Université de Montréal

Le Diabète Maternel Influence la Morphogenèse Rénale et la Programmation Périnatale

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Résumé

Le diabète maternel est un facteur de risque majeur pour le développement de malformations congénitales. Dans le syndrome de l'embryopathie diabétique, l'exposition prolongée du fœtus à de hautes concentrations ambientes de glucose induit des dommages qui peuvent affecter plusieurs organes, dont les reins. Les malformations rénales sont la cause de près de 40 pourcent des cas d'insuffisance rénale infantile. L'hyperglycémie constitue un environnement utérin adverse qui nuit à la néphrogenèse et peut causer l'agenèse, la dysplasie (aplasie) ou l'hypoplasie rénale. Les mécanismes moléculaires par lesquels les hautes concentrations ambientes de glucose mènent à la dysmorphogenèse et aux malformations demeurent toutefois mal définis.

Le diabète maternel prédispose aussi la progéniture au développement d'autres problèmes à l'âge adulte, tels l'hypertension, l'obésité et le diabète de type 2. Ce phénomène appelé 'programmation périnatale' a suscité l'intérêt au cours des dernières décennies, mais les mécanismes responsables demeurent mal compris.

Mes études doctorales visaient à élucider les mécanismes moléculaires par lesquels le diabète maternel ou un environnement *in utero* hyperglycémique affecte la néphrogenèse et programme par la suite la progéniture a développer de l'hypertension par des observations *in vitro*, *ex vivo* et *in vivo*. Nous avons utilisé les cellules MK4, des cellules embryonnaires du mésenchyme métanéphrique de souris, pour nos études *in vitro* et deux lignées de souris transgéniques (Tg) pour nos études *ex vivo* et *in vivo*, soient les souris HoxB7-GFP-Tg et Nephrin-CFP-Tg. Les souris HoxB7-GFP-Tg expriment la protéine fluorescente verte (GFP) dans le bourgeon urétérique (UB), sous le contrôle du promoteur HoxB7. Les souris Nephrin-CFP expriment la protéine fluorescente cyan (CFP) dans les glomérules, sous le contrôle du promoteur nephrin spécifique aux podocytes.

Nos études *in vitro* visaient à déterminer si les hautes concentrations de glucose modulent l'expression du gène Pax2 dans les cellules MK4. Les cellules MK4 ont été traitées pendant 24h avec du milieu contenant soit 5mM D-glucose et 20mM D-mannitol ou 25mM D-glucose et avec ou sans antioxydants ou inhibiteurs de p38 MAPK, p44/42 MAPK, PKC et NF-kB. Nos résultats ont démontré que le D-glucose élevé (25mM) augmente la génération des espèces réactives de l'oxygène (ROS) dans les cellules MK4 et induit spécifiquement l'expression du gène Pax2. Des analogues

du glucose tels le D-mannitol, L-glucose ou le 2-Deoxy-D-glucose n'induisent pas cette augmentation dans les cellules MK4. La stimulation de l'expression du gène Pax2 par le D-glucose dans les cellules MK4 peut être bloquée par des inhibiteurs des ROS et de NF-kB, mais pas par des inhibiteurs de p38 MAPK, p44/42 MAPK ou PKC. Ces résultats indiquent que la stimulation de l'expression du gène Pax2 par les concentrations élevées de glucose est due, au moins en partie, à la génération des ROS et l'activation de la voie de signalisation NF-kB, et non pas via les voies PKC, p38 MAPK et p44/42 MAPK.

Nos études *ex vivo* s'intéressaient aux effets d'un milieu hyperglycémique sur la morphogenèse de la ramification du bourgeon urétérique (UB). Des explants de reins embryonnaires (E12 à E18) ont été prélevés par micro-dissection de femelles HoxB7-GFP gestantes. Les explants ont ensuite été cultivés dans un milieu contenant soit 5mM D-glucose et 20mM D-mannitol ou 25mM D-glucose et avec ou sans antioxydants, catalase ou inhibiteur de PI3K/AKT pour diverses durées. Nos résultats ont démontré que le D-glucose stimule la ramification du UB de manière spécifique, et ce via l'expression du gène Pax2. Cette augmentation de la ramification et de l'expression du gène Pax2 peut être bloquée par des inhibiteurs des ROS et de PI3K/AKT. Ces études ont démontré que les hautes concentrations de glucose altèrent la morphogenèse de la ramification du UB via l'expression de Pax2. L'effet stimulant du glucose semble s'effectuer via la génération des ROS et l'activation de la voie de signalisation Akt.

Nos études *in vivo* visaient à déterminer le rôle fondamental du diabète maternel sur les défauts de morphogenèse rénale chez la progéniture. Dans notre modèle animal, le diabète maternel est induit par le streptozotocin (STZ) chez des femelles HoxB7-GFP gestantes (E13). Les souriceaux ont été étudiés à différents âges (naissants et âgés de une, deux ou trois semaines). Nous avons examiné leurs morphologie rénale, nombre de néphrons, expression génique et les événements apoptotiques lors de cette étude à court terme. La progéniture des mères diabétiques avait un plus faible poids, taille et poids des reins, et possédait des glomérules plus petits et moins de néphrons par rapport à la progéniture des mères contrôles. La dysmorphogenèse rénale observée est peut-être causée par l'augmentation de l'apoptose des cellules dans la région du glomérule. Nos résultats ont montré que les souriceaux nés de mères diabétiques possèdent plus de podocytes apoptotiques et plus de marquage contre la caspase-3 active dans leurs tubules rénaux que la

progéniture des mères contrôles. Les souriceaux des mères diabétiques montrent une augmentation de l'expression des composants du système rénine angiotensine (RAS) intrarénal comme l'angiotensinogène et la rénine, ainsi qu'une augmentation des isoformes p50 et p65 de NF-kB. Ces résultats indiquent que le diabète maternel active le RAS intrarénal et induit l'apoptose des glomérules, menant à une altération de la morphogenèse rénale de la progéniture.

