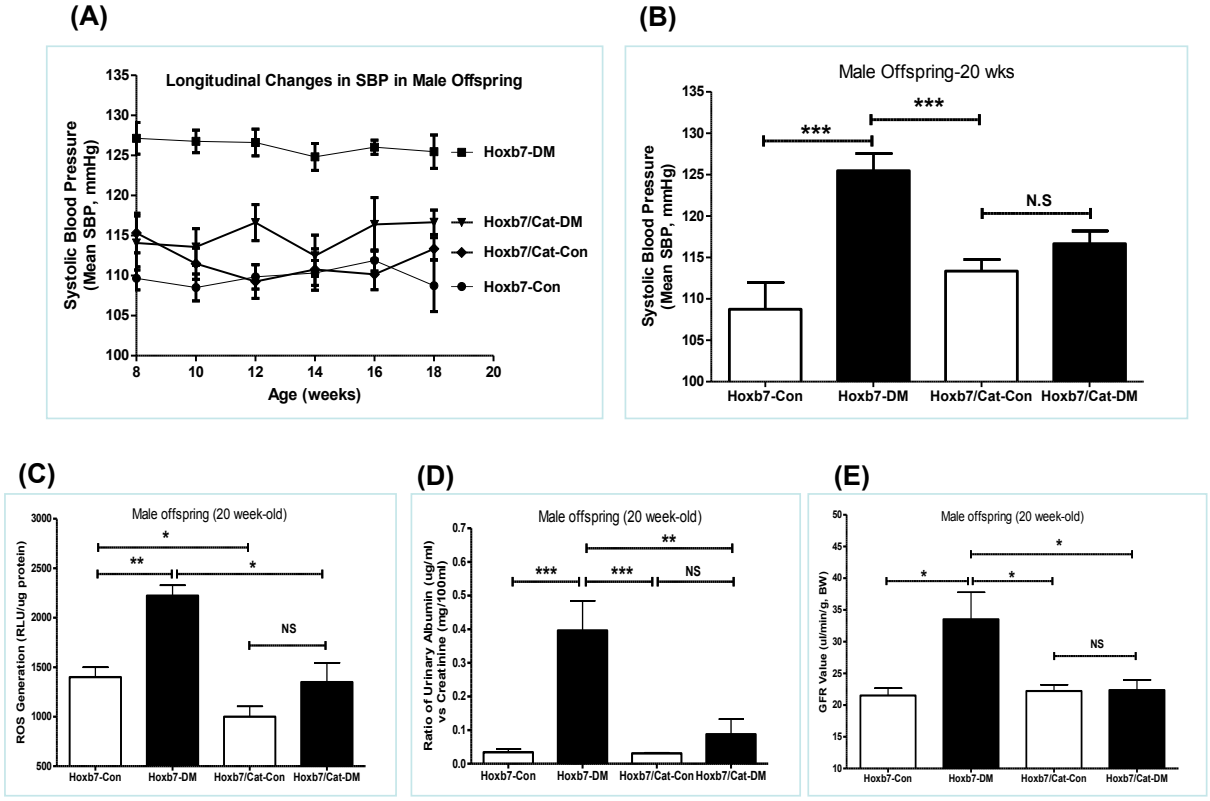

Figure 2-4: Mean SBP, ROS generation and renal function measurement in male offspring at age of 20 week-old. (A) Longitudinal changes in mean SBP in the male offspring from age 8 to 20 weeks. (B) Mean SBP in the male offspring at age of 20 week-old. (C) ROS generation. ROS production was normalized with protein concentration and expressed as relative light units (RLU) per μg protein. (D) Ratio of urinary albumin (μg/ml)/creatinine (mg/100 ml) (ACR) measurement. (E) GFR measurement. □, non-diabetic offspring (Hoxb7-Con; Hoxb7/Cat-Con); ■, diabetic offspring (Hoxb7-DM; Hoxb7/Cat-DM); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; N.S., non-significant;

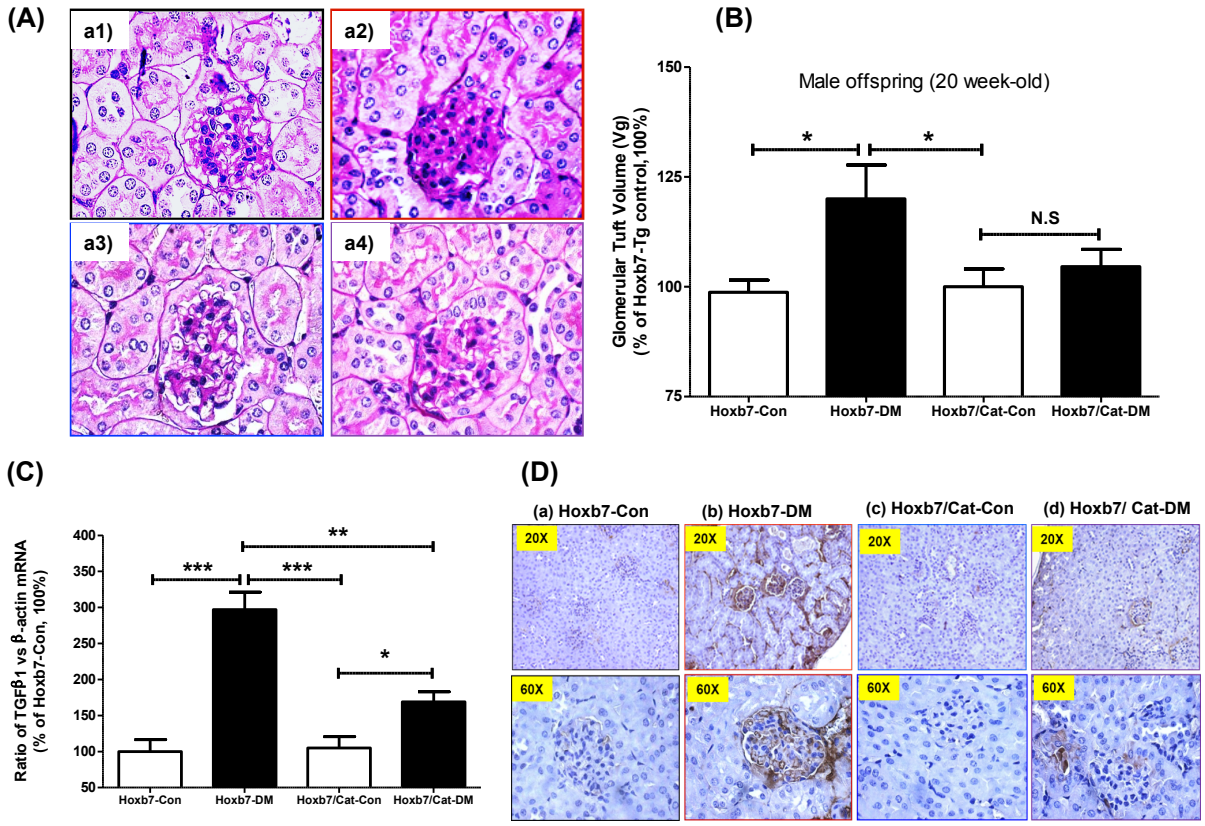

Figure 2-5: Renal morphology and TGF-β1 gene expression in the male offspring at age of 20 week-old. (A) PAS staining (Magnification, 60X). (a1) Hoxb7-Con; (a2) Hoxb7-DM; (a3) Hoxb7/Cat-Con; (a4) Hoxb7/Cat-DM. (B) Quantification of Vg value [Hoxb7-Con (N=13); Hoxb7-DM (N=9); Hoxb7/Cat-Con (N=7); and Hoxb7/Cat-DM (N=6)]. The y-axis shows the percentage of Vg compared with Hoxb7-Con (100%). (C) RT-qPCR of renal TGF-β1 mRNA. The relative densities of TGF-β1 in the renal cortex were compared with their own ß-actin mRNA. Hoxb7-Con values were considered as 100%. Each point represents the mean ± SD of 3 independent experiments. (D) TGF-β1-IHC expression (magnification, 20X, 60X). □, non-diabetic offspring (Hoxb7-Con; Hoxb7/Cat-Con); ■, diabetic offspring (Hoxb7-DM; Hoxb7/Cat-DM); *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; N.S., non-significant;

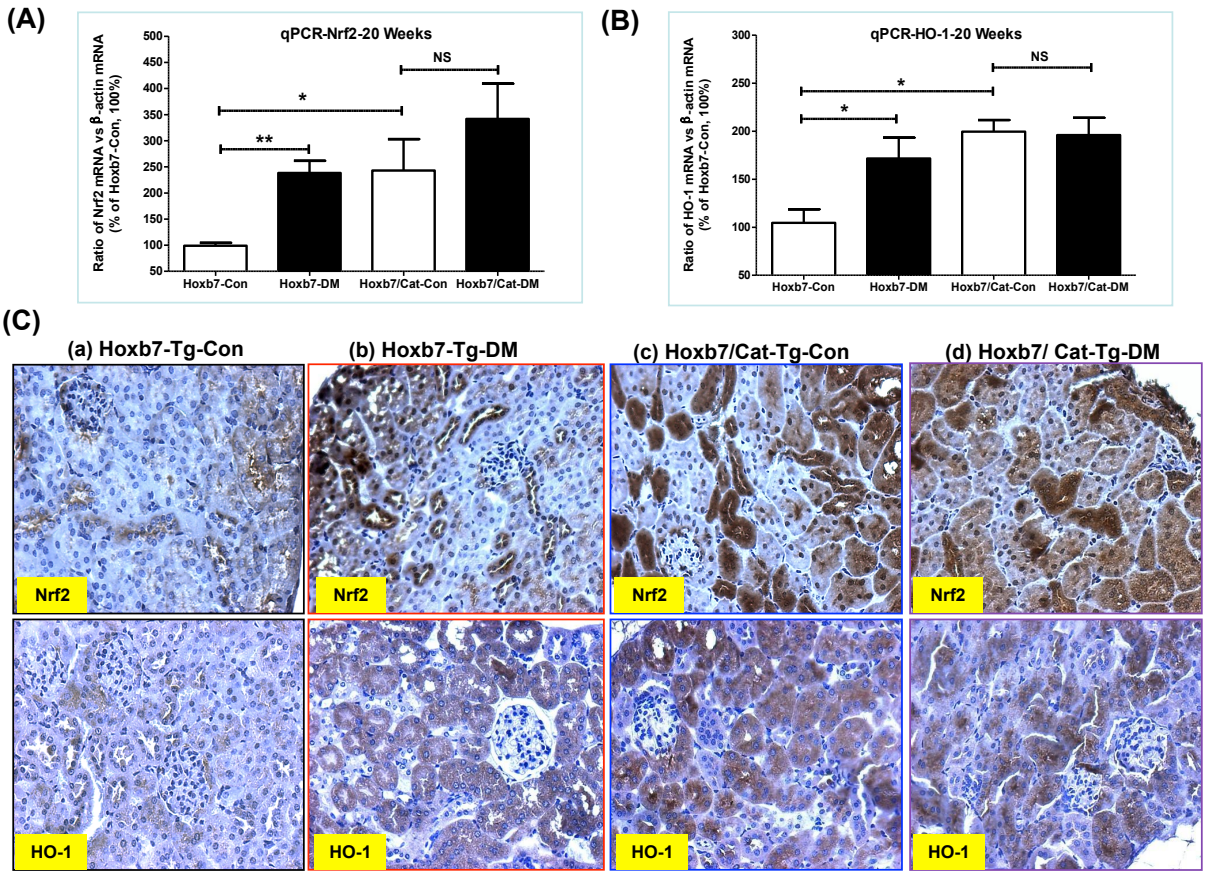

Figure 2-6: Nrf2 and HO-1 gene expression in the male offspring at the age of 20-week-old. (A-B) RT-qPCR of Nrf2 (A) and HO-1 mRNA (B). The relative densities of Nrf2 and HO-1 in the renal cortex were compared with their own ß-actin mRNA. Hoxb7-Con values were considered as 100%. Each point represents the mean ± SD of 3 independent experiments. (C) Nrf2 and HO-1-IHC expression (magnification, 20X). □, non-diabetic offspring (Hoxb7-Con; Hoxb7/Cat-Con); ■, diabetic offspring (Hoxb7-DM; Hoxb7/Cat-DM); *, $P \leq 0.05$; **, $P \leq 0.01$; N.S., non-significant;

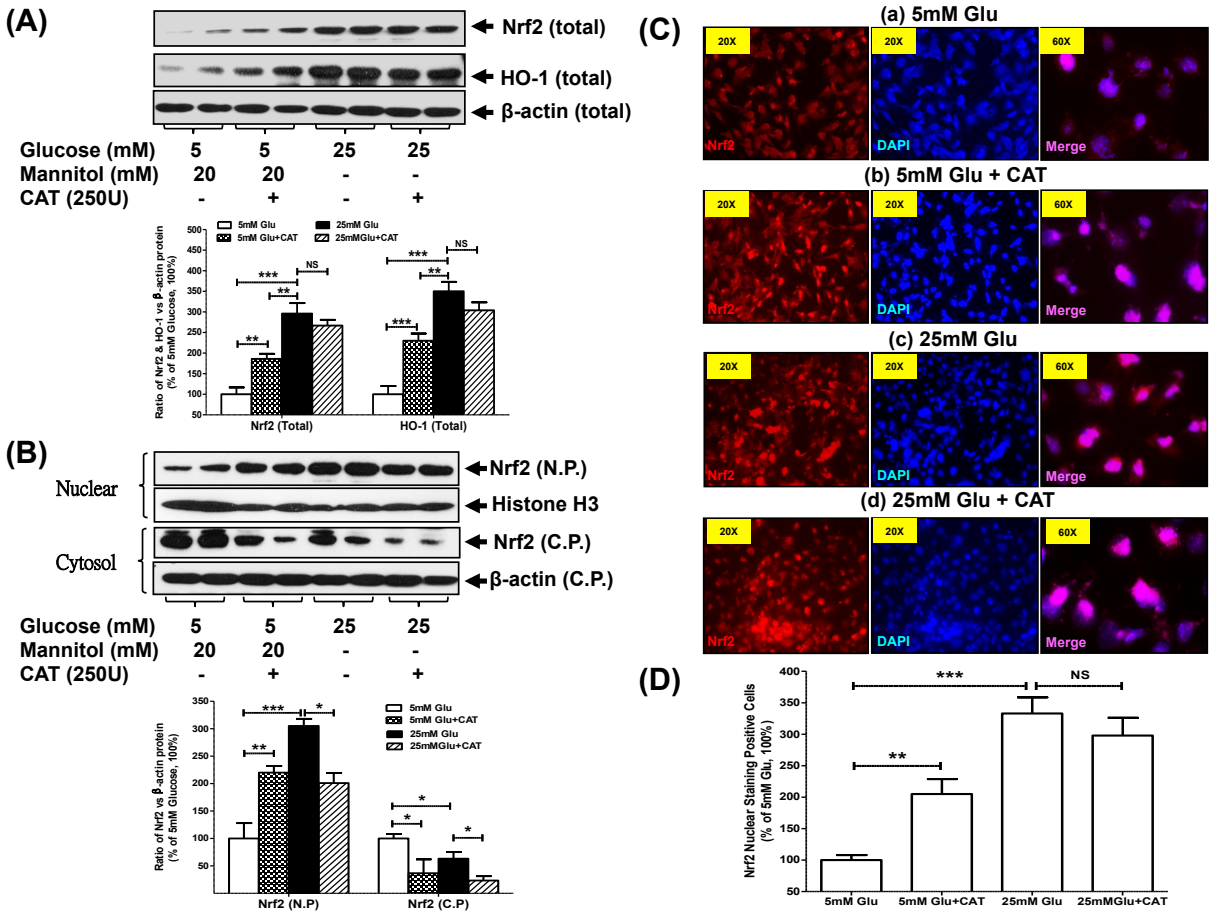

Figure 2-7: High glucose effects on Nrf2 and/ or HO-1 protein expression as well as Nrf2 nuclear translocation analyzed by WB (A and B) and IF staining (C and D) in IRPTCs *in vitro*. (A) WB performed on the total cell lysis; (B) WB performed on isolated nuclear protein (N.P.) and cytosolic protein (C.P.) extracts; (C) IF images (Magnification 20X and 60X); (D) Semi-quantification of Nrf2 IF-nuclear positive cells. The relative blot densities of Nrf2 and HO-1 protein expression in IRPTCs were compared with their own ß-actin or Histone H3. The values in 5mM glucose medium were considered as 100%. Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; N.S., non-significant;

CHAPTER 3: PUBLISHED ARTICLE

Overexpression of Angiotensinogen Downregulates Aquaporin 1 Expression via Modulation of Nrf2-HO-1 Pathway in Renal Proximal Tubular Cells of Transgenic Mice

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Overexpression of Angiotensinogen Downregulates Aquaporin 1 Expression via Modulation of Nrf2-HO-1 Pathway in Renal Proximal Tubular Cells of Transgenic Mice

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Short Title: AQP-1 and Nrf2 in Hypertension and Kidney Injury

Key words: Aquaporin-1, Nrf2, Intrarenal Renin-Angiotensin System, Hypertension, Kidney Injury

3.1 Abstract

Introduction: The present studies aimed to examine the regulation of aquaporin 1 (AQP1) expression in angiotensinogen (Agt) transgenic (Tg) mouse model, focusing on underlying molecular mechanisms.