En conclusion, nos études ont permis de démontrer que le glucose élevé ou l'environnement *in utero* diabétique altère la morphogenèse du UB, qui résulte en un retard dans la néphrogenèse et produit des reins plus petits. Cet effet est dû, au moins en partie, à la génération des ROS, à l'activation du RAS intrarénal et à la voie NF-kB.

Nos études futures se concentreront sur les mécanismes par lesquels le diabète maternel induit la programmation périnatale de l'hypertension chez la progéniture adulte. Cette étude à long terme porte sur trois types de progénitures : adultes nés de mères contrôles, diabétiques ou diabétiques traitées avec insuline pendant la gestation. Nous observerons la pression systolique, la morphologie rénale et l'expression de divers gènes et protéines. Nous voulons de plus déterminer si la présence d'un système antioxydant (catalase) peut protéger la progéniture des effets néfastes des ROS causés par l'environnement *in utero* hyperglycémique. Les souris Catalase-Tg expriment la catalase spécifiquement dans les tubules proximaux et nous permettrons d'explorer notre hypothèse sur le rôle des ROS dans notre modèle expérimental de diabète maternel.

Mots-clés: diabète maternel; hyperglycémie; néphrogenèse; système rénine angiotensine; Pax2; apoptose; espèces réactives de l'oxygène; programmation périnatale

Abstract

Maternal diabetes is a major risk factor for congenital malformations. When the fetus is exposed to high, sustained, ambient glucose levels, widespread fetal damage may affect multiple organs, including the kidneys, evoking diabetic embryopathy syndrome. Renal malformations account for up to 40% of childhood renal failure cases. Hyperglycemia constitutes an adverse *in utero* environment that dynamically impairs nephrogenesis, resulting in renal agenesis, dysplasia, aplasia or hypoplasia. However, the molecular mechanisms by which high, ambient glucose levels lead to renal dysmorphogenesis and birth defects have not yet been delineated.

Maternal diabetes also programs the offspring to develop other problems later in life, such as hypertension, obesity and type 2 diabetes. This phenomenon, called 'perinatal programming', has attracted worldwide attention in recent decades, yet the mechanisms by which it occurs are incompletely understood.

My PhD studies are designed to elucidate the underlying molecular pathways by which maternal diabetes or hyperglycemic environments *in utero* impair nephrogenesis and subsequently make the offspring develop perinatal programming of hypertension i*n vitro*, *ex vivo* and *in vivo*. We employed mouse embryonic metanephric mesenchyme cells, namely MK4 cells, for our *in vitro* experiments, and 2 transgenic (Tg) mouse lines, Hoxb7-GFP-Tg and Nephrin-CFP-Tg mice, for *ex vivo* and *in vivo* investigations. Hoxb7-GFP-Tg mice specifically express green fluorescent protein (GFP) in ureteric buds (UB), driven by the Hoxb7 promoter. Nephrin-CFP-Tg mice express cyan fluorescent protein (CFP) in glomeruli, driven by the podocyte-specific nephrin promoter.

In our *in vitro* studies, we examined whether high glucose alters Pax2 gene expresson in MK4 cells. The cells were treated with either 5 mM D-glucose plus 20 mM D-mannitol or 25 mM D-glucose media with or without reactive oxygen species (ROS) blockers (DPI, rotenone), and inhibitors of p38 mitogen-activated protein kinase (MAPK) (SB203580), p44/22 MAPK (PD98059), protein kinase C (PKC) (GF109203X), or nuclear factor kappa B (NK-kB) (PDTC) for 24-hr incubation. Our data showed that high D-glucose (25 mM) increased ROS generation and specifically induced Pax2 gene expression, but not other glucose analogs such as D-mannitol, L-glucose or 2-deoxy-D-glucose in MK4 cells. The stimulatory effect of high D-glucose on Pax2 gene expression could be blocked by ROS and NF-kB inhibitors in

MK4 cells but not by inhibitors of p38 MAPK (SB203580), p44/22 MAPK (PD98059), and PKC (GFX) in MK4 cells. These data indicated that the stimulatory effect of high glucose on Pax2 gene expression is mediated, at least in part, via ROS generation and activation of NF-κB, but not via the PKC, p38 MAPK and p44/42 MAPK signalling pathways.

In our *ex vivo* studies, we investigated the influence of a high-glucose milieu on UB branching morphogenesis. Kidney explants (E12 to E18) were microdissected from timed-pregnant Hoxb7-GFP mice and cultured with either 5 mM D-glucose plus 20 mM D-mannitol or 25 mM D-glucose media with or without ROS blockers (DPI, rotenone), catalase and phosphoinositide-3-kinase (PI3K)/AKT inhibitor at different time points, depending on the experiment. We found that high D-glucose specifically stimulated UB branching in a time-dependent manner. High D-glucose stimulation of UB branching morphogenesis was mediated via Pax2 gene expression. High D-glucose-induced UB branching and Pax2 gene expression could be blocked by ROS and PI3K/AKT inhibitors. These studies demonstrated that high glucose alters UB branching morphogenesis via Pax2 gene and protein expression. The stimulatory effect of high glucose seems to be mediated via ROS generation and activation of the AKT signalling pathway.

In our *in vivo* studies, we explored the fundamental role of maternal diabetes on renal morphogenesis impairment in offspring. In our experimental model, maternal diabetes was induced by streptozotocin in pregnant Hoxb7-GFP mice at embryonic day 13. The offspring were examined at several time points after birth (neonatal, 1 week, 2 weeks, and 3 weeks) with follow-up of kidney morphology, nephron number, gene expression, and apoptotic events in this short-term postnatal experiment. We observed that the offspring of diabetic mice had lower body weight, body size, kidney weight, small volume of glomeruli and a reduced number of nephrons in comparison to non-diabetic control offspring. Renal dysmorphogenesis may have been the result of increased cell apoptosis in glomeruli. Our findings showed that the offspring of diabetic mice displayed significantly more apoptotic podocytes as well as augmented active caspase-3 immunostaining in renal tubules compared to control mice offspring. Diabetic mice offspring presented heightened expression of intrarenal renin-angiotensin system (RAS) components, such as angiotensinogen and renin, with

upregulation of p50 and p65 NF-kB isoforms. These data indicated that maternal diabetes activates the intrarenal RAS and induces glomerular apoptosis, resulting in impairment of renal morphogenesis in diabetic offspring.

In conclusion, our findings indicated that a high-glucose milieu *in utero* or maternal diabetic alters UB morphogenesis, culminating in retardation of nephrogenesis with smaller kidney size. The underlying mechanism(s) is mediated, at least in part, via ROS generation and activation of the intrarenal RAS and NF-kB pathways.

In the future, we aim to investigate the underlying mechanism(s) of how maternal diabetes induces perinatal programming of adult hypertension in offspring *in vivo*. This long-term postnatal study will be undertaken in 3 groups: adult offspring (20 weeks) of control mice, adult offspring of diabetic pregnant mice, and adult offspring of insulin-treated, diabetic, pregnant mice. We will follow-up by tracking hypertension, kidney morphology, and gene expression. Furthermore, we also plan to determine whether an antioxidant system (catalase) can protect against an hyperglycemic environment *in utero* that affects embryonic organogenesis via an increase in ROS generation. Catalase-Tg mice that specifically overexpress catalase in proximal tubules will be tested. Such Tg mice with catalase overexpression represent a model for exploring our hypothesis on the role of ROS in gestational diabetes.

Keywords : maternal diabetes, hyperglycemia, nephrogenesis, renin-angiotensin system, Pax2, Apoptosis, perinatal programming

Table of Contents

List of Tables

Chapter 1

Chapter 3

Chapter 4

Chapter 6

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 7

Chapter 8

Chapter 9

Chapter 12

List of Abbreviations

$^{\cdot}O_2^-$	Superoxide Radical
$^{\cdot}OH^-$	Hydroxyl Radical
$^{\cdot}R\text{-}OO^-$	Peroxyl Radical
ACE	Angiotensin Converting Enzyme
ACE2	Angiotensin Converting Enzyme 2
AGE	Advanced Glycation End-product
Agt	Angiotensinogen
Ang I	Angiotensin I
Ang II	Angiotensin II
AP-1	Activated Protein-1
AT1R	Ang II subtype I Receptor
AT2R	Ang II subtype II Receptor
ATM	Ataxia Teleangiectasia Mutated
ATR	A-T-Related
Bax	Bcl-2–associated X protein
BB	BioBreeding
BMI	Body Mass Index
Bmp	Bone morphogenetic protein
BW	Body Weight
CAKUT	Congential Abnormalities of Kidney and Urinary Tract
CFP	Cyan Fluorescent Protein
CKD	Chronic Kidney Disease
CLG	Capillary Loop Glomeruli
CNS	Central Nervous System
CSR	Caudal Regression Syndrome
DN	Diabetic Nephropathy
DNA	deoxyribonucleic acid
DNA-PK	dsDNA-activated Protein Kinase
DNI κ B α	Dominant negative IκBα
DPI	diphenyene iodinium
DR5	Death Receptor 5

E13	Embryonic day 13
ECM	extracellular matrix
EDG	Early Developing Glomeruli
ERK	Extracellular signal-Regulated Kinase
ESRD	End-Stage Renal Disease
Eya 1	Eyes-absent-1
FAS	Fetal Alcohol Syndrome
FGFR2	Fibroblast Growth Factor Receptor 2
FITC	Fluorescien isothiocyanate
Foxc1	Forkhead box c 1
GATA3	GATA binding protein 3
GDM	Gestational Diabetes Mellitus
GDNF	Glial cell-line-Derived Neurotrophic Factor
GFP	Green Fluorescent Protein
GFR	Glomerular Filtration Rate
GF α 1	GDNF family receptor alpha 1
GMSA	Gel Motility Shift Assay
GPI	glycosyl-phosphatidylinositol
GSH	glutathione
H_2O_2	Hydrogen Peroxide
HbA1c	Hemoglobin A1c
HBW	High Birth Weight
HE	Hematoxylin/Eosin
HOCl	Hydrochlorous acid
hPGH	human placental growth hormone
HPK	hypoplastic kidney
hPL	human Placental Lactogen
IDMs	Infants of Diabetic Mothers
IkB	Inhibitor of NF-kB
IKK	IkB kinase
IKKγ	IkB kinase gamma
IP	intraperitoneal

IRPTCs	Immortalized Renal Proximal Tubular Cells
IUGR	intrauterine growth restriction
JG	Juxta-Glomerular
KAP2	Kidney Androgen-regulated protein Promoter 2
KEEP	Kidney Early Evaluation Program
Krd	Kidney and retinal defects
LBW	Low Birth Weight
LFB3	Liver-enriched factor 3
Lim 1	LIM homeobox protein 1
MAPK	Mitogen-Activated Protein Kinases
MEK	MAPK kniase
MET	Mesenchyme-to-Epithelial Transformation
MG	Mature Glomeruli
MM	Metanephric Mesenchyme
mRNA	Messenger ribonucleic acid
NADPH	nicotinamide-adenine dinucleotide phosphate
NF-κB	Nuclear Factor kappa B
NOD	Non-Obese Diabetic
NOS	Nitric Oxide Synthase
ODMs	Offspring of Diabetic Mothers
OMN	oligomeganephronia
p53	protein 53
Pax2	paired box gene 2
Pax3	paired box gene 3
Pax4	paired box gene 4
Pax5	paired box gene 5
Pax6	paired box gene 6
Pax7	paired box gene 7
Pax8	paired box gene 8
Pax9	paired box gene 9
PDTC	Pyrolidine dithiocarbamate
PERP	p53 apoptosis effector related to PMP-22