Methods: Male Tg mice specifically overexpressing rat Agt in their renal proximal tubular cells (RPTCs) (Agt-Tg) and rat immortalized RPTCs (IRPTCs) stably transfected with rat Agt cDNA were used.

Results: Agt-Tg mice developed hypertension and nephropathy, changes that were either partially or completely attenuated by treatment with losartan or dual RAS blockade (losartan and perindopril), respectively, while hydralazine prevented hypertension but not nephropathy. Decreased expression of AQP1 and heme oxygenase-1 (HO-1) and increased expression of nuclear factor-erythroid 2-related factor 2 (Nrf2) and sodium–hydrogen exchanger 3 (NHE3) were observed in RPTCs of Agt-Tg mice and in Agt-transfected IRPTCs. These parameters were normalized by dual RAS blockade. Both in vivo and in vitro studies identified a novel mechanism(s) in which Agt overexpression in RPTCs enhances the cytosolic accumulation of Nrf2 via the phosphorylation of pGSK3β Y216. Consequently, lower intranuclear Nrf2 levels are less efficient to trigger HO-1 expression as a defense mechanism, which subsequently diminishes AQP1 expression in RPTCs.

Conclusions: Our data suggest that Agt mediated-downregulation of AQP1 and Nrf2 signaling may play an important role in intrarenal RAS-induced hypertension and kidney injury.

3.2 Introduction

Aquaporin-1 (AQP1) is the major water channel in the renal proximal tubule and the loop of Henle (1). These two nephron segments are responsible for reabsorbing 80% of the glomerular filtrate (1). Since renal proximal tubular cells (RPTCs) reabsorb 60% to 70% of filtered sodium (Na) and fluid, changes in the way in which RPTCs reabsorb water (i.e., AQP1) and Na (via increased Na transporter expression (2)) can have profound effects on renal and body fluid balance. AQP1 deficient mice (Aqp1-null) displayed normal phenotypes with respect to survival, physical appearance and organ morphology, but these mice became severely dehydrated after water deprivation, indicating that AQP1 is required for the formation of a concentrated urine (3). Aqp1-null mice had a relatively low blood

pressure phenotype, which can be explained by several possibilities—e.g., polyuria (4), impaired nitric oxide signaling (5) and reduced renin cell recruitment (6).

It has been observed that AQP1 expression is up-regulated in the kidneys (7) and brain (8) of spontaneously hypertensive rats (7, 8). In contrast, recent studies reported that renal and cardiac AQP1 expression was down-regulated and associated with renal fibrosis (9) and high-salt diet induced hypertension (10). Thus, it remains unclear whether AQP1 expression can directly or indirectly affect blood pressure and kidney injury.

The intrarenal renin angiotensin system (RAS) plays a key role in blood pressure regulation and renal hemodynamics, and all RAS components are expressed in RPTCs (11). To date, how intrarenal RAS influences AQP1 expression in either patho- and physiological conditions are poorly understood. Bouley et al. reported that angiotensin II (Ang II) rather than osmolality may be more important in regulating AQP1 levels in renal proximal tubules (RPTs) (12). Ang II at low concentrations (10-9 and 10-8 M) or infusion of Ang II at 80 ng/min/kg increased AQP1 expression in cultured rat immortalized renal proximal tubular cells (IRPTCs) in vitro and in rat kidneys in vivo, respectively. In contrast, Ang II at high concentration (10-7 M) inhibited AQP1 expression in IRPTCs. Thus, the intrarenal RAS appears to regulate AQP1 expression, influencing water reabsorption and body fluid homeostasis.

Our lab has established that transgenic (Tg) mice specifically overexpressing angiotensinogen (Agt, the sole precursor of all angiotensins) in their RPTCs developed hypertension and nephropathy with elevated intrarenal reactive oxygen species (ROS) production (13-15). In the present study, we aimed to determine whether intrarenal RAS-induced hypertension and kidney injury in our Agt-Tg mice could be mediated, at least in part, via alteration of AQP1 expression and whether RAS blockade in this transgenic model could reverse this effect. We further aimed to define the underlying molecular mechanisms both in vivo and in vitro.

3.3 Materials and Methods

Animal Models & Ethics Statement

Agt-Tg mice overexpressing renal rat Agt (rAgt) were generated by employing the kidney-specific, androgen-regulated protein promoter (KAP2) linked to rAgt cDNA as reported previously (16). There is no need to administer exogenous androgen since the circulating level of testosterone in adult male Agt-Tg mice (from 12 weeks of age, as is the case here) is sufficiently high to drive KAP2 promoter to express the transgenes (13, 14, 16). Thus, male Agt-Tg mice were employed and studied starting at 10 weeks of age and treated with or without hydralazine (15mg/kg/day, in drinking water), losartan (losartan 30 mg/ kg/ day, in drinking water) and /or dual RAS blockers (losartan 30 mg/ kg/ day plus perindopril 4 mg/ kg/ day, in drinking water) from week 13 until week 20 (13-15) (8-15 mice per group). Non-Tg littermates served as controls. All animals had ad libitum access to standard mouse chow (Diet #2918, Harlan Teklad, Montreal, Canada) and water.

The animal study was carried out in strict accordant with the recommendation in the Guide for the Case and Use of Laboratory Animals of the National Institutions of Health. Animal care and procedures were approved by the Animal Care Committee from the Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM). Mice were euthanized by sodium pentobarbital overdose [75mg/kg of BW (body weight)] and efforts were made to minimize suffering.

Physiological Studies

Mean systolic blood pressure (SBP) was monitored by the tail-cuff method with the Visitech BP-2000 Blood Pressure Analysis System for mice (Visitech System Inc., Apex, NC, USA), as reported elsewhere (13-15). Animals in each group were acclimated to longitudinal SBP measurement (2 weeks period of pre-training starting at 11 weeks of age, followed by actual measurement of SBP thrice-weekly from 13 weeks until 20 weeks of age) to minimize stress to the animals. While the technique of tail-cuff measurement is generally considered less sensitive than telemetry, our SBP data includes a 2 weeks pre-study training period and substantial numbers of animals (N= 8 to 15 mice per group) and longitudinal measurement (8 weeks excluding the 2 week pre-study training period).

Twenty-four hours before the mice were euthanized, body weight (BW) was recorded and mice were individually housed 24 hours in metabolic cages. Blood was collected

individually via intracardiac exsanguination before death and then centrifuged to obtain serum. Urine was collected and assayed for albumin (Alb) and creatinine (Cre) ratio (Alb/Cre, μg/mg) (ELISA, Albuwell and Creatinine Companion, Exocell, Inc., Philadelphia, PA, USA), Agt and Ang II measurement [i.e., C18 Sep-Pak columns (Waters, Mississauga, ON); extraction kits (Bachem Americas, Torrance, CA) ELISAs (Bachem Americas)] as reported previously (13-15). Kidney weight (KW) were rapidly recorded. The left kidney was utilized for renal histology and the right kidney was reserved for renal proximal tubules (RPTs) isolation by the Percoll gradient method for protein expression experiments as previously reported (13-15).

Renal Morphology, Immunohistochemistry and Immunofluorescence

The renal morphology and immunostaining (immunohistochemistry (IHC) and immunofluorescence (IF)) were performed as described previously (14, 16). Briefly, the kidney morphology was studied with periodic-acid Schiff (PAS) and Masson's trichrome staining. The antibodies were used for IHC and IF including: anti-AQP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-nuclear factor-erythroid 2p45 (NF-E2) related factor-2 (Nrf2) and anti-kelch-like ECH-associated protein 1 (Keap1) antibodies (Abcam, Cambridge, MA, USA); anti-heme oxygenase-1 (HO-1) (Assay Designs, Ann Arbor, MI); anti-catalase (Cat) and anti-β-actin antibodies (Sigma-Aldrich, Oakville, ON, Canada); anti-collagen type IV (Chemicon International, Temecula, CA, USA); anti-glycogen synthase kinase 3β (GSK3β) (27C10) and anti-phospho-GSK3β (Ser 9) (pGSK S9, an inactive form) (5B3) as well as anti-histone H3 (3H1) antibodies (Cell Signaling, Boston, MA, USA); anti-TGFβ1 and anti-β-catenin (total) antibodies (R&D Systems, Inc., Burlington, Canada); anti-GSK3β (pY216, an active form) (BD Transduction Laboratories™, Mississauga, ON, Canada); anti-phospho-β-catenin (Ser33/37/Thr41) (Cell signaling, ON, Canada); anti-phospho-β-catenin (Ser552) (Thermo Fisher Scientific, Rockford, IL, USA). The sodium–hydrogen exchanger 3 (NHE3) antibody was a gift from Dr. Orson Moe (University of Texas Southwestern Medical Center, Dallas, TX, USA). A rabbit polyclonal antibody against rAgt was generated in our laboratory (17) and is specific for intact rat and mouse Agt (55–62 kDa) and does not cross-react with pituitary hormone preparations or other rat or mouse plasma proteins, as described elsewhere (13-15).

Rat Immortalized Renal Proximal Tubular Cells (IRPTCs)

The IRPTC cell line (11, 18) and an IRPTC stable clone that has been stably transfected with the control plasmid pRC/RSV (designated as "pRSV-IRPTC") or with a plasmid pRC/RSV containing the rAgt cDNA (designated as "pRSV/rAgt-IRPTC") (13, 19) were employed for our in vitro studies. We have previously reported that as compared to naïve IRPTC and pRSV-IRPTC, pRSV/rAgt-IRPTC express significantly high amount of rAgt mRNA and protein as well as significant higher amount of Ang II secreted into the culture medium (13, 19). All in vitro studies were performed in the normal 150 nM NaCl final concentration with an osmolarity of 415 mOsm/kg in normal glucose (5 mM D-Glucose) DMEM as reported by Bouley R et al (12).

Nuclear protein (NP) and cytosolic protein (CP) extracts were prepared using the NE-PER nuclear and cytoplasmic extraction kit (Thermo scientific, Burlington, Ontario, Canada) (18). Cobalt protoporphyrin (CoPP, an activator of HO-1 expression) was purchased from Sigma-Aldrich Canada (Oakville, ON, Canada).

Statistical Analysis

Statistical significance between the experimental groups was analyzed by 1-way ANOVA, followed by the Bonferroni test using Graphpad Software, Prism 5.0 (La Jolla, CA, USA, http://www.graphpad.com/prism/Prism.htm). A probability level of $P \leq 0.05$ was considered to be statistically significant and followed by a Bonferroni analysis with adjustment for multiple comparisons (13-15).

3.4 Results

Physiological parameters

We measured biological parameters in five subgroups of animals at the age of 20 weeks --non-Tg littermates as controls (Con, N=12); Agt-Tg (N=14); Agt-Tg + RAS blockade (losartan and perindopril treatment, Agt-Tg + L/P, N=15); Agt-Tg + Losartan (Agt-Tg + L, N=14) and Agt-Tg + Hydralazine (Agt-Tg + H, N=8) as shown in Fig 1. There were no significant differences in BW, KW and KW/BW ratio (Fig 1A) among the 5 groups. However, as compared to the control group, SBP (Fig 1A, a cross-sectional measurement at week 20; Figure 1B, a longitudinal measurement (week 13 to 20)), urinary Alb/Cre ratio

(ACR, μg/mg) (Fig 1C), Agt/Cre ratio (ng/mg) (Fig 1D) and Ang II/Cre ratio (ng/mg) (Fig 1E) were relatively increased in Agt-Tg mice; these changes were prevented by the treatment of losartan alone and/or dual RAS blockade in Agt-Tg mice. It appears that dual RAS blockade was more effective than losartan alone in decreasing the urinary ACR (Fig 1C). In contrast, although hydralazine treatment was able to decrease SBP in Agt-Tg mice over the follow-up period (Figs 1A and 1B), the urinary ACR was unchanged (Fig 1C). The serum level of Agt (Fig 1E) or Ang II (Fig 1F) did not differ between the groups.

Renal Morphology and Extracellular Matrix Protein

Both PAS staining (Fig 2A) and Masson's trichrome staining (Fig 2B) of kidney sections revealed enhanced extracellular matrix (ECM) protein accumulation in the glomerulo-tubular areas in hypertensive Agt-Tg, a finding that was confirmed by collagen type IV (Fig 2C) and TGFβ1-IHC-staining (Fig 2D). The degree of oxidative stress was confirmed by lower Cat expression in kidneys of hypertensive Agt mice (Fig 2E). Semi-quantitative analysis revealed that dual RAS blockade was more effective in preventing ECM accumulation and collagen type IV/TGF-β1 expression as well as in normalizing catalase expression in Agt-Tg mice as compared to losartan treatment alone. Given the greater effectiveness of dual blockade, the remainder of our mechanistic experiments were done with Agt-Tg mice treated with dual RAS blockade.

Renal Agt, AQP1 and HO-1 Protein Expression

We assessed Agt, AQP1, and HO-1 gene expression in the renal cortex by IHC (Fig 3A) and in isolated RPTs by western blot (WB) (Fig 3B). AQP1 shows a two-band WB pattern (glycosylated (38 kDa) and non-glycosylated AQP1 fractions (28 kDa)), matching its original described character as an N-proteoglycan (20). The functional significance of AQP1 glycosylation is unknown but it could play a role in AQP1 oligomerization (21), removal of sugars from the AQP1 molecule seems not to influence AQP1 water transport function (22). Thus, in the current study, we evaluated the change of total AQP1 including both glycosylated and non-glycosylated AQP1. Compared to non-Tg control littermates, increased Agt, but decreased AQP1 and HO-1 protein expression were observed in RPTs of Agt-Tg mice and these changes were normalized with dual RAS blockade. These data indicate an inverse relationship between Agt expression and AQP1 and HO-1 expression in

RPTs of Agt-Tg mice.

To establish a functional relationship among Agt, AQP1 and HO-1 expression, we performed in vitro studies by using IRPTCs (11, 18). In the presence of CoPP, an activator of HO-1, both HO-1 and AQP1 protein expressions were increased (Fig 3C) while the Agt protein expression was reduced (Fig 3D) in a dose-dependent manner. Furthermore, these effects seem selective since CoPP did not affect Nrf2 expression (Fig 3E). We further confirmed those results in naïve IRPTCs and IRPTCs transiently transfected with rat Agt cDNA followed by the stimulation of 2 uM CoPP (Fig 3F).

Renal Agt, AQP1 and Nrf2-Keap1 Expression

As compared to controls, there was augmented Nrf2 protein expression in kidneys of both Agt-Tg and Agt-Tg treated mice with dual RAS blockade (Fig 4A), and that expression pattern was further confirmed in the fresh isolated RPTs by WB (Fig 4B). However, higher magnification of IHC staining revealed that the augmented Nrf2 was mostly localized to the cytosolic portion in RPTCs of Agt-Tg mice with some Nrf2 staining in the nuclei of RPTCs (Fig 4A). In contrast, in the kidneys of Agt-Tg treated with dual RAS blockade, the majority of positive IHC-Nrf2 was localized in the nuclei of RPTCs (Fig 4A). Keap1, a protein involves in Nrf2 degradation showed no change in expression in the kidneys among 3 groups by either IHC staining (Fig 4A) or WB (Fig 4B).

Next, we validated the renal Nrf2 translocation pattern in our pRSV/rAgt-IRPTC stable transformants (13, 19). As compared with naïve IRPTC and pRSV-IRPTC control transformants, the pRSV/rAgt-IRPTC stable transformants expressed high amounts of rat Agt and Nrf2 protein without any change in Keap1 protein expression (Fig 5A). Also, AQP1 expression was dramatically suppressed in pRSV/rAgt-IRPTC stable clone (Fig 5A). Similar to the in vivo observation, the higher and lower Nrf2 expression was observed in the cytosolic fraction and nuclear fraction of pRSV/rAgt-IRPTC stable transformants, respectively, as compared to pRSV-IRPTC controls (Fig 5B). Moreover, the lower AQP1 expression pattern in the pRSV/rAgt-IRPTC stable clone was further confirmed by IF-AQP1 staining (Fig 5C).

Renal Agt and Phosphorylation of GSK3β and β-catenin

Since studies have reported that phosphorylated (p) GSK S9 increases Nrf2 nuclear translocation whereas pGSK Y216 enhances Nrf2 nuclear export, we investigated the expression of pGSK S9 and pGSK Y216 in vivo and in vitro. As compared to control animals, the expression of pGSK S9 was decreased whereas pGSK Y216 expression was increased in kidneys of Agt-Tg mice, and dual RAS blockade treatment reversed these changes in Agt-Tg mice (Fig 5D). The expression pattern of pGSK S9 and pGSK Y216 was further confirmed by WB in the fresh isolated RPTs (Fig 5E). The similar expression pattern of pGSK S9 and pGSK Y216 was also observed in our pRSV-IRPTCs and pRSV/rAgt-IRPTC stable transformants (Fig 6A).

Studies indicate that both Ang II and AQP1 can interact with the GSK3β and β-catenin pathways to trigger renal injury. Thus, we studied these interactions in vitro. Our data indicated that the phosphorylation of β-catenin (Ser33/37/Thr41 and Ser552) was significantly inhibited in pRSV/rAgt-IRPTC stable transformants as compared to naïve IRPTC and/or pRSV-IRPTC control transformants (Fig 6A).

Renal Agt and NHE3 Expression

Co-IF staining of AQP1 and NHE3 revealed that Agt-Tg mice expressed less AQP1 protein and augmented NHE3 protein in their RPTCs as compared to control littermates and that the treatment with dual RAS blockade reversed these changes (Fig 6B). These observations were further confirmed by WB for AQP1 and NHE3 in isolated RPTs of these mice (Fig 6C). The increased NHE3 expression was also confirmed in pRSV/rAgt-IRPTC stable transformants (Fig 6D).

3.5 Discussion

The present report identifies novel mechanism(s) by which Agt overexpression inhibits AQP1 expression in RPTCs, resulting in renal injury and hypertension (see our concept of a molecular model in Fig 7). In brief, Agt overexpression in RPTCs enhances cytosolic accumulation of Nrf2 via the phosphorylation of pGSK3β Y216. Consequently, less intranuclear Nrf2 is available to trigger HO-1 expression as a defense mechanism. As a result, AQP1 expression in RPTCs is subsequently diminished. The depleted AQP1 expression through β-catenin-dependent signaling further contributes to hypertension that

involves the intrarenal RAS (via NHE3) and nephropathy.

In this study, we are focusing on the functional interaction between Agt and AQP1 in RPTCs, given the fact of that Aqp1-null mice appear not to develop homeostasis disturbances although they have slight dehydration (3). In concert with our previous findings (13-15), we observed that Agt-Tg mice specifically overexpressing rat Agt in their RPTCs developed hypertension and nephropathy. Since we only detect significant increased urinary Agt/Cre ratio and Ang II/Cre ratio in Agt-Tg mice, while serum levels of Agt and Ang II remain unchanged, it suggests that Agt derived predominantly from RPTCs rather than other sources (23, 24) plays the key role in this phenomenon.

The use of combination treatment with an ACE inhibitor and angiotensin-receptor blocker (ARB) to ameliorate the progression of kidney disease has been controversial because of concern about an increased risk of hyperkalemia or acute kidney injury (25, 26). However, a recent meta-analysis published in Lancet (27) reported a benefit of dual RAS blockade in the prevention of chronic kidney disease with or without diabetes. Our current data lend to support these observations. We found that as compared to the treatment with losartan alone, dual RAS blockade (losartan and perindopril) was more effective in preventing hypertension induced by activation of the intrarenal RAS and nephropathy progression in Agt-Tg mice. Moreover, although hydralazine decreased systemic hypertension in Agt-Tg mice over the follow-up period, it had no impact on ACR (a marker of renal function), suggesting that intrarenal RAS activation contributed to the development of nephropathy independent of systemic hypertension (and possibly associated with elevated ROS production in RPTCs in Agt-Tg mice, as reported previously (14)).

Both Agt and AQP1, which are mainly expressed in RPTCs, are important for maintaining normal fluid homeostasis; however, how they interact has not been fully delineated. Whether their interaction has a regulatory role in the development of hypertension and nephropathy remains elusive. Notably, substantial inhibition of AQP1 and HO-1 protein expression in the RPTCs was observed in the kidney of Agt-Tg mice, implicating their possible role in the pathogenesis of hypertension and nephropathy. This possibility is supported by the observation that significantly decreased renal AQP1 content was observed in the obstructed kidneys of rats with unilateral ureteral obstruction (UUO),

suggesting that down-regulation of AQP1 might be associated with tubulointerstitial fibrosis (9). Moreover, in the mouse model of hypertension induced by high-salt diet, the reduction of cardiac AQP1 might be associated with hypertension and cardiac injury, since ARB treatment (valsartan) partially reversed the effects of high-salt diet on hypertension with cardiac damage (fibrosis and inflammatory cell infiltration) and normalized cardiac AQP1 expression (10).

Our in vitro studies demonstrated that CoPP, an activator of HO-1, dose-dependently stimulates HO-1 and AQP1 and inhibits Agt protein expression in IRPTCs, suggesting an inverse relationship between the expression of Agt and HO-1/AQP1 in RPTCs. How HO-1 and AQP1 interact is not fully understood. A possible link between AQP1 and HO-1 might be via the Kruppel-like protein, since the AQP1 promoter contains Kruppel-like sequences (28), and Kruppel-like factor 2 dependently induced HO-1 expression (29).

HO-1 is a stress-inducible protein that induces cellular protection in the event of injury, inflammation, oxidative stress, etc. Exogenous induction of HO-1 has been shown to have renal and/or cardiovascular protective functions (30, 31) and to attenuate the development of hypertension and to decrease blood pressure in models of established hypertension (30, 31). HO-1 expression is modulated by Nrf2, a transcription factor that is highly expressed in the kidney (32, 33). It is thought that the Nrf2/Keap1-HO-1 defense system is renoprotective and that its induction might even improve kidney function (32, 33). Thus, we tested the intrarenal expression pattern of Nrf2/Keap1 in our three groups of animals.

Via elevated ROS generation, Agt-Tg mice displayed augmented RPTC Nrf2 accumulation, primarily in the cytosol, with less nuclear staining, and this Nrf2 translocation pattern was further confirmed in our pRSV/rAgt-IRPTC stable transformants. These data suggested that while overexpression of Agt in RPTCs resulted in activated Nrf2 expression, it still failed to promote sufficient HO-1 and AQP1 expression in RPTCs to prevent or diminish ROS-induced kidney damage and hypertension occurring in Agt-Tg mice. Compelling studies suggested that Nrf2 accumulation/activation is countered by two major Nrf2 degradation mechanisms—e.g., Keap1-induced Nrf2 proteasomal degradation in the cytosol; and/or GSK3β- mediated nuclear export and degradation of Nrf2 (32-36). Since

renal Keap1 expression did not differ among the three groups of animals, Keap1-associated Nrf2 degradation appears to be normal in our model. In contrast, the activated GSK3β (i.e., pGSK Y216 phosphorylation (33, 34, 37)) has been reported to phosphorylate Fyn tyrosine kinase, leading to enhanced nuclear export of Nrf2 and proteasomal degradation, likely via the adaptor protein β-TrCP independent of Keap1 (33, 34, 38, 39). By conducting both in vivo and in vitro experiments, we observed that overexpression of Agt in RPTCs indeed promoted and inhibited the phosphorylation of pGSK Y216 (active form) and pGSK S9 (inactive form), respectively. This suggests that the enhanced nuclear export of Nrf2 was associated with an accumulation of Nrf2 in the cytosol, while lower nuclear levels of Nrf2 failed to trigger HO-1/AQP1 induction-mediated renoprotection in RPTCs in Agt-Tg mice.

Evidence suggests that β-catenin might be one of mediators that links AQP1 and Ang II functionally (40-42). For example, AQP1 acts as a scaffold in interaction with GSK3β to promote β-catenin degradation by increasing β-catenin phosphorylation; vice versa, loss of AQP1 inhibits β-catenin degradation and facilitates the translocation of free β-catenin to the nucleus to enhance Wnt signaling, consequently triggering cystic dilation of RPTs in polycystic kidney disease (42). Additionally, Ang II via AT1R appears to promote the accumulation of β-catenin protein, correlated with GSK3β phosphorylation, contributing to the development of renal fibrosis and hypertension (40, 41). In the current study, we observed the depleted AQP1 in our Agt-Tg and pRSV/rAgt-IRPTC stable transformants activated and inhibited phosphorylation of GSK3β (Y216) and β-catenin (Ser33/37/Thr41 and Ser552), respectively, suggesting that loss of AQP1 might trigger the Wnt/β-catenin pathway, resulting in RPTs damage and hypertension.

Finally, our data both in vivo and in vitro also suggest that overexpression of Agt in RPTCs and the related hypertension might be due to decreased water absorption via AQP1 and increased sodium reabsorption via NHE3. Indeed, this observation is in line with recent findings--e.g., when compared with WT mice (NHE3+/+), AQP1 significantly increased in RPTCs of NHE3 KO mice (NHE3-/-) which completely blunt Ang II–induced hypertension, underscoring the importance of AQP1 and NHE3 interaction (43). In fact, Ang II-dependent hypertension mediated by an increased NHE3 abundance in RPTCs has been reported in AT1a receptor-deficient mice (44-46) and in oxidative stress-modulated

AT1R signaling in Sprague-Dawley rats (47, 48), though other Ang II infusion models have provided variable results (49-51) (NHE3 has been reported as increased (50) or decreased (49) or no changed (51)).

In conclusion, our data suggest that Agt/Nrf2 mediated-downregulation of AQP1 and HO-1 expression in the proximal tubule plays a key role in Ang II -induced hypertension and kidney injury.

Author Contribution

S.Y.C, C.S.L, X.P.Z, M.C.L and I.C. performed all experiments and contributed to discussion. R.B., J.R.J, J.S.D.C and S.L.Z participated in the interpretation of the results and reviewed/edited the paper. S.L.Z. drafted the manuscript. All authors have read and critically revised the final version of the manuscript.

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Abbreviations

ACR, albumin and creatinine ratio; Agt, angiotensinogen; Ang II, angiotensin II; AQP1, aquaporin 1; BW, body weight; Cat, Catalase; CoPP, cobalt protoporphyrin; CP, cytosolic protein; Cre, creatinine; GSK3β, glycogen synthase kinase 3β; HO-1, heme oxygenase-1; IF, immunofluorescence; IHC, immunohistochemistry; Keap1, kelch-like ECH-associated protein 1; KW, kidney weight; IRPTCs, rat immortalized renal proximal tubular cells; Na, sodium; NHE3, sodium–hydrogen exchanger 3; NP, nuclear protein; Nrf2, nuclear factor-erythroid 2p45 (NF-E2) related factor-2; PAS, periodic-acid schiff; RAS, renin angiotensin system; RPTs, renal proximal tubules; RPTCs, renal proximal tubular cells; SBP, systolic blood pressure; Tg, transgenic; WB, western blot;

Disclosures

None

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3.7 Figure legends

(A)

20 Week-Old	Con	Agt-Tg	Agt-Tg + L/P	Agt-Tg+L	Agt-Tg+H
Systolic Blood Pressure (SBP, mmHg)	108.8 ± 0.70	118.4 ± 1.22 **	90.5 ± 4.23 ***,†††	111.1± 1.91	95.6± 4.22*,†
Body Weight (BW, g)	34.38 ± 0.96	34.63 ± 1.07	32.98 ± 1.73	33.08± 4.17	34.75± 3.18
Kidney Weight (KW, g)	0.37 ± 0.008	0.37 ± 0.022	0.38 ± 0.014	0.35±0.022	0.38± 0.001
KW/BW ratio (g/g)	0.0108 ± 0.00047	0.0113 ± 0.000872	0.0118 ± 0.000336	0.0108 ±0.001530	0.0114± 0.000329

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ vs. Con; † $P \leq 0.05$; †† $P \leq 0.01$; ††† $P \leq 0.001$ vs. Agt-Tg

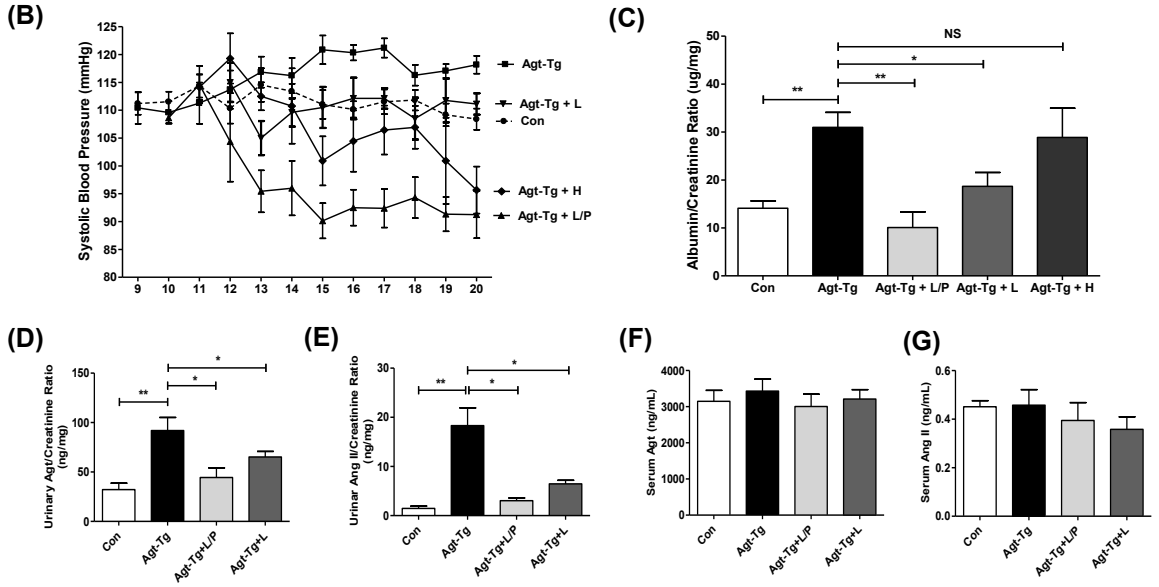

Fig 3-1: Physiolgical measurements. (A) Biological parameters in 5 groups of mice (Con, Agt-Tg, Agt-Tg + L/P, Agt-Tg + L, and Agt-Tg + H) at 20 week-old. *, p 0.05, **, p ≤ 0.01, ***, p ≤ 0.001 vs. Con; †, p 0.05, ††, p ≤ 0.01, †††, p ≤ 0.001 vs. Agt-Tg; (B) Longitudinal SBP (mmHg) measurement in 5 groups of mice from age 9 to 20 weeks; (C) Urinary Albumin/Creatinine ratio (ACR, ug/mg) in 5 groups of mice. (D) Urinary Agt/Creatinine ratio (ng/mg). (E) Urinary Ang II/Creatinine ratio (ng/mg). (F) Serum level of Agt (ng/ml). (G) Serum level of Ang II (ng/ml); *, p≤ 0.05, **, p ≤ 0.01, ***, p ≤ 0.001 vs. Con; NS, non-significant;