PGDM	pregestational diabetes mellitus
PI3K	Phosphoinositide-3-kinase
PKC	Protein Kinase C
PKD1	Polycystic Kidney Disease 1
PKD2	Polycystic Kidney Disease 2
PKHD1	Polycystic Kidney and Hepatic Disease 1
PMP-22	peripheral myelin protein 22
RAR	Retinoic Acid Receptor
RAS	Renin-Angiotensin System
RCS	Renal-Coloboma Syndrome
Ret	Rearranged during Tranfection proto-oncogene
Robo2	Roundabout homologue 2
ROS	Reactive Oxygen Species
RPF	Renal Plasma Flow
RPTCs	Renal Proximal Tubular Cells
Sall1	Sal-like 1
Six1	Sine oculis homeobox homolog 1
Six4	Sine oculis homeobox homolog 4
Slit2	Slit homologue 2
SM	Stromal Mesenchyme
Spry1	sprouty 1
STZ	streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
Tg	transgenic
TGF-β	Transforming Growth Factor-beta
TK	Tyrosine Kinase
TNF-α	Tumor Necrosis Factor-alpha
TP53	Tumor Protein 53
TUNEL	Terminal deoxynucleotidyl transferase DUTP nick end labeling
UB	Ureteric Bud
USA	United States of American

UV	Ultraviolet
WD	Wolffian Duct
WHO	World Health Organization
WT-1	Wilms' Tumor suppressor 1
XO	Xanthine Oxidase

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CHAPTER 1 : MATERNAL DIABETES

1. Maternal Diabetes or Gestational Diabetes Mellitus

Diabetes during pregnancy is associated with an increased risk of maternal and neonatal morbidities. Maternal diabetes includes pre-gestational diabetes mellitus (PGDM) and gestational diabetes mellitus (GDM). PGDM refers to pre-existing diabetes (Type 1 or Type 2) when women become pregnant. GDM occurs only during pregnancy.

1.1. PGDM

PGDM is defined as carbohydrate intolerance diagnosed prior to pregnancy. About 0.2-0.5% of all pregnancies are complicated by PGDM and are associated with adverse maternal and neonatal outcome (1;2).

1.2. GDM

GDM is formally defined as any degree of carbohydrate intolerance with onset or first recognition during pregnancy (3). It is classically diagnosed by oral glucose challenge during weeks 24-28 of gestation (4), because insulin resistance increases during the second trimester of pregnancy, and glucose levels will rise in women who do not have the ability to produce enough insulin to adapt to this resistance. GDM is a problem that affects a significant number of women during pregnancy. It could have lasting health impacts on both mother and fetus (as discussed below).

1.3. Prevalence and Epidemiological Studies of GDM

GDM affects 3-10% of pregnancies, depending on the population studied, and ranges from 1% to 14% among different racial/ethnic groups (5). Most commonly, it has been demonstrated that GDM complicates nearly 5-7% of all pregnancies,

accounting for more than 200,000 pregnancies annually in the U.S.A. (5;6). Several studies have provided evidence to support the assertion that the prevalence of GDM is strongly related to race, as consistently higher rates have been reported in African-Americans, Native Americans, Hispanics, Pacific islanders, and South and East Asians (7-10). In a Los Angeles survey, Baraban et al. (11) observed that the prevalence of GDM increased from 14.5 cases per 1,000 women in 1991 to 47.9 cases per 1,000 women in 2003. In another study, it was estimated that, between 1994 and 2002, among women of varied ethnic/racial backgrounds living in Colorado, the overall prevalence of GDM doubled from 2.1% in 1994 to 4.1% in 2002 (12). The prevalence of GDM was 12.8% among James Bay Cree women in northern Quebec in 1995-96 (13).

1.4. Risk Factors Related to GDM

The traditional and most common risk factors for GDM include high maternal age, obesity, parity, previous delivery of a child weighing more than 9 lbs at birth or with birth defects, stillbirth (fetus death in uterus), previous pregnancy with GDM, impaired glucose tolerance, and family history of diabetes. These and other reported risk factors are summarized in Table 1-1 (7). Being overweight or obese before pregnancy has also been associated with a high prevalence of GDM (14;15). Some studies have revealed a positive correlation between increasing pre-pregnancy body mass index (BMI) and the augmented rate of GDM (16;17). In a meta-analysis, the risk of developing GDM doubled in overweight women (BMI 25.0-30.0 kg⁄m^2), quadrupled in obese women (BMI 30.0-35.0 kg⁄m^2), and rose 8-fold in women with severe obesity (BMI >35.0 kg⁄m^2) (18). Women with a history of GDM have an heightened risk of recurrent GDM in subsequent pregnancies. It has been found that GDM recurs in 30-69% of subsequent pregnancies after a pregnancy with GDM

(19). MacNeill et al. (20) demonstrated that the rate of GDM recurrence was 35.6% in a retrospective, longitudinal study performed in Nova Scotia, Canada. In addition, twin pregnancy has also been associated with a high prevalence of GDM (21).

Table 1-1. Risk Factors for Gestational Diabetes (7).