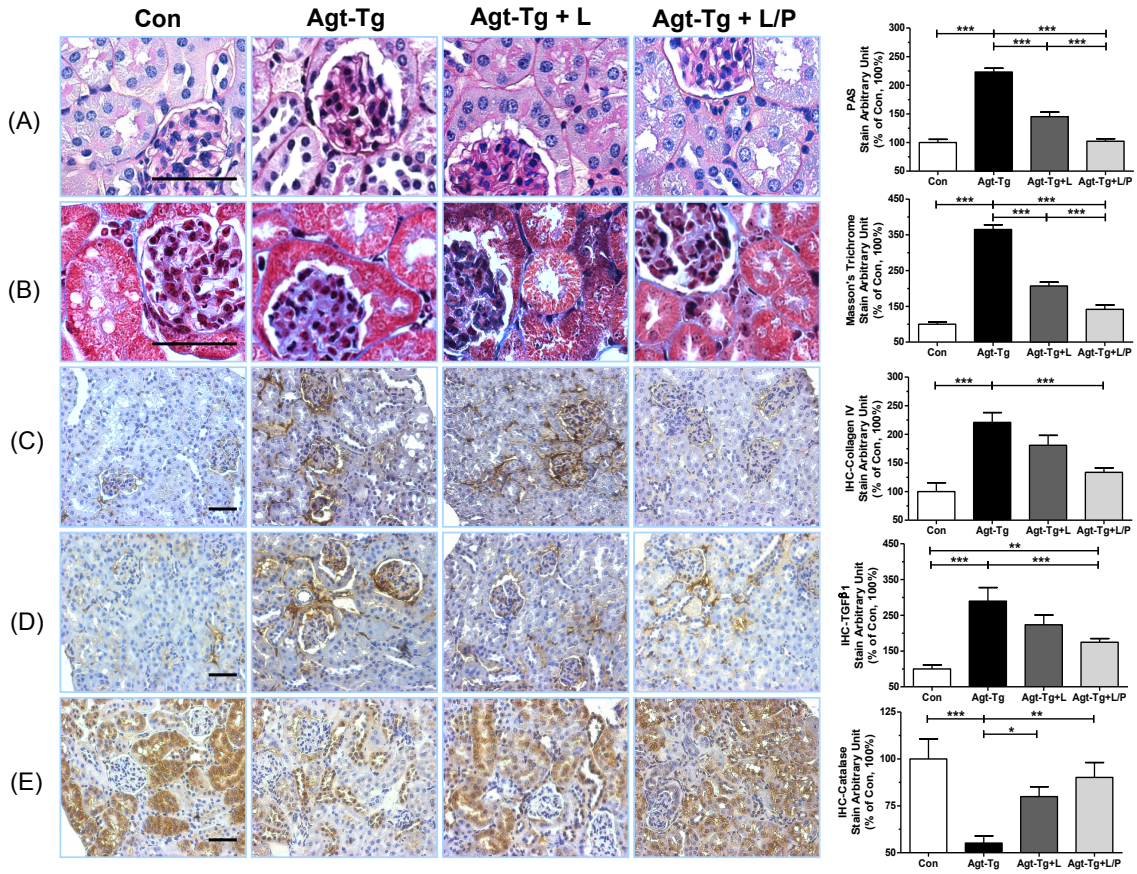

Fig 3-2: Renal morphology and IHC, (A) PAS staining (magnification, 600X); (B) Masson's trichrome staining (magnification, 600X). (C) IHC-Collagen type IV (magnification, 200X); (D) IHC-TGFβ1 (magnification, 200X); and (E) IHC-Catalase (magnification, 200X), in 4 groups of mice (Con, Agt-Tg, Agt-Tg + L and Agt-Tg + L/P) at 20 week-old. Scale bar=50μm. *, p≤ 0.05, **, p ≤ 0.01, ***, p ≤ 0.001 vs. Con; NS, non-significant;

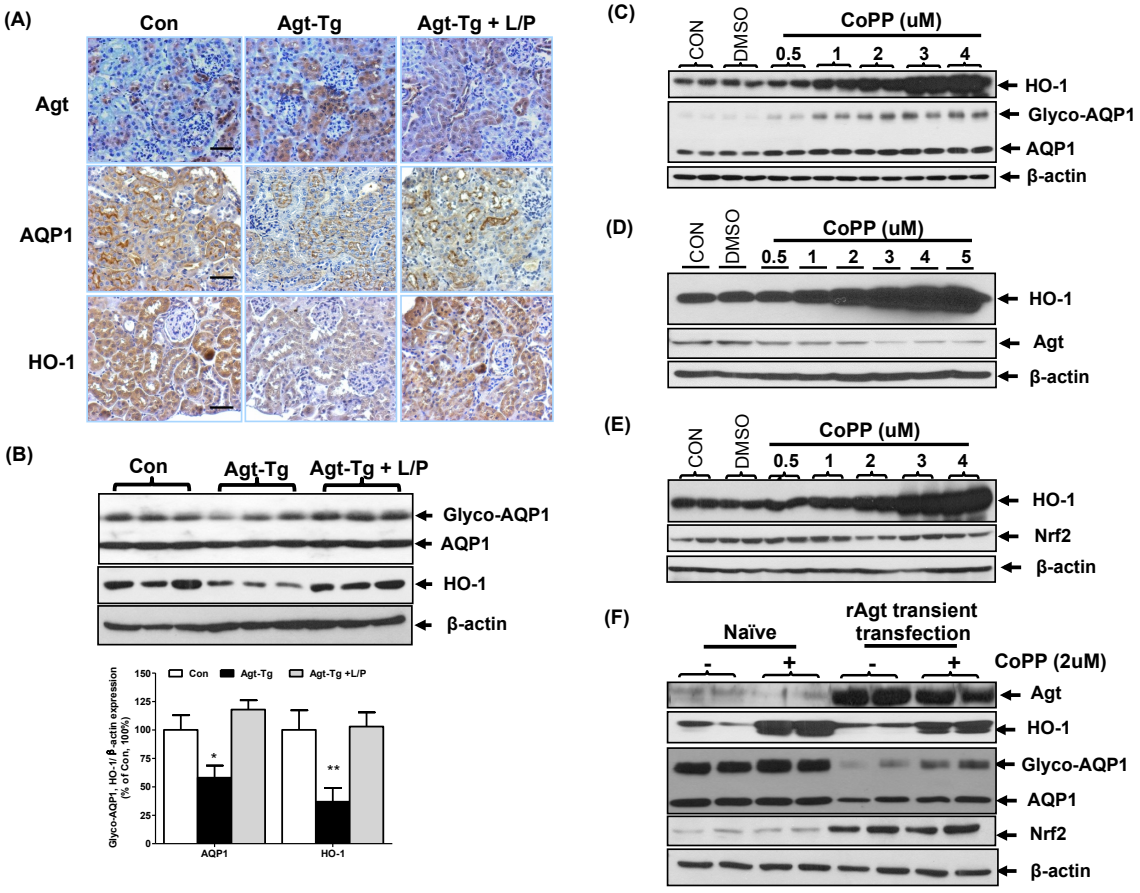

Fig 3-3: Agt, HO-1 and AQP1 expression in vivo and in vitro. (A-B) AQP1 and HO-1 protein expression in 3 groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 week-old. (A) IHC staining (magnification 200X). Scale bar=50μm. (B) Western blot (WB) in the isolated RPTCs. The relative densities of AQP1 and HO-1 were compared with its own ß-actin. Control values were considered as 100%. Each point represents the mean ± S.E.M of 3 independent experiments.; **, p 0.01 vs. Con; (C-F) CoPP effect analyzed by WB in vitro. (C) CoPP dose-dependent effect on HO-1 and AQP1 protein expression in naïve IRPTCs; (D) CoPP dose-dependent effect on HO-1 and Agt protein expression in naïve IRPTCs; (E) CoPP dose-dependent effect on HO-1 and total Nrf2 protein expression in naïve IRPTCs; (F) CoPP (2uM) effect on naïve IRPTCs and IRPTCs transient transfection of rat Agt cDNA.

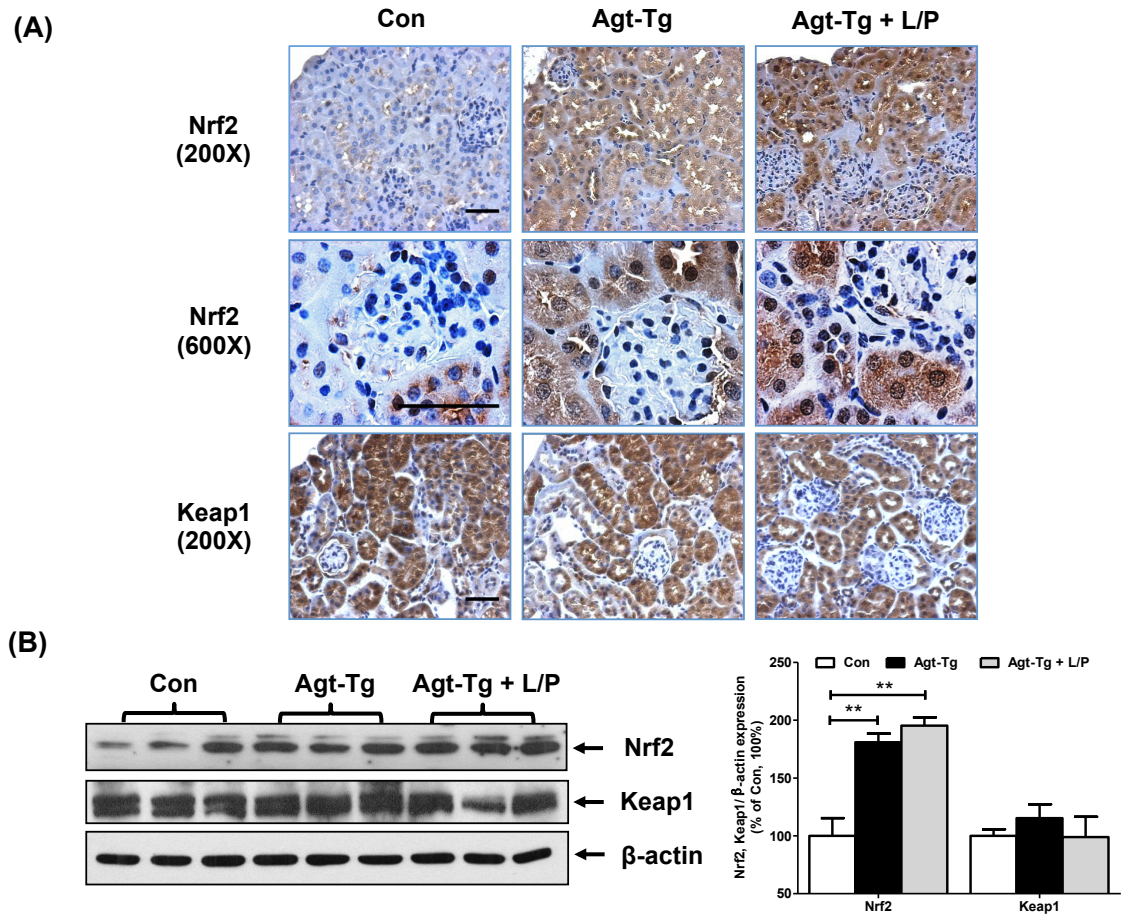

Fig 3-4: Renal Nrf2-Keap1 expression in vivo. (A) IHC staining (magnification 200X and 600X) Scale bar=50μm. (B) WB in the isolated RPTCs. Con (white bar); Agt-Tg (black bar); Agt-Tg+L/P (shadow bar); The relative densities of Nrf2 and Keap1 were compared with its own ß-actin. Control values were considered as 100%. Each point represents the mean ± S.E.M of 3 independent experiments. **, **, p≤0.01 vs. Con;

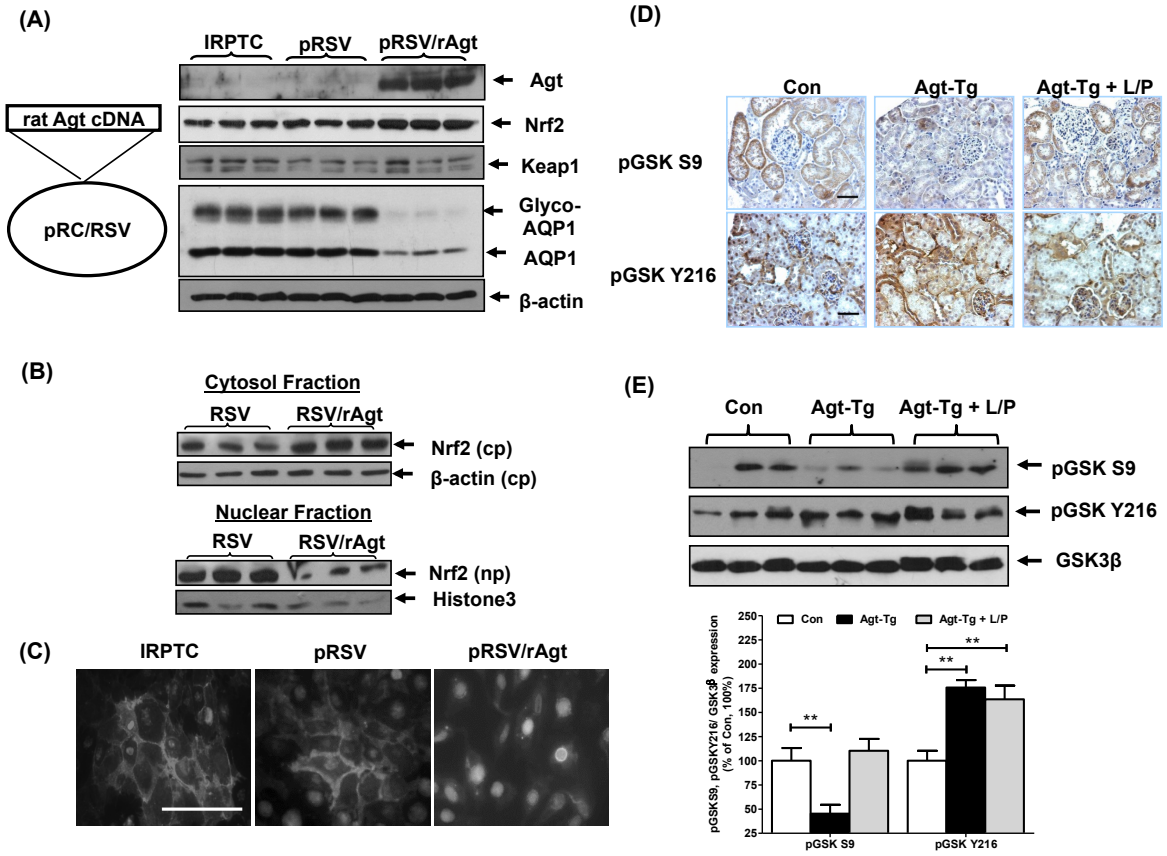

Fig 3-5: The phosphorylation of GSK3β expression in vivo and in vitro. (A) Agt, AQP1 and Nrf2/Keap1 protein expression in IRPTC stable transformants analyzed by WB. (B) Nrf2 translocation in the isolated cytosol and nuclear fraction analyzed by WB; (C) IF-AQP1 (magnification 200X); (D-E) Phosphorylation of GSK3β in the kidney of 3 groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 week-old. (D) IHC staining (magnification 200X) Scale bar=50μm. (E) WB in the isolated RPTCS. The relative densities of pGSK3β S9 and pGSK3β Y216 were compared with total GSK3β. Control values were considered as 100%. Each point represents the mean ± S.E.M. of 3 independent experiments. **, p≤0.01 vs. Con;

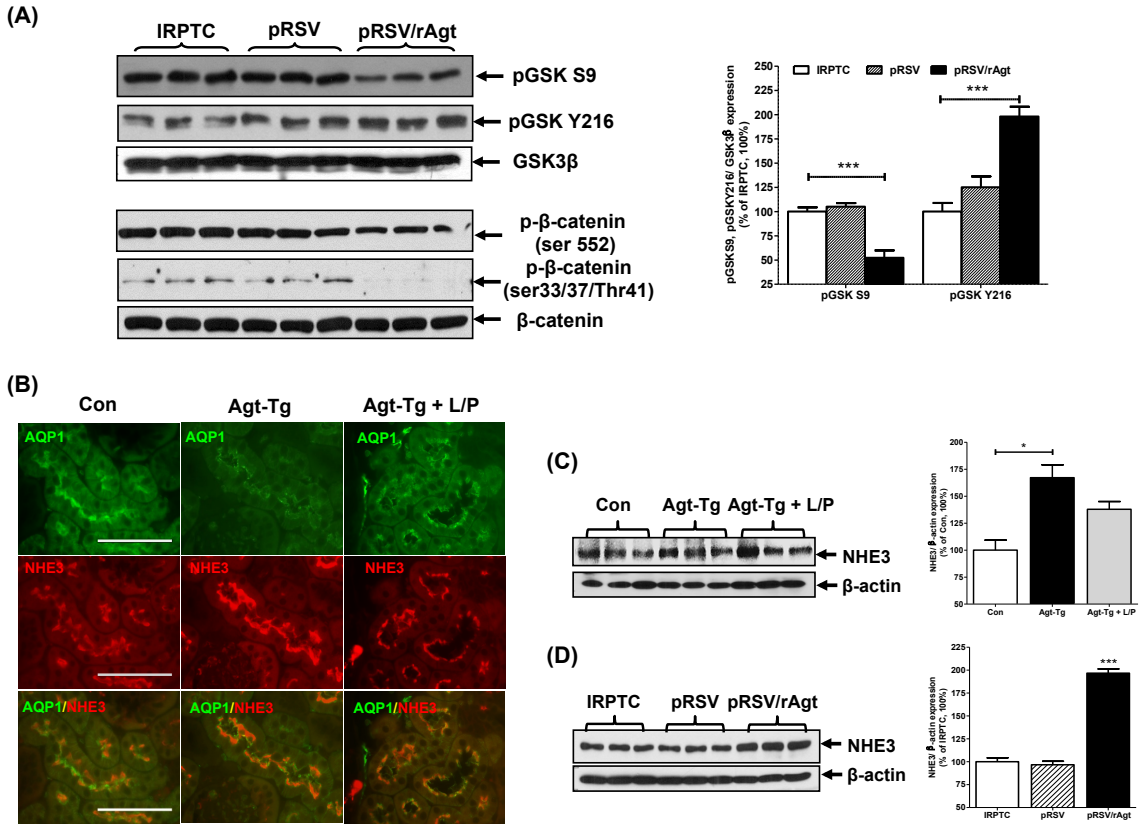

Fig 3-6: The phosphorylation of β-catenin and NHE3 expression in vivo and in vitro. (A) Phosphorylation of GSK3β and β-catenin in IRPTC and IRPTCs stable transformants analyzed by WB. The relative densities of pGSK3β S9 and pGSK3β Y216 were compared with total GSK3β. The values in naïve IRPTC cells were considered as 100%. Each point represents the mean ± S.E.M of 3 independent experiments.; ***, p 0.001 vs. naïve IRPTC; (B) Co-localization of IF-AQP1 and IF-NHE3 (magnification 200X) in 3 groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 week-old. Scale bar=50μm. (C) WB in the isolated RPTCS in 3 groups of mice (Con, Agt-Tg and Agt-Tg + L/P) at 20 week-old. The relative density of NHE3 was compared with its own ß-actin. Control value was considered as 100%. Each point represents the mean ± S.E.M. of 3 independent experiments. *, p≤0.05 vs. Con; (D) NHE3 protein expression in IRPTC stable transformants analyzed by WB. The relative density of NHE3 was compared with its own ß-actin. The value in naïve IRPTC cells was considered as 100%. Each point represents the mean ± ± S.E.M. of 3 independent experiments. ***, p≤0.001 vs. naïve IRPTC.

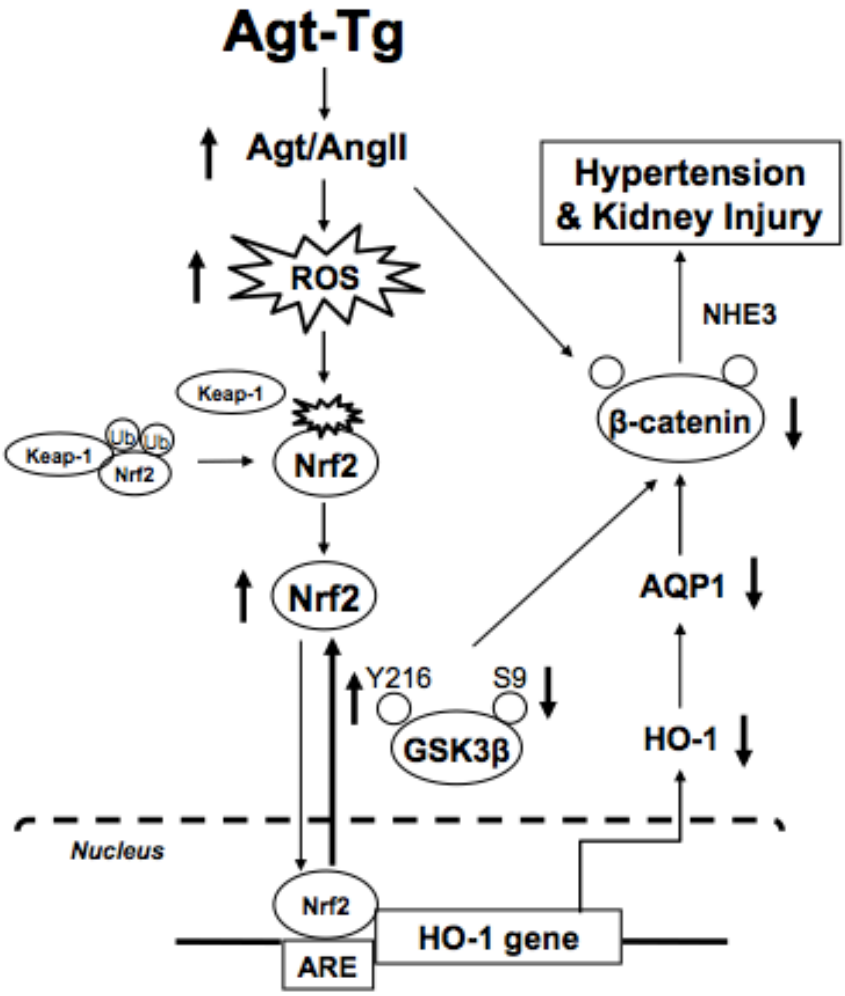

Fig 3-7: Our working model. In brief, overexpression of intrarenal Agt gene in RPTCs via elevated ROS generation mediates Nrf2 activation with an impaired Nrf2 translocation pattern. Agt overexpression in RPTCs promotes and inhibits the phosphorylation of pGSK Y216 (active form) and pGSK S9 (inactive form), respectively. Consequently, nuclear export of Nrf2 activity is enhanced, resulting in the accumulation of Nrf2 in the cytosol, and decreased Nrf2 expression in nuclei, which fails to trigger HO-1 expression as a defense mechanism and subsequently diminishes AQP1 expression in RPTCs. Concomitantly, the depleted AQP1 expression through β-catenin-dependent signaling further contributes to inrenal RAS-induced nephropathy and hypertension (via NHE3)."

CHAPTER 4: DISCUSSION

Article 1

4.1 Summary

Our data suggests that overexpression of catalase (CAT) in mouse renal proximal tubular cells (RPTCs) prevents the offspring from maternal diabetes intrauterine programmed hypertension and kidney injury. Male offspring from two transgenic (Tg) lines were used in this study: Hoxb7-GFP-Tg as controls, and Hoxb7/CAT-GFP-Tg, which overexpress CAT in their RPTCs.

Diabetes was induced in pregnant dams with STZ to create maternal diabetes murine model. We examined nephrogenesis in mouse embryonic kidney on embryonic day E15 and measured parameters such as systolic blood pressure (SBP), glomerular filtration rate (GFR) and kidney injury, as well as generation of ROS in adult. To delineate the underlying mechanism involved, we tested renal gene expression of TGF-β1 (a marker of kidney fibrosis), nuclear factor-erythroid 2p45 (NF-E2) related factor-2 (Nrf2, a transcription factor of antioxidant genes), and heme oxygenase (HO) -1 (an antioxidant enzyme), both *in vitro* and *in vivo*.

We observed renal dysmorphogenesis in the offspring from diabetic Hoxb7-GFP-Tg dams. At adulthood, these offspring developed hypertension, generated higher ROS in their kidneys, as well as increased renal TGF-β1 protein expression. We found that those observed alterations were improved in the offspring of diabetic Hoxb7/Cat-GFP-Tg dams that overexpressing CAT in their RPTCs. The underlying mechanism(s) is via activation of the offspring's renal Nrf2-HO-1 defense system. Both protein and gene expression of Nrf2 and its downstream target HO-1 were observed to be upregulated in RPTCs of the offspring, and Nrf2 protein was translocated normally into the nuclei of these cells, indicating that it was both activated and functional. The *in vitro* study of CAT-treated cultured RPTCs also induced Nrf2 and HO-1 protein expression, and promoted Nrf2 nuclear translocation. In conclusion, in our maternal diabetes murine model, we demonstrated that overexpression of CAT in RPTCs, improved maternal diabetes-programmed hypertension and kidney injury in the adult offspring, at least in part, by activating their kidney Nrf2-HO-1 defense system.

4.2 Maternal diabetes intrauterine environment causes elevated ROS in the kidney, impairs kidney development and leads to perinatal programing of hypertension and kidney injury in the offspring

Oxidative stress, via elevated ROS, is the major cause of adverse events in the kidneys of human and mouse infants/pups of diabetic mothers [48]. Fetal growth relies on maternal nutrient availability and the placenta's ability to transport nutrients from the maternal circulation to the fetus. Maternal blood does not have direct contact with fetal blood in the placenta. Therefore, the transporters, gradients and diffusion channels for maternal nutrients to exchange across the placenta are very important [293]. Glucose is the primary energy substrate required for fetal growth because fetal gluconeogenesis is minimal [294]. Transplacental passage of glucose transport is accomplished by facilitated diffusion through glucose transporter proteins (GLUTs) [295]. Pregnancy represents a natural state of maternal insulin resistance and this facilitates the maternal-fetal glucose concentration gradient and fetal glucose uptake [296]. In the case of maternal diabetes, high glucose from the mother is transmitted to the fetus constantly, causing an unbalanced redox environment in the developing fetus [136]. Excess glucose in the fetus generates increased ROS, which directly impairs nephrogenesis in the offspring, reducing their nephron number and kidney size [48, 136].

Previous studies from our lab using the Hoxb7-GFP-Tg maternal diabetes mouse model demonstrated that increased ROS due to high glucose negatively affects nephrogenesis, both *in vivo* and *ex vivo* [138]. Also maternal diabetes induces perinatal programming of hypertension, kidney injury, glucose intolerance and the activation of the intrarenal RAS gene expression in the offspring [135]. The present study continued this work, and showed that elevated ROS in the kidney of fetuses from diabetic mouse dams, caused retarded nephrogenesis and reduced nephron numbers in their pups, as well as perinatal programmed hypertension and kidney injury in the adult offspring. We demonstrated that overexpression of CAT in the offspring's RPTCs prevents the reduction in nephron number in the embryonic stage and the development of hypertension and kidney injury in the adult offspring.

4.3 Overexpressed CAT in the offspring's RPTCs can normalize reduced nephron number in mouse offspring exposed to maternal diabetes *in utero*

In our previous study of adult *db/db* mice that develop diabetes spontaneously, we reported that the overexpression of CAT in their RPTCs had therapeutic effects on renal complications [290, 297]. Eriksson *et al* assessed *ex vivo* cultures of rat embryos (at day E9) from diabetic dams, to delineate the relationship between ROS and teratogenic potential during embryonic life. They found that catalase treatment of the cultured embryos reduced the frequency of malformed embryos [4]. Since the ROS is the primary mediator of maternal diabetes intrauterine programmed hypertension and kidney injury, we hypothesized that overexpression of CAT in fetal and adult offspring RPTCs could inhibit excess ROS production and prevent perinatal programmed hypertension and kidney injury.

In our mouse model of STZ-induced maternal diabetes, we found that Hoxb7-GFP-Tg fetal kidney development in a high glucose intrauterine milieu, showed reduced green fluorescent GFP expression in ureteric bud (UB) tips by 40% ($n=5\text{-}11$; $p<0.001$) on day E15. In contrast, the Hoxb7/Cat-GFP-Tg offspring of diabetic dams had normal UB branching and UB tip numbers. Similarly, morphologic examination showed that the Hoxb7-GFP-Tg newborns had kidneys with significantly reduced nephron numbers (40%; $n=7\text{-}9$; $p<0.001$) and kidney size, whereas the Hoxb7/Cat-GFP-Tg offspring had normal kidneys.

Overexpressed CAT in offspring RPTCs of diabetic dams can improve glomerulogenesis. CAT was overexpressed in tubular cells locally, so it was not clear how this could normalize the reduced glomeruli (nephron numbers) and smaller kidney size that were induced by the hyperglycemic environment *in utero*. The explanation is that during nephrogenesis, renal tubules (though proximal to distal tubules) and glomeruli, arise from the same group of ancestor cells [298]. Due to this close spatial and temporal proximity, it is likely that the overexpressed CAT in RPTCs could also influence glomeruli development.

The expression and regulation of the CAT transgene (driven by kidney androgen-regulated protein (KAP) promoter) appears to be normally regulated and functional in fetal Tg

mouse RPTCs. Our results indicated that the CAT transgene was expressed normally during embryonic development, since its expression reversed the abnormal nephrogenesis phenotype induced by increased ROS in the offspring of diabetic dams. The neonatal kidneys examined by IHC showed very high CAT expression in the RPTCs of Hoxb7/Cat-GFP-Tg mice compare to the Hoxb7-GFP-Tg controls.