Maternal factors
Older age
High parity
Prepregnancy weight
Pregnancy weight gain
BMI = 27
Short stature
Low birth weight
α-Thalassaemia trait
Polycystic ovary syndrome
High intake of saturated fat
Family history
Family history of diabetes
GDM in woman's mother
Previous obstetric outcome
Congenital malformation
Stillbirth
Macrosomia
Caesarean section
Previous GDM
Pregnancy factors
High blood pressure in pregnancy
Multiple pregnancy
Increased iron stores
Protective factors
Young age
Alcohol use

1.5. Pathophysiology of GDM

The precise mechanisms underlying GDM remain unknown. Chronic insulin resistance is a central element in the pathophysiology of GDM. Insulin resistance during pregnancy stems from various factors, including alteration of placental steroid and peptide hormone levels (e.g., estrogens, progesterone), human placental lactogen (hPL) and human placental growth hormone (hPGH) (22). During the 2nd and 3rd trimesters of pregnancy, rising estrogen and progesterone levels confer

increasing tissue insulin resistance, contributing to the disruption of glucose-insulin balance (23). hPL, the key hormone mediating insulin resistance in pregnancy, is produced by the placenta, affects fatty acid and glucose metabolism, promotes lipolysis, and decreases glucose uptake (24). However, hPL increases up to 30-fold during normal pregnancy and induces insulin release from the pancreas in pregnancy (25). Brelje et al. (25) reported that hPL stimulated the growth of pancreatic islets and insulin secretion during pregnancy, suggesting that it influences beta-cell function and peripheral tissue sensitivity to insulin.

hPGH is another major hormone that may be involved in the pathogenesis of insulin resistance during pregnancy (26). It increases 6- to 8-fold during pregnancy and replaces normal pituitary growth hormone in the maternal circulation (24). hPGH overexpression in transgenic (Tg) mice causes severe peripheral insulin resistance (27). It has been demonstrated that the cytokine tumor necrosis factor-alpha (TNF-α), produced in the placenta, is one of the potential mediators of insulin resistance during pregnancy (28). Incubation of human tissue explants, such as the placenta and adipose tissue, in different glucose concentrations, showed that women with GDM release greater amounts of TNF-α in response to a glucose stimulus than those with normal glucose tolerance (29). Kirwan et al. (30) determined that TNF-α levels were higher in women with GDM than in those with normal glucose tolerance in late pregnancy. TNF-α levels were inversely correlated with insulin sensitivity. Studies have also disclosed increased circulating levels of leptin and decreased adiponectin in women with GDM (31;32). Autoimmune destruction of pancreatic beta cells is another potential cause of GDM. Type 1 diabetes mellitus (T1DM) resulting from pancreatic beta cell destruction is characterized by circulating immune markers against pancreatic islets, such as anti-islet cell antibodies or beta cell antigens, such as glutamic acid decarboxylase.

A small minority of women with GDM have been shown to present the same markers in their circulation, indicating that they most likely have inadequate insulin secretion due to autoimmune destruction of pancreatic beta cells (33;34).

1.6. Maternal diabetes Complications

Maternal diabetes complications affecting mother and fetus have been well demonstrated. This risk is largely related to high blood glucose and its consequences. Maternal complications include preterm labor and T2DM development, among others. Fetal complications comprise congenital anomalies, macrosomia, etc.

1.6.1. Maternal Complications of Maternal Diabetes

The maternal complications of maternal diabetes are diabetic retinopathy, diabetic nephropathy (DN), hypertension, preterm labor, infections, cesarean section, T2DM development, and so on.

1.6.1.1. Diabetic Retinopathy

Diabetic retinopathy is one of the diabetic microangiopathy complications generally occurring in T1DM and T2DM. It is the result of microvascular retinal changes. Hyperglycemia-induced pericyte death and basement membrane thickening lead to incompetence of the vascular walls. This damage changes the formation of the blood-retinal barrier and makes the retinal blood vessels more permeable. Studies have demonstrated worsening of retinopathy in diabetes during pregnancy (35;36). The risk factors for diabetic retinopathy during pregnancy include the duration of diabetes mellitus, metabolic control before and during pregnancy, and the presence of coexisting hypertension, as summarized in Table 1-2 (37).

Table 1-2. Risk factors for worsening of diabetic retinopathy during pregnancy

1. Coexisting hypertension or preeclampsia
2. Severity of retinopathy before conception
3. Duration of diabetes before conception
4. Poor glycemic control before conception
5. Rapid institution of glycemic control

1.6.1.2. DN

DN is one of the diabetic complications generally occurring in T1DM and T2DM. DN, the leading cause of end-stage renal disease (ESRD), complicates approximately 5% of insulin-dependent diabetic pregnancies (38). Normal pregnancy is marked by increases of approximately 30-50% in the glomerular filtration rate (GFR) and proteinuria. Generally, women with underlying nephropathy can expect varying degrees of renal function deterioration during pregnancy. For example, an elevated GFR accompanying pregnancy may cause microvascular renal injury leading to glomerular changes; enhanced protein excretion can also cause renal damage. Kitzmiller et al. (39) reviewed 35 pregnancies complicated by nephropathy and found that proteinuria increased in 69% of all cases with a decline in 65% of them after delivery. Other complications, including preeclampsia, premature labor and hypertension, are all significantly more common in women with DN during pregnancy. The prevalence of preeclampsia in pregnant women with T1DM is higher than in pregnant women without diabetes (40). Pregnancy-induced hypertension is also twice more frequent in patients with T1DM than in non-diabetic women (41). Kidney disease can significantly raise the risk of high blood pressure problems during pregnancy.