KAP promoter is reported to be regulated by sex steroids and KAP protein is expressed specifically in the epithelial cells of RPTCs [212]. In our transgenic mice, we showed that the KAP promoter starts to be induced earlier than day E15, on which we observed the reversal of the nephron number reduction caused by oxidative stress. Thus, it appears that the KAP promoter can be induced in embryos, and not only in adult male mice. Consistent with our finding, Kasik *et al* screened KAP mRNA expression in mouse fetuses, and found that KAP is abundant and highly expressed specifically in the fetal kidney during the latter third of pregnancy, indicating that the KAP promoter is functional during at this time [299].

4.4 Overexpressed CAT in the offspring's RPTCs can prevent hypertension and kidney injury in mouse offspring exposed to maternal diabetes *in utero*

After characterizing our offspring of diabetic dams in Hoxb7/Cat-GFP double transgenic mouse model, we confirmed that the offspring's altered nephrogenesis was due to the intrauterine hyperglycemia environment and was able to be normalized by overexpression of CAT in their RPTCs. We next focused on the development of hypertension and kidney injury in the adult offspring. We performed a longitudinal study of systolic blood pressure (SBP), from age 8 to 20 weeks. Also we did kidney physiology and pathology examinations at age 20 weeks. We found that the offspring of diabetic dams developed: 1) hypertension from age 8 weeks and reduced kidney function at age 20 weeks, characterized by renal hyperfiltration and increased ACR; and, 2) kidney pathology, characterized by glomerular expansion and the accumulation of TGFb1 (a marker for inflammation), in the interstitial compartment. In this mouse model, overexpression of CAT specifically in the offspring's RPTCs prevented the reduction of nephron number and subsequent development of the adverse effects (hypertension and kidney pathology) caused by high glucose exposure *in utero*.

4.5 20-week-old adult offspring from diabetic dams have high RPTC ROS

Our *ex vivo* studies showed that adult offspring of diabetic dams (age 20 weeks) had high levels of ROS in their RPTCs, compared to control adult offspring of non-diabetic dams. This was remarkable because the adult offspring of diabetic dams had only been exposed to the hyperglycemic environment *in utero* for about 6 days just prior to birth, and then were separated from their dams after weaning (age 3 weeks). They had normal glycaemia, and their kidney/body weight ratios were also normal, indicating no kidney hypertrophy. The explanation of their sustained RPTCs ROS is that their subnormal nephron number (caused by hyperglycemia *in utero*) was not sufficient to handle the workload. Over time, the RPTCs became damaged and generated excess ROS, which contributed to their programmed late-onset hypertension and kidney injury eventually.

4.6 The underlying mechanism: role of the renal Nrf2-HO-1 defense system in offspring born to diabetic dams

The renal protection function of the Nrf2-HO-1 defense was reported in studies of various kidney disease models [183, 300, 301]. Constant exposure to a high glucose environment *in utero* can impair Nrf2 function, reduce its downstream gene expression, and lead to pathology changes in many cell types of offspring from diabetic mothers (reviewed by Chapple *et al* [302]). For example, a proteomic analysis on epithelial cells isolated from human umbilical cords of infants from normal and GDM pregnancies (n=44-55), reported that the proteins involved in redox homeostasis were significantly altered in the offspring of diabetic pregnancies and associated with increased mitochondrial superoxide generation, protein oxidation and DNA damage. Furthermore, Nrf2 mRNA, protein levels, and nuclear translocation failed to respond to a Nrf2 stimulant [303].

We hypothesized that the Nrf2-HO-1 defense system plays important role in our offspring of maternal diabetes mice model. To delineate the underlying mechanism mediated by Nrf2, we examined Nrf2 and HO-1 expression *in vivo* and *in vitro* of 20 weeks old offspring. In our kidney IHC staining, we observed increased Nrf2 protein expression as well as enhanced Nrf2 nuclear translocation in offspring from diabetic dams compared to offspring of normal glycemic dams, along with increased HO-1 expression. Real-time PCR results confirmed that Nrf2 and HO-1 mRNA levels were increased in the kidneys of

offspring of diabetic dams. These data suggested that the kidney Nrf2-HO-1 defense system is activated more strongly in adult offspring of diabetic dams, compared to control offspring of normal glycemic dams.

However, as mentioned earlier, the offspring of diabetic dams showed increased ROS in their RPTCs while their kidney Nrf2-HO-1 defense system was activated at age 20 weeks, and this antioxidant defense system was suppose to protect the kidney from generating excess ROS. The possible explanation for why this activated Nrf2-HO-1 defense system failed to normalize the excess kidney ROS observed in our offspring of diabetic dams is that although the antioxidant defense system is functional, it is not able to counterbalance the greatly increased amount of ROS production due to decreased nephron number and increased workload, so the excess ROS accumulates in the kidney and causes progressive kidney injury.

We stimulated IRPTC with high glucose (25mM) for 24 hours in vitro to see the response of Nrf2. Western blots showed that Nrf2 protein expression was elevated in these cells in response to high glucose. Normal Nrf2 nuclear translocation was confirmed by western blots of separated nuclei and cytoplasm of the IRPTC. Immunofluorescence staining showed that more Nrf2 remained in the nuclei vs. the cytoplasm with high glucose compared to normal glucose (5mM). Furthermore, we treated IRPTC with CAT and found that CAT could promote Nrf2 entering the nuclei. This is consistent with our *in vivo* observations and suggests although Nrf2 is an upstream activator of CAT, overexpressed CAT can trigger Nrf2 nuclear translocation and therefore induces its target gene HO-1 expression.

4.7 Could CAT be used in the clinic to prevent offspring of diabetic mothers from developing hypertension and kidney injury?

The role of Nrf2 in the kidney has been extensively studied because oxidative stress is a critical mediator of progressive CKD (i.e. glomerulosclerosis and diabetic nephropathy) [141]. It has been shown that in diseased kidneys, Nrf2 activity is impaired and its downstream target gene expression is reduced [183, 304, 305]. Therefore, administration of an Nrf2 activator is a potential solution for the treatment of CKD that has been reported

to be effective in animal studies [306]. The current experimental and clinical strategy for ROS-mediated diseases is to activate the Nrf2 signaling pathway using Nrf2 activator compounds, which bind to the inhibitory protein Keap1, releasing its partner Nrf2 to translocate into the nucleus [307]. Nrf2 then induces transcription of multiple antioxidant genes that protect the cells.

Bardoxolone methyl, a synthetic Nrf2 activator, was tested in 2011 in a small clinical trial (the US phase 2b BEAM study; n=227), in patients with stage II diabetic nephropathy. It showed efficacy in attenuating the progression of nephropathy and improved estimated glomerular filtration rate (eGFR) [308]. The international BEACON trial in 2013 tested the same compound in patients with T2DM and stage 4 CKD (n=2185) and was terminated due to increased adverse effects and mortality rate (96 patients in the bardoxolone methyl group were hospitalized or died from heart failure as compared with 55 in the placebo group) [309]. Research is focussed on discovering novel inducers without these undesirable side effects.

What is the possible reason(s) lead to failure of the bardoxolone methyl trial? Evidence from animal studies suggested that the Nrf2 activator also induced other adverse pathways at the same time. For instance, a study investigated the effect of dh404, which is a derivative of bardoxolone methyl, on the kidney of STZ-induced diabetic apolipoprotein $E^{-/-}$ mouse model, showed that low dose dh404 reduced urinary ACR, glomerular injury and kidney injury. High dose dh404 stimulated the production of proinflammation mediators in the kidney, such as MCP-1 and the p65 subunit of NF-kB [306]. A proteomic screen of the human embryonic kidney 293 (HEK293) cell line which was stimulated by CDDO-Im (an analog of bardoxolone methyl and known Nrf2 activator), identified 577 target proteins, including mammalian target of rapamycin (mTOR) and other transcription factors [310]. These findings revealed the complexity of the Nrf2 downstream network, and the possibility that Nrf2 activators have unwanted consequence due to this fact.

Since overexpression of CAT in the kidneys is beneficial to offspring of diabetic dams, and the CAT gene is downstream of Nrf2, the idea to administer a Nrf2 activator to offspring to prevent hypertension and kidney injury seems to be feasible. However, the failure of

bardoxolone methyl trial suggests great challenges and more studies need to be engaged in the future.

4.8 Other organs affected by maternal diabetes perinatal programming

As mentioned earlier in Introduction, epidemiologic studies showed a strong correlation between the presence of diabetes during pregnancy and increased rates of adulthood onset T2D, obese and CVD in children born to diabetic mothers, compared to non-diabetic mothers. Therefore, organs that are related to energy metabolism, cardiovascular and insulin signaling pathways are likely affected by hyperglycemic intrauterine environment. Experimental studies of intrauterine hyperglycemic impact on various organs are described bellow.

4.8.1 Liver

The liver has a central role in regulating global energy metabolism, including glucose synthesis, lipid metabolism, and ketogenesis [311]. Excessive hepatic triacylglycerol (TG) accumulation can result in non-alcoholic fatty liver disease (NAFLD), which is associated with the development of insulin resistance [312]. It has shown that the liver TG content of adolescents correlated with insulin sensitivity, independent of whole body and visceral fat mass [313], hence the effects of maternal overnutrition or diabetes induced perinatal programming on the liver of the offspring could be a significant determinant of insulin sensitivity in youth [314].

Excess accumulation of hepatic lipids may lead to the formation of intracellular lipid droplets in cytoplasm, which is called microsteatosis. Moreover, severe lipid accumulation may result in larger hepatocellular lipid droplets in the nucleus, termed macrosteatosis. Experimental study using insulin receptor substrate-1 haploinsufficient (IRS1) mice, which displayed hyperinsulinemia and overall insulin resistance while maintaining normal blood glucose levels throughout pregnancy as maternal hyperglycemic model. The author observed that maternal insulin resistance increased liver fat accumulation in the male offspring compared to WT control male offspring, despite no changes in adiposity between the groups [315].

4.8.2 Heart

One study using STZ-induced maternal prenatal diabetes rat model demonstrated that the maternal environment has programming effects directly on the cardiovascular system of the offspring. Although the fetus had no cardiac malformations, cardiac hypertrophy at adulthood has been reported in the offspring of STZ-diabetic dams [316]. A similar study using same model suggests that perinatal development in a hyperglycemic intrauterine milieu does not result in hypertension in early adulthood but leads to subtle changes in the cardiovascular system which may predispose to overt cardiovascular disease [317]. In an STZ-induced mild pregestational diabetes rat model, the author found that the fetus accumulates lipids in different fetal heart [318].

4.8.3 Brain

The developing brain is susceptible to the damaging effects of metabolic disturbances throughout pregnancy. Study showed that inducing maternal diabetes by administration of STZ to pregnant dams could cause increased food intake in the offspring [319]. The result appeared to suggest that maternal hyperglycemia is a factor that can program feeding behavior in the offspring. However, unlike rodents which develop leptin-mediated feeding circuit postnatally, in primates it occurs *in utero* [320]. That explains maternal influences during the suckling period are also important factors driving the effects on food consumption in the rodent offspring. For example, cross-fostering the offspring of obese dams by lean dams prevented elevated caloric intake [321].

Article 2

4.9 Summary

In this study we investigated the role of Aquaporin 1 (AQP1), a major water channel in renal proximal tubular cells (RPTCs) of the kidney, in the development of angiotensinogen (Agt)/angiotensin II (Ang II)-induced hypertension and renal injury. We used two complementary experimental systems: transgenic mice overexpressing rat Agt (rAgt-Tg mice) in their RPTCs driven by kidney androgen-regulated protein (KAP) promoter [322], and cultured immortalized rat renal proximal tubule cells (IRPTCs) that overexpress (rAgt)

by stable transfection pRSV/rAgt plasmid [323]. Compared to their non-transgenic littermates, rAgt-Tg mice develop hypertension and kidney injury, was prevented by dual RAS blockade (losartan plus perindopril). The smooth muscle relaxant, hydralazine normalized blood pressure without affecting albumin/creatinine ration (ACR), a biomarker for kidney injury. We observed that both renal AQP1 and heme oxygenase (HO) -1 were decreased, and renal Nrf2 and sodium transporter NHE3 were increased, in both experimental systems, and these changes were reversed by RAS dual blockade. To understand why elevated Nrf2 downregulates HO-1 expression and how overexpressed rAgt downregulated AQP1 expression, we examined a candidate gene that possibly is a mediator of Nrf2 translocation (GSK3β).

The present study identified a novel mechanism for intrarenal Agt-induced hypertension and kidney injury: overexpression of Agt in RPTCs causes Nrf2 cytosolic accumulation by initiating the GSK3β pathway that exports Nrf2 from the nucleus to the cytosol. Nrf2 is an upstream transcription factor of HO-1, thus a deficiency of Nrf2 in the nucleus causes decreased HO-1 expression and downregulates AQP1. Reduced AQP1 is associated with β-catenin-mediated kidney damage. In conclusion, our data suggest that decreased AQP1 and HO-1 expression in renal proximal tubules induced by excess Agt and activated intrarenal RAS, plays a role in the progression of hypertension and kidney injury.

4.10 Characterization of the rAgt-Tg mouse model and determination of drug treatment

In transgenic mice that overexpress rat Agt (rAgt), immunohistochemistry (IHC) staining revealed that the rAgt was specifically overexpressed in RPTCs. ELISA tests confirmed that the levels of Agt and Ang II were above normal in the urine, and normal in the serum, and indicated that the rAgt was correctly cleaved by the RAS of mouse kidney. In these mice, the rAgt (along with the endogenous mouse Agt) was activated and fully functional, as their blood pressure was on average higher by approximately 10 mm Hg compare to non-Tg littermates. The rAgt-Tg mice had a high urinary ACR, revealing a loss of kidney function compare to non-Tg littermates. Renal morphological staining such as PAS and Masson trichrome of the transgenic mouse kidney revealed kidney structure changes that are associated with loss of kidney function, including glomerular expansion, proximal

tubule structural damage and dilation, interstitial accumulation of inflammatory factor TGF-β1, as well as collagen IV, a biomarker for renal fibrosis. An increase in renal ROS is associated with renal RAS activation in kidney pathology progression [324, 325]. Thus we hypothesize that increased ROS plays a role in Agt-mediated AQP1 downregulation. We therefore examined catalase (CAT) expression (an antioxidant enzyme) in the rAgt-Tg mouse kidney by IHC, and found the expression of CAT is lower compare to non-Tg littermates, therefore confirming an impairment in the ROS defense system.

The effect of administration of losartan (ARB) (losartan 30 mg/ kg/day) alone or in combination with perindopril (ACEI) (losartan 30 mg/ kg/day plus perindopril 4 mg/kg/day) in adult rAgt-Tg mice, from week 13- 20 of age, was assessed. The treatment partially or largely ameliorated the urinary rAgt and Ang II concentrations, and had no effect on their serum levels. Blood pressure measurement indicated that RAS dual blockade treatment lowered the elevated rAgt-Tg mouse blood pressure to non-Tg littermates level. The CAT IHC showed that RAS dual blockade treatment normalized kidney CAT level, suggesting that it repaired the defective antioxidant defense mechanism. Histology examination showed that RAS dual blockade treatment restored kidney morphology and inhibited the characteristic progression of kidney injury seen in the rAgt-Tg mouse model. Hence the RAS dual blockade treatment was found to be the most effective and was used in subsequent experiments.

To distinguish the effect of blockade of the RAS signaling pathway from that of lowered blood pressure, we treated adult rAgt-Tg mice (13-20 weeks old) with the smooth muscle relaxant hydralazine (15mg/kg/day), which normalized blood pressure but did not restore renal function (the elevated ACR remained unchanged). These results clarified that in the rAgt-Tg mouse model, the rAgt, which is overexpressed specifically in mouse RPTCs, is the major mediator of hypertension and kidney pathology, via alteration of kidney structure and physiology. This experiment ruled out the possibility that the increased rAgt-Tg mouse blood pressure *per se* was a significant contributor to the kidney injury observed in this mouse model.

4.11 Mechanisms involved in AngII-regulated AQP1 expression

4.11.1 Results of the rAgt-Tg mouse study

We studied the underlying mechanism of how excess of Ang II in the kidney causes a decrease in AQP1, using the rAgt-Tg mouse model. AQP1, as described in my thesis Introduction, is an essential kidney water channel, present in plasma membranes of the proximal tubules and descending limb of the loop of Henle [252, 253]. AQP1 expression was found to be reduced in rAgt-Tg mouse kidneys. We previously showed that the ROS defense system is impaired in rAgt-Tg mice. We decided to examine renal HO-1 and Nrf2 expression in more detail. First, we confirmed that AQP1 and HO-1 expression are downregulated in the kidneys of 20-week-old rAgt-Tg mice, and this can be rescued by treatment with the RAS dual blockade described in the previous section. To determine whether the reduction of HO-1 is due to inactivation of its upstream regulator, we analyzed Nrf2 expression (a master regulator of the antioxidant response which relieves oxidative stress) and its associate protein Keap1, an Nrf2 repressor, which binds to and anchors Nrf2 in the cytoplasm, facilitating its ubiquitination and proteolysis.

We observed that Nrf2 was induced but remained in the cytoplasm of the rAgt-Tg mouse kidney cells, and was not translocated into the nucleus as normally occurs in non-Tg littermates. Insufficient nuclear Nrf2 could be causing reduced HO-1 expression, which would be expected if Keap1 protein expression were increased, however, Keap1 levels were normal. This result provides evidence for our hypothesis that Nrf2 translocation from the cytoplasm into the nucleus is defective in this mouse model, perhaps because another (as yet unidentified) Nrf2 regulation pathway exists which leads to reduced HO-1 expression, or the Keap1-Nrf2 interaction is more complex and requires additional factors and/or a Nrf2 conformational change which may be missing and required for translocation into the nucleus.

Glycogen synthase kinase (GSK)3β is a serine/threonine protein kinase involved in many different signaling pathways, including glycogen metabolism, gene transcription, apoptosis and microtubule stability [326-329]. We tested our hypothesis that GSK3β plays a role in Nrf2 translocation by studying GSK3β expression in our rAgt-Tg mouse model. We confirmed the presence of two forms of phosphorylated GSK3β by western blot on isolated

renal proximal tubules: 1) S9 (the inactivate form), which stabilizes Nrf2 in the cytoplasm; and, 2) Y216 (the active form), which is responsible for Nrf2 export from the nucleus to the cytoplasm. We found that while total GSK3β protein levels remained unchanged, there was a decrease in the inactive form and an increase in the inactive form of GSK3β in rAgt-Tg mouse RPTCs compare to non-Tg littermates. This result also provides support for our earlier hypothesis that Nrf2 transport into the nucleus is defective in kidney proximal tubules of rAgt-Tg mice.

Sodium-hydrogen exchanger 3 (NHE3) is the major apical sodium transporter in the RPTCs that controls sodium reabsorption [246]. We found by western blotting that in rAgt-Tg mouse RPTCs, NHE3 protein expression was increased compared to non-Tg littermates. Furthermore, by immunofluorescence staining, we found that NHE3 is colocalized with AQP1. These data suggest our Agt-induced hypertension hypothesis that overexpression of rAgt in RPTCs leads to decreased water absorption via AQP1 and increased sodium reabsorption via NHE3, which together likely causes hypertension.

4.11.2 Results of the *in vitro* studies

We employed for our *in vitro* experiments three stable clonal cell lines: 1) IRPTC, a rat renal proximal tubule cell line immortalized by SV40 DNA [208]; 2) pRC/RSV plasmid that contains rAgt cDNA stably transected into IRPTC (pRSV/rAgt-IRPTC); and, 3) control pRC/RSV plasmid stably transfected into IRPTC (pRSV-IRPTC) [323]. We characterized these cell lines by detecting rAgt, Nrf2, Keap1 and AQP1 protein expression in western blots. rAgt was overexpressed in pRSV/rAgt-IRPTC, as expected. Consistence with the experiments described on RPTCs of rAgt-Tg mice, Nrf2 and AQP1 levels were found to be decreased, while Keap1 stayed unchanged, in pRSV/rAgt-IRPTC compared to IRPTC and pRC/RSV-IRPTC. We found AQP1 was express predominantly at cell membranes in IRPTC by immunofluorescence staining, and was dramatically decreased in pRSV/rAgt-IRPTC. The results supported our hypothesis that Agt plays a role downregulating AQP1 expression.

To clarify if the reduced HO-1 expression in our rAgt-Tg mouse model leads to decreased AQP1 expression, we stimulated HO-1 protein expression *in vitro* using CoPP, a chemical

inducer of HO-1. We found that CoPP restored AQP1 protein expression in a dose-dependent manner. Induction of HO-1 by CoPP also inhibited rAgt expression in IRPTC, while Nrf2 expression remained unchanged. These results show that HO-1 plays a role on AQP1 induction.

Nrf2 cytosolic accumulation was observed in RPTCs of rAgt-Tg mice, and this was further confirmed in pRSV/rAgt-IRPTC and pRSV-IRPTC cells by separating the cytosolic and nuclear fractions. Overexpression of Agt in IRPTC indeed caused Nrf2 to be retained in the cytosol, possibly via the GSK3β export pathway. NHE3 expression was stimulated when Ang II concentration was high, consistent with our observations *in vivo*.

β-catenin was reported as a transcriptional co-regulator candidate factor that mediates Ang II-induced kidney injury via an as yet unidentified mechanism (detailed in following section). We hypothesized that β-catenin may be involve in the process of Ang II regulated AQP1 expression, and therefore examined β-catenin status in IRPTC. We found that the phosphorylated form of β-catenin was decreased in the total cell lysate of pRSV/rAgt-IRPTC, indicating that high levels of rAgt caused activation of the Wnt/β-catenin pathway.

4.12 Nrf2 cytoplasmic accumulation of pRSV/rAgt-IRPTC and RPTC of rAgt-Tg mice

The Keap1-Nrf2-ARE pathway is the main pathway that responds to and reduces oxidative stress, via activating a large number of antioxidant genes such as HO-1, NQO1 and catalase [180, 181]. Emerging evidence has indicated an alternative regulatory mechanism involved in Nrf2 signaling that is independent of Keap1, named by the glycogen synthase kinase 3β (GSK3β)/Fyn pathway [274, 330]. Briefly, activated GSK3β (that is phosphorylated at tyrosine 216) phosphorylates Nrf2 at tyrosine 568 and/or regulates it indirectly through phosphorylation of Fyn kinase. This leads to nuclear localization of Fyn, which further phosphorylates Nrf2 on tyrosine 568, resulting in the export of Nrf2 from the nucleus back to the cytoplasm [274, 331]. Conversely, inactivation of GSK3β by the inhibitory phosphorylation of GSK3β at serine 9 leads to Nrf2 nuclear accumulation.

Overexpression of rAgt in cells and kidney is expected to increase the level of AngII and activate AT1R signaling, thus inducing downstream reactions, such as inflammation and oxidative stress. In response to increased cellular ROS, Nrf2 is assumed to disassociate with KEAP-1 and translocate into the nucleus. In the current study, in RPTCs both *in vivo* and *in vitro*, we did not detect any changes in the level of Keap1. Nrf2 remained in the cytoplasm and failed to translocate to the nucleus. Thus, we hypothesize that GSK3β mediates Nrf2 accumulation in the cytoplasm. Indeed, we detected the upregulation of the active form phosphorylate GSK3β Y216 in the cytoplasm, which exports Nrf2 out of the nucleus, and downregulation of the inactive form phosphorylate GSK3βS9, in both rAgt-Tg mice and the pRSV/rAgt-IRPTC stable clone.

4.13 β-catenin is a mediator of Agt-induced kidney injury – *in vivo* results from literatures match our *in vitro* finding

We showed that overexpressed rAgt in the rat kidney causes hypertension and kidney injury and are partly due to decreased AQP1, probably mediated by the β-catenin signaling pathway. The effects of the activated kidney RAS on hypertension and kidney injury have been widely studied [207, 332]. In our study, we discovered a novel pathway by which the renal RAS elicits hypertension and kidney injury.

The Wnt/β-catenin signaling cascade plays a pivotal role during kidney development [276, 333] and is nearly silenced in adulthood under normal conditions. Recently, activation of the canonical Wnt/β-catenin pathway was observed in many different types of renal pathology [280, 334, 335]. When this pathway is activated, β-catenin, a co-transcriptional factor, translocates into the nucleus and cis-acts on its target genes, which promote cell proliferation and differentiation [336]. Without Wnt signaling, β-catenin stays in the cytoplasm and is phosphorylated by multiple kinases, including GSK3β. Recruiting and binding to other components, the complex targets β-catenin for degradation. Thus, decreased GSK3β, or dissociation of β-catenin with the destructive structure, prevents phosphorylation and degradation of β-catenin and results in Wnt pathway activation.

In disease conditions, the renal RAS is activated in parallel with the Wnt/β-catenin pathway. For example, study of Zhou *et al* using the adriamycin (ADR)-induced

nephropathy model found multiple RAS genes upregulated (i.e. renin, Ang, ACE, AT1R). Giving pharmacological blockade of the Wnt/β-catenin pathway suppressed RAS gene induction and ameliorated kidney injury of ADR mice [337]. In contrast, other studies suggest that activated RAS leading to proteinuria and kidney injury is regulated by the upstream regulator Wnt/β-catenin pathway [338]. Taken together, those evidences show that crosstalk between the RAS and Wnt/β-catenin pathways occur.

AQP1 also plays a role in the Wnt/β-catenin pathway that causes kidney damage. Wang *et al* found that in a mouse model of polycystic kidney disease, membrane-bound AQP1 could retard cyst formation by interacting with multiple elements, include GSK3β and phosphorylated β-catenin, to form a destruction complex. The complex is finally targeted for degradation by proteasomes, leading to inhibition of Wnt signaling [281]. This finding is consistent with our observations in conditions of rAgt overexpression (pRSV/rAgt-IRPTC), where AQP1 is markedly downregulated. With the presence of very low levels of AQP1, β-catenin is dephosphorylated, and this likely causes activation of the Wnt signaling pathway, which contributes to kidney damage.

4.14 The role of NHE3 in Agt-induced hypertension

NHE3 is thought to be the major cotransporter present in kidney proximal tubules and the loop of Henle [339, 340]. The function of NHE3 is to secret H^+ out of the tubule cell into the lumen, and exchange sodium absorbed from the lumen into the tubule cell on its apical site, contributing approximately 75% of sodium reabsorption [341]. In a recent study, Li *et al* reported that NHE3 is essential for developing Ang II-dependent hypertension [342]. By infusing Ang II into mice with normal or mutant NHE3 expression in the kidney, they observed that the mouse blood pressure was elevate only slightly in the kidney-NHE3 null mice compare to WT mice. We also observe increased NHE3 in the proximal tubules of our rAgt-Tg mice. Taken together, the results suggest that increased NHE3 and sodium reabsorption may be an important factor that leads to hypertension, and if AQP1 expression does not increase coordinately, this enhances the development hypertension.

4.15 How does HO-1 regulate AQP1 expression?

We observed in IRPTC that HO-1 induction in vitro upregulates AQP1 protein expression.

Our finding is consistent with a study on H_2O_2-induced oxidative damage of rat alveolar epithelial cells (a primary culture isolated from lung tissue), which found that AQP1 expression was significantly reduced in the H_2O_2-injured group and apoptosis rate was increased. Administration of HO-1 recombinant protein protected the rat alveolar epithelial cells from apoptosis and upregulated AQP1 expression, suggesting that HO-1 increases AQP1 expression in H_2O_2-injured cells due to its antioxidant property [343]. In our present study, it is likely that decreased AQP1 expression, both in rAgt-Tg mice proximal tubules and pRSV/rAgt-IRPTC, is due to insufficient HO-1 triggered by the defect of nuclear Nrf2 translocation. Whether the AQP1 stimulation effect of CoPP is through oxidative stress clearance by HO-1 or another pathway remains unknown and needs further experiments to answer the question.

4.16 Glycosylation of AQP1

In our western blot experiments using antibodies directed against AQP1, we always detected two proteins: one of 28 kDa, which matches its estimated molecule weight, and a second protein around 35-40 kDa, which is thought to be corresponding to the glycosylated form. Many renal AQPs contain a N-link consensus glycosylation site and some have been investigated [344]. For instance, mutation in AQP2 has been shown to lead to nephrogenetic diabetes insipidus, due to a defect in covalent modification during N-linked glycosylation [345]. However, whether glycosylation can affect the activity of AQP1, or has other functional significance, is currently unknown. A study on cardiomyocytes performed enzymatic deglycosylation and successfully eliminated the upper 35-40 kDa AQP1 protein, suggesting that the lower 28 kDa protein is glycan-free AQP1 [346]. AQP1 was first characterized as a N-proteoglycan [347], however, removal of its glycosyl group did not affect its ability to assemble into tetramers or affect its water transport efficiency [348]. We assumed that glycosylation increases the stability and half-life of AQP1. In our rAgt-Tg RPTCs preparations and in the pRSV/rAgt-IRPTC, both the unmodified as well as glycosylated form of AQP1 were found to be be decreased by western blotting, indicating there may be the existence of additional mechanisms that regulate AQP1 expression by modulating the speed of its degradation and/or expression.

4.17 Dramatic decrease of AQP1 in pRSV/rAgt-IRPTC but modest decrease of AQP1 in rAgt-Tg mice RPTC

Interestingly, comparing naive IRPTC (having normal rAgt expression) with a stable cell line that overexpresses rAgt (pRSV/rAgt-IRPTC), we observed dramatic decreased AQP1 in the latter. In contrast, the difference between RPTCs' AQP1 protein expression in mice that overexpress rAgt vs. non-Tg littermates is relatively smaller. The explanation may be due to the divergence of single cell colony *(in vitro)* or system crosstalk (*in vivo*). This is difficult to study because unlike kidney tissue, our IRPTC cell line only expresses AQP1 and not AQP2. This could be explained if the AQP1 and AQP2 water channel systems interact, and counterbalancing mechanisms among tubule segments exist *in vivo*.

Different levels of rAgt expression might be an explanation. In our western blot analysis, rAgt in rAgt-Tg mouse RPTCs protein extracts (data not shown) is about 2-fold higher compare to non-Tg littermates. In our *in vitro* experiments, rAgt expression was around 20-fold higher in the pRSV/rAgt-IRPTC compare to the IRPTC cell lines. This extremely high and long-term constitutive overexpression of rAgt is likely to completely suppress AQP1 protein expression in Agt/RSV-IRPTC. Using transient transfection of pRSV/rAgt plasmids into IRPTC, we found that the magnitude of the decrease in AQP1 expression was smaller.

In conclusion, in this study we identified a novel pathway of how overexpressed Agt in RPTCs induces hypertension and kidney injury. Our data suggest that high Ang II levels impaired Nrf2 function and subsequence decrease HO-1 and AQP1 expression. Reducing HO-1 and AQP1 play a role in developing hypertension and kidney injury.