1.6.1.3. Hypertension

Hypertensive disorders complicating 5-10% of all pregnancies (42) are classified into 4 categories, as recommended by the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy: 1) chronic hypertension, 2) preeclampsia-eclampsia, 3) preeclampsia superimposed on chronic hypertension, and 4) gestational hypertension (43). Like pregnancy-induced hypertension, GDM is also relatively common and affects 3-5% of pregnancies (44). Chronic hypertension complicates nearly 1 in 10 diabetic pregnancies, and diabetic pregnant women with underlying renal or retinal vascular disease are at high risk (45). Women with diabetes and preexisting chronic hypertension are prone to developing preeclampsia. Preeclampsia and gestational hypertension are obviously more frequent in women with GDM. In a large population-based cohort in Washington State reported by Bryson et al. (46), GDM was more common in each of the pregnancy-induced hypertension case groups than in the controls. Another cohort study of 10,666 women in Sweden disclosed a significantly increased risk of preeclampsia among mothers with GDM compared to mothers without GDM (47). In Canada, a cohort study in Alberta showed that women with GDM are at 2-fold higher risk of presenting with preeclampsia than normal pregnant women (48). Recently, Lykke et al. (49) demonstrated that women who had severe preeclampsia in a first pregnancy ran a 7.58-fold increased risk of subsequent hypertension compared to women with no hypertensive disorder.

1.6.1.4. Development of T2DM

GDM affects nearly 5 to 7% of all pregnancies and increases the risk for women with a history of GDM of developing T2DM later in life. The factors that are associated with T2DM include increased BMI, history of impaired glucose tolerance,

gestational age at diagnosis of GDM, and insulin use during pregnancy. During pregnancy, women with GDM display metabolic abnormalities similar to those of people with T2DM, such as insulin resistance and reduced beta cell compensation (50). In a population-based study assessing all deliveries in the province of Ontario, Canada, Feig et al. (51) found that T2DM developed within 9 years after the index pregnancy in 18.9% of women with a history of GDM compared to 2.0% of women without GDM (Figure1-1) (51).

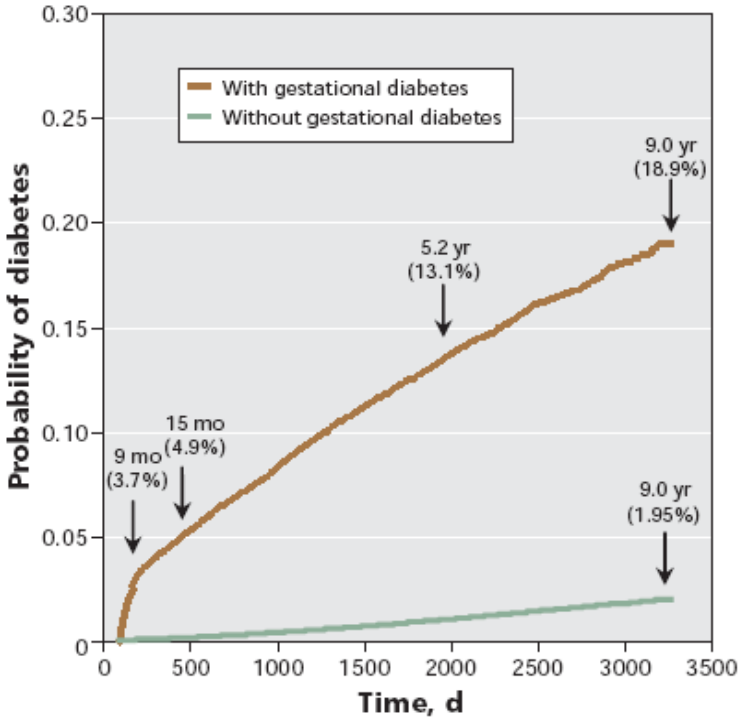

Figure 1-1 Cumulative incidence rate of diabetes mellitus (51)

Recently, in their meta-analysis of cohort studies, Bellamy et al. (52) reported an approximately 7.5-fold increased risk of T2DM in women who had gestational diabetes during pregnancy. In addition, Järvelä et al. (53) showed that about 10% of Finnish women with a history of GDM will incur diabetes over the next 6 years, and nearly half of them acquire T1DM while the other half develop T2DM. Their findings revealed that a small percentage of women with GDM may suffer from

T1DM postpartum. Taken together, the relationship between GDM and the risk of T2DM suggests that GDM can serve as a window to T2DM predisposition.

1.6.1.5. Premature Labor

Premature labor is defined as the beginning of labor before 37 weeks of gestation. Premature labor continues to be the leading cause of perinatal morbidity and mortality. It is estimated that approximately 6% of infants in the general population are born prematurely (54). Preterm labor occurs in approximately 12% of pregnancies and is the leading cause of neonatal mortality in the United States (55). Most women with GDM have normal labor and delivery but some with GDM may experience early or premature labor due to overstretching of the uterus by a macrosomic fetus or too much amniotic fluid (56). The rate of premature labor is 4 to 5 times higher among pregnant women with GDM compared to the general population (40). In addition, the preterm delivery rate is significantly higher in pregnant women with PGDM than in a control population (57;58).

1.6.1.6. Infection

Women with GDM have twice the number of urinary tract infections compared to women without GDM (22). This increased incidence of infection is thought to result from the high amount of glucose in urine during pregnancy. Nowakowaka et al. (59;60) found that the prevalence of fungi in the vagina is significantly higher in diabetic pregnant women with poor glycemic control compared to the controls.

1.6.1.7. Cesarean Section

An increasing number of newborns are being delivered by cesarean section, and GDM is among the high risk factors. Pregnancy weight gain, an important and modifiable risk factor for GDM, is also independently associated with elevated rates

of cesarean delivery (61). In the Tunisian population, Denguezli et al. (62) showed that the total cesarean rate was significantly higher in diabetic pregnancies (50.2%) than in the control group (28.2%). Fetal macrosomia was significantly correlated with the risk of cesarean delivery in diabetic women. In fact, women with diabetes often have macrosomic babies, and delivery can be difficult for both babies and mothers. In addition, the odds of requiring cesarean delivery can be much higher when babies are found to be macrosomic. Siggelkow et al. (63) noted a significantly higher number of secondary cesarean sections in the macrosomic group (27.4 vs. 16.7% in the normal-weight group; $P<0.002$). Furthermore, complications, such as preterm labor and infections, may also increase the risk of cesarean delivery.