CHAPTER 5: FUTURE WORK

Article1

To further study the role of ROS and Nrf2 signaling in maternal diabetes perinatal programmed hypertension and kidney injury, Nrf2KO mice is a good model to use in our following study. Postnatal variables are also known as important factors to influence offspring development during sensitive period after birth. Our lab published very recently a study regarding post weaning high fat (HF) diet can accelerating kidney injury progress in maternal diabetes model. We are interested to see if the phenotype induced by perinatal programming can be reversed in CAT-Tg offspring.

5.1 Does absence of Nrf2 in offspring kidney accelerate hypertension and kidney injury development in response to maternal diabetes condition?

Conventional Nrf2 knockout (Nrf2KO) mice that previously created and characterized by other research teams is a good model to study the role of Nrf2 in various disease conditions [349]. The embryos of Nrf2KO mice were reported to be fertilized, viable and developed normally under standard laboratory conditions, and the author concluded that Nrf2 is dispensable for mouse development. Nevertheless, the mice were found to be susceptible to environmental toxins and oxidative stress, such as carcinogens [350] and hypoxia [351] in adulthood. Parallel to our study, a number of other studies highlighted the important role of Nrf2 in protecting fetal organ development against adverse environments *in utero*, such as toxin exposure, preeclampsia and diabetes [302]. However, long-term effect of Nrf2 in perinatal programming is not clear.

In our present study, since we have demonstrated that ROS plays an important role in maternal diabetes programmed hypertension and kidney injury in offspring at adulthood, this Nrf2KO mouse model provides a good tool to deduce the role of Nrf2 in perinatal programmed hypertension and kidney injury. To study the influence of maternal hyperglycemia environment with the presence or absence of Nrf2 in offspring, Nrf2 heterozygous knockout ($Nrf2^{+/-}$) dams will be mated with $Nrf2^{+/-}$ males to generate three genotypes of offspring: $Nrf2^{+/+}$, $Nrf2^{+/-}$ and $Nrf2^{-/-}$ (Figure 5-1). Our previous STZ-induced maternal diabetes model will be adapted to these dams. As mentioned in figure 5-1, offspring of all six groups (A to F) will be studied. Blood pressure will be monitored by tail-cuff method thrice weekly from 8 weeks until 18 weeks of age. GFR will

be estimated in 20 week-old offspring prior to sacrifice. Animals will be euthanized at 20 weeks of age. Body weight and kidney weight will be recorded. Left kidney will be utilized for renal morphology assessment and IHC while the right kidney cortex will be reserved for ROS generation and gene expression experiments.

It has been reported that in STZ-induced diabetic mice, administration of Nrf2 activator, significantly decreased blood glucose levels through the improvement of insulin secretion [352]. We have no idea if maternal glycemic level will go higher in $Nrf2^{-/-}$ or $Nrf2^{+/-}$ dams compare to $Nrf2^{+/+}$ dams after induction of diabetes by STZ, and eventually enhance perinatal programming. Hence we intend to collect offspring from same genotype of dams to control the possible variation.

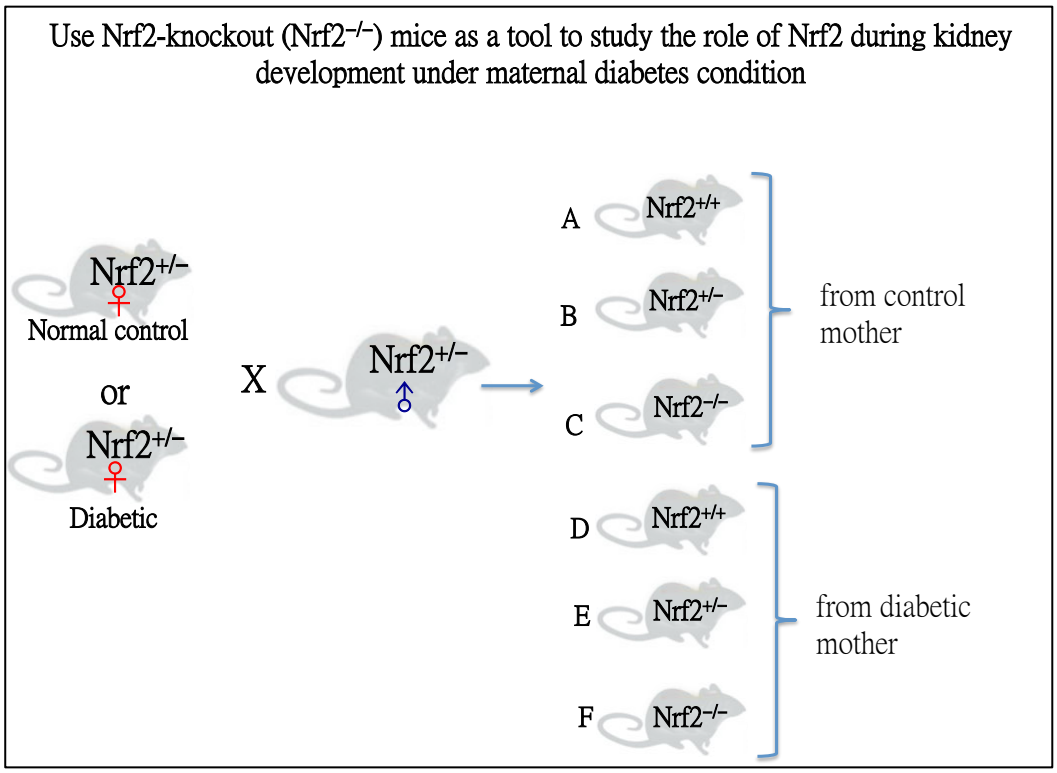

Figure 5-1: Experimental design using Nrf2KO heterozygous to create three kinds genotypes of offspring: Nrf2 WT, Nrf2KO heterozygous and Nrf2-null mice.

We hypothesize that the deficiency of Nrf2 in offspring can predispose offspring to oxidative damage under maternal hyperglycemia environment. We expect to see in $Nrf2^{-/-}$ offspring kidney, nephron number loss is exacerbated compare to $Nrf2^{+/+}$ offspring in response to maternal diabetic pregnancy during developmental stage. At adulthood, these $Nrf2^{-/-}$ offspring have greater ROS generation and develop hypertension and kidney injury. $Nrf2^{+/-}$ offspring may show an intermediate phenotype between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ offspring, or no change because only one copy of Nrf2 gene could function well. However, there is a weak point in this experimental design. This Nrf2 KO mouse model is Nrf2 ablated in all organs; include cardiovascular system, which largely involves in blood pressure regulation. Reports indicate that down-regulation Nrf2 contributes to vascular dysfunction in hypertension [353]; and maternal hyperglycemic environment is known to grogram alterations in hearts and induce cardiovascular dysfunction [317]. Therefore it is difficult to exclude the role of heart Nrf2 in the developing of maternal diabetes perinatal programmed hypertension. A kidney tissue specific Nrf2 ablation line is required to dress the role of Nrf2 in kidney in response to intrauterine hyperglycemic environment.

5.2 Does overexpression CAT in RPTCs in offspring protect or delay progression of kidney injury programed by maternal diabetes and postnatal overnutrition?

Perinatal programmed adult hypertension and kidney diseases may develop with rapid postnatal catch-up growth, which constitutes a second hit. Therefore it is important to take postnatal growth into account in experimental models of perinatal programmed adult diseases [354].

Catch-up growth refers to a period after birth in growth-restricted neonates when they accelerate growing to optimize the growth of certain organs as well as body weights [354]. A study using low protein diet maternal IUGR rat model indicated that early postnatal overfeeding can induce rapid postnatal catch-up growth, improves postnatal nephrogenesis. However, the aging male offspring manifested sustained systemic hypertension and CKD [354]. “Mismatch pathway” was proposed to explain this phenomenon: there is a mismatch between the postnatal nutritional condition and the condition that predicted by nutrient status during pregnancy, As a result, energy balance regulation in offspring is disturbed and later physical function problems related to metabolism are predisposed [355].

In our previous publication we used STZ-induced maternal diabetes model and fed the offspring with high fat diet (HFD) since 4 weeks of age, and looked into potential underlying mechanisms [356]. We observed that the offspring born to severe diabetic dams and on normal diet (ND) possessed IUGR phenotypes, as well as developed hypertension and kidney injury in adulthood. Nevertheless, IUGR offspring exposed to HFD showed rapid weight gain, catch-up growth and intense kidney injury in adulthood, accompany with increased TGFβ1, collagen type IV expression and oxidative generation in kidneys. Further data suggested HFD in a form of fatty acid increase CD36 and fatty acid–binding protein 4 (Fabp4) expression and contribute synergistically to kidney injury.

It appears ROS is one of the targets of CD36 and Fabp4 in maternal diabetes plus post-weaning HFD-induced perinatal programmed kidney injury. We can take the advantage of our CAT-Tg mice and see whether overexpression CAT in offspring RPTCs can reverse kidney injury induced by prenatal and postnatal insults. The same strategy using STZ to induce diabetes and feed offspring HFD from four to 20 weeks of age will be applied. Six groups of animal will be collected: control non-Tg; control non-Tg feed HFD; diabetic non-Tg; diabetic non-Tg feed HFD; diabetic CAT-Tg; diabetic CAT-Tg feed HFD. We expect to see that the progression of kidney injury can be attenuated by overexpression CAT in RPTCs.

We can add two more groups of animal: diabetic non-Tg feed antioxidant; diabetic non-Tg feed HFD and antioxidant, to see whether administration of antioxidant systemically (by adding in drinking water) or locally (overexpress CAT in kidney) is more beneficial. As it has been wildly studied, maternal diabetes also programmed CVD and can complicated hypertension and kidney injury eventually. Giving antioxidant systemically can degrade ROS in multiple affected organs and tissues are expected to have better result. Nonetheless, in our previous publication, we have already demonstrated that intrauterine hyperglycemic environment programmed nephron number loss via ROS generation in offspring kidney, and lead to kidney failure at adulthood. Overexpression CAT in the fetus can prevent from nephron number loss caused by high glucose milieu and may have a better shape compare to fetus of non-Tg diabetic dams. Perhaps give the antioxidant systemically as a treatment to affected offspring after the most critical window is closed, the benefit would be limited.

Article2

Our next goal is to prove our hypothesis by *in vivo* induction of the kidney Nrf2/HO-1defense system pathway to see whether it could increase kidney tubular AQP1 expression, reduce blood pressure and prevent kidney injury induced by overexpression of Agt/Ang II.

5.3 Does kidney-specific treatment with an HO-1 inducer (CoPP) normalize blood pressure and AQP1 expression in rAgt-Tg mice?

Studies of experimental hypertensive mouse models demonstrated that systemic induction of HO-1 by chemical inducers, such as hemin or CoPP, could prevent the development of hypertension [357-359]. Despite increasing production of CO, which functions as a vasodilator in vascular smooth muscle, the mechanism involved is not yet known, nor is the role of kidney HO-1 induction. Vera *et al* have developed a method of delivering HO-1 inducers specifically to kidneys using intrarenal medullary interstitial (IRMI) catheters implanted 1.5 to 2.0 mm into the left kidney [360]. Saline is infused through the catheter for a period of 3 days, followed by either CoPP (250 μg/mL; at 50 μL/h) or vehicle (0.1 NaOH pH 8.0) for 2 days. Two days later, mice were implanted with osmotic minipumps, which infused Ang II or vehicle (saline) at 1 μg/kg/minute. Blood pressure was measured by carotid artery implanted five days after Ang II infusion. The kidney-specific HO-1 induction prevented the development of Ang II-dependent hypertension and highlighted the importance of intrarenal HO-1 induction in the regulation of blood pressure. However, the underlying mechanism remains unknown.

We will use Vera's method to test kidney-specific CoPP treatment of our rAgt-Tg mice. Vera reported that two days of CoPP infusion induces high intrarenal HO-1 levels for 11 days. In our future study, we plan to implant catheters in the medullary interstitial region of rAgt-Tg mouse kidneys and those of the control non-Tg littermates. Five days after CoPP or vehicle infusion, blood pressure will be measured with the tail cuff method daily for five days. Mice will be sacrificed after 10 days since CoPP infusion. Before sacrifice, the mice will be housed in metabolic cages for 24 hours, and the amount of water and food consumed, as well as the amount of urine will be monitored. AQP1 and HO-1 expression will be examined by western blot and immunostaining. We expect that this kidney-specific

CoPP treatment will normalize both blood pressure and the rAgt-induced reduction in AQP1.

5.4 Does kidney-specific treatment with an Nrf2 activator normalize blood pressure and AQP1 expression in rAgt-Tg mice?

Since Nrf2 is an upstream transcription factor of HO-1, we hypothesize that the kidney-specific Vera method could similarly be used to test whether an Nrf2 activator may also normalize blood pressure and AQP1 expression in our rAgt-Tg mice. In these mice, transgenic rAgt is persistently expressed in their RPTCs and this may permanently change the profile of their kidney Nrf2 import/export system. The HO-1 levels of this experiment may be unchanged or increased, and Agt-induced hypertension may be maintained or reduced.

5.5 To prove that GSK3β regulate Nrf2 nuclear translocation and decreases HO-1 and AQP1 expression in rAgt-Tg mice and pRSV/rAgt-IRPTC

We detected both upregulation of the active form of phosphorylated GSK3β (Y216), which exports Nrf2 out of the nucleus into the cytoplasm, and downregulation of the inactive form phosphorylate GSK3β (S9), in both rAgt-Tg mouse RPTCs and pRSV/rAgt-IRPTC (a stable clone). We hypothesized that Agt regulates Nrf2 nuclear translocation via GSK3β. To further verify the causal relation between GSK3β and downstream consequences, we will next block the GSK3β pathway using LiCl, a chemical inhibitor of GSK3β reported by Salazar *et al* to cause nuclear accumulation and stabilization of Nrf2 in human hepatoma (HepG2) cells [274]. The same research team also found inhibition of GSK3β could increase HO-1 levels in human embryonic kidney (HEK) 293T cells [361]. These studies were only done *in vitro* and demonstrated that LiCl could prevent Nrf2 nuclear export mediated by GSK3β. We hope to demonstrate that LiCl treatment of our rAgt-Tg mice normalizes HO-1 and AQP1 expression, ameliorate blood pressure, and prevent kidney injury. *In vitro*, we plan to clarify the mechanism involved by blocking GSK3β expression using siRNA and LiCl in pRSV/rAgt-IRPTC and examine Nrf2 distribution inside these cells by immunofluorescence.

5.6 To further investigate if β-catenin interacts with AQP1 and plays a role in kidney injury progression induced by Agt overexpression

In the present study, we observed that β-catenin was dephosophorylated in pRSV/rAgt-IRPTC, which overexpresses rAgt. We hypothesized that overexpression of Agt likely causes activation of the Wnt/β-catenin signaling pathway, mediated by the failure of AQP1 to interact with β-catenin, which further contributes to kidney damage [281]. To show physiological relevance, we will next test whether administration of a Wnt/β-catenin pathway inhibitor can reverse hypertension and nephropathy in rAgt-Tg mice.

We hypothesize that AQP1 siRNA will inhibit AQP1 expression in IRPTC and will activate the Wnt/β-catenin pathway. Several genes that correlate with kidney injury were found to be Wnt/β-catenin target genes, such as Snail1, which has a key role in driving epithelial–mesenchymal transition (EMT) in glomerular podocytes; and fibronectin, a major interstitial matrix component. Other target genes of Wnt/β-catenin that were studied are Cyclin D1 and Myc, which are involved in cell cycle regulation and cell proliferation, although their role in kidney disease is not yet clear [362]. In the future, we plan to examine gene expression of all the above genes in IRPRC, to confirm if the Wnt/β-catenin pathway is activated when AQP1 is absent due to AQP1 siRNA.

CHAPTER 6: REFERENCES

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