1.6.2. Impact of Maternal Diabetes Complications on the Progeny

The maternal diabetic environment *in utero* has a serious impact on the fetus, neonate, child and adult, as shown in Figure 1-2 (64). I will briefly review the short-term (perinatal) and long-term complications that occur in the offspring of diabetic mothers (ODMs).

SHORT- AND LONG-TERM COMPLICATIONS IN OFFSPRING OF DIABETIC MOTHERS

Age at Expression	Period of Exposure	Complications
Fetus	1st trimester	Early growth delay Congenital anomalies
	2nd trimester	Macrosomia
		Nervous system development delay
	3rd trimester	Stillbirth
Neonate	Delivery	Birth injury
		Respiratory distress syndrome Hypoglycemia Hypomagnesemia Hypocalcemia
Child/adult		Obesity ↓ Impaired glucose tolerance ↓ Diabetes mellitus

Figure 1-2. Short- and long-term complications in the offspring of diabetic mothers (modified from Weintrob et al.: J Diabetes Complications) (64)

1.6.2.1. Short-term Impact of maternal diabetes Complications on ODMs

1.6.2.1.1. Macrosomia

Fetal macrosomia is defined as birth weight of more than 4,000 g (8 lb, 13 oz) or birth at greater than the 90th percentile for gestational age. Based on these definitions, macrosomia affects 1-10% of all deliveries (65). Macrosomia arises in approximately 20% of GDM pregnancies with prevalence rates ranging from 10% to 32% in Caucasian populations (66). Among North American Pima Indians, a population with high diabetes prevalence in pregnancy, fetal macrosomia rates as high as 80% have been found in women with GDM, 94% in those with impaired gestational glucose tolerance, 43% in those with pre-gestational diabetes, and 23% in women who were nondiabetic (67). Fetal macrosomia is associated with high perinatal morbidity, including shoulder dystocia, birth trauma, and neonatal asphyxia (68).

Maternal diabetes is the most common cause of macrosomic babies. Augmented maternal plasma glucose levels produce fetal hyperglycemia, resulting in fetal hyperinsulinemia and elevated insulin-like growth factor values, which stimulate glycogen synthesis, fat deposition, and excessive fetal growth. Apart from glycemic control, various other factors, such as maternal overweight and obesity are likely to influence macrosomia. Olmos et al. (69) reported that the macrosomia in the infants of type-1 pregestational diabetics is mostly due to suboptimal glycemic control. On the other hand, the macrosomia in type-2 pregestational diabetic patients, who already have a near-optimal glycemic control, is related to maternal pre-pregnancy overweight/obesity.

1.6.2.1.2. Microsomia

Fetal microsomia is defined as birth weight of less than 2,500 g (5 lb, 8 oz) or birth at less than the 10th percentile for gestational age. Some babies are small due to genetics, but most microsomic babies are small because of fetal growth problems occurring during pregnancy. Although most fetuses of diabetic mothers exhibit fetal macrosomia, fetal microsomia is significantly more frequent in pregnancies complicated by preexisting T1DM. A high frequency of intrauterine growth restriction (IUGR) has been demonstrated in 44% of fetuses from T1DM mothers, compared to 3% in the controls (70;71). In Pima Indians, maternal diabetes during pregnancy usually produces low birth weight (LBW) infants (72). Fetal microsomia carries an increased risk of morbidity and mortality both in the perinatal period and in later life. IUGR fetuses tend to have more congenital malformations at birth and are at heightened risk of cardiovascular complications, T2DM, and ESRD in later life (73;74). In African-Americans, LBW rates are 2.3 times greater than in Caucasians, with severe LBW being 3.1 times higher and paralleling the increased risk of ESRD (75).

Many microsomic babies have a condition called IUGR. Severe maternal diabetes is associated with IUGR, resulting from nutrient limitation associated with chronic maternal hypertension, advanced diabetic retinal or renal vasculopathy (76-78). The elevated glucose concentration in the mother, accompanying hyperglycemia in the fetus, leads to degranulation of the fetal β-cells, resulting in fetal hypoinsulinemia. Severe hyperglycemia in maternal rats results in hyperglycemia and hypoinsulinemia of the fetuses and IUGR. IUGR is most commonly caused by reduced uteroplacental circulation or by maternal malnutrition. Increased IUGR rates in women with diabetic vasculopathy are likely due to impaired and decreased uterine blood flow (79).

1.6.2.1.3. Stillbirth

Stillbirth is the death of a baby still in the uterus. Every year, about 25,000 babies are stillborn in the U.S.A. (80). Dunne et al. (81) reported a high rate of stillbirth in PGDM. Similarly, Goldenberg et al. (82) found that the rate of stillbirths was no different in women with either T1DM or GDM, but it was 5-fold higher in women with T2DM.

The pathophysiology of stillbirth in diabetic pregnancies is unclear. It is generally accepted that unexplained stillbirths in diabetic pregnancies are associated with poor glycemic control. Rackham et al. (83) reported that women with pregestational diabetes have higher risk of stillbirth, which was related to mean daily blood glucose values. Stillbirth can also result from severe placental insufficiency and fetal growth restriction, especially in the presence of maternal vascular complications.

1.6.2.1.4. Birth Defects

Birth defects, also called “congenital anomalies” or “congenital abnormalities”, are defined as aberrations of structure, function, or body metabolism present in a baby at or before birth and affecting many organs. The prevalence of birth defects is 1-2% in the general population (45). In the U.S.A., 1 in 33 infants is born with a birth defect, the leading cause of infant deaths (84). The etiologies of most birth defects remain unknown. Environmental substances *in utero* that evoke birth defects are called teratogens, including alcohol, certain drugs/medications, cigarette smoking, and hyperglycemia. Hyperglycemia is a prominent teratogen for birth defects in diabetic pregnancies (85). When the fetus is exposed to high, sustained glucose concentrations, widespread damage to the fetus may affect multiple organs, including the cardiovascular, nervous, skeletal and urogenital systems, resulting in diabetic embryopathy syndrome (86). Experimental studies have shown a positive

correlation between the level of hyperglycemia during embryogenesis and the risk of birth defects (87). Cardiac anomalies occur 4 to 5 times more frequently in infants of diabetic mothers than in those of control mothers. The risk of neural tube defects in diabetic pregnancies is nearly 3 times greater than in control pregnancies (88). There is approximately a 200-fold increase in the rate of caudal regression syndrome (CSR) in diabetic offspring compared to control offspring (89). Clinical studies have reported high rates of congenital malformation in T1DM and T2DM pregnancies (90;91). GDM has also been associated with birth defects (91;92). The risk of birth defects is 2-4 times higher in PGDM compared to 3% in the general population. Becerra et al. (93) noted that infants of mothers with GDM who required insulin during the 3rd trimester of pregnancy were 20.6 times more likely to have major cardiovascular system defects than infants of non-diabetic mothers. Hemoglobin A1c (HbA1c) levels are a direct measure of glucose control in diabetic mothers: high HbA1c values are predictive of risk for congenital complications (94). Recently, Reece et al. (95) observed high HbA1c levels in association with an increased rate of infants with major malformations, indicating that birth defects correlate very strongly with blood glucose levels (Figure 1-3). It has been shown that good glycemic control in diabetic mothers reduces the prevalence of birth defects to that in the general population (96).

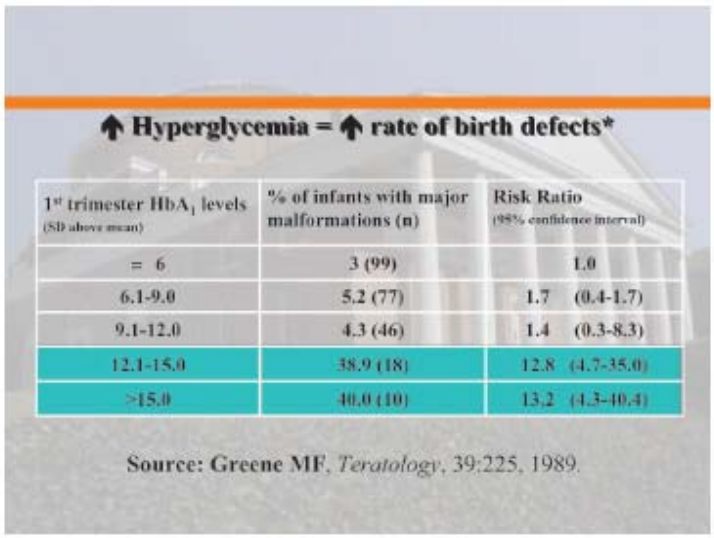

Figure 1-3. Blood glucose levels correlate inversely to birth defect risk (adapted from Reece EA: J Matern Fetal Neonatal Med) (95)

In addition, some women diagnosed with GDM for the first time may actually have undiagnosed T2DM. Pre-pregnancy obesity, which is a risk for T2DM, has been found to be associated with birth defects (97). Maternal obesity and diabetes appear to increase the risk of birth defects through shared causal mechanisms. Studies have also shown that maternal smoking and alcohol intake during pregnancy are significant environmental causes of birth defects (98;99).

1.6.2.1.5. Perinatal Morbidity

Perinatal morbidity in diabetic pregnancies has decreased since the discovery of insulin in 1922. However, current perinatal mortality rates in ODMs remain high compared to the general population. I will briefly review several perinatal morbidities that occur in ODMs.

1.6.2.1.5.1. Neonatal Hypoglycemia

Neonatal hypoglycemia, defined as blood glucose values less than 40 mg/dl in

the baby in the first few days after birth, is a common neonatal problem in ODMs (100). The causes of neonatal hypoglycemia are many, such as premature birth, small or large for gestational age, maternal diabetes, and maternal hypertension, occurring more frequently in infants of diabetic mothers (IDMs). The most common risk factor for hypoglycemia in IDMs is hyperinsulinemia, a condition in which plasma insulin concentration exceeds 30 pmol/L. The pancreas of IDMs produces extra amounts of insulin in response to the high blood glucose levels of mothers with poorly-controlled diabetes mellitus. Since glucose supply from the mother is no longer present when the baby has been delivered, extra insulin remains and blood glucose can drop too low (hypoglycemia). About 10-25% of all IDMs have transient hypoglycemia during the first 4-6 hours after delivery. The frequency of neonatal hypoglycemia has been found to increase significantly if the maternal glucose level is greater than 90 mg/dl during delivery (101).

1.6.2.1.5.2. Other Neonatal Metabolic Problems

In addition to hypoglycemia, hypocalcemia and hypomagnesemia have been demonstrated to affect infants of women with T1DM (102). Neonatal hypocalcemia is a frequent event in the first hours of life of newborns from mothers with diabetes mellitus. Strict blood glucose control of diabetes during pregnancy seems to reduce the risk of neonatal hypocalcemia. Poor diabetic control leads to glucose excretion in urine with consequently increased urinary loss of magnesium, followed by low maternal blood magnesium concentration. Maternal hypomagnesemia causes fetal hypomagnesemia with consequent impairment of neonatal parathormone secretion, resulting in abnormally low calcium levels in blood of the fetus (103).

1.6.2.2. Long-term Impact of Maternal Diabetes Complications on ODMs

The long-term impact of maternal diabetes complications on ODMs has been well studied in animal models and clinical trials. There is increasing evidence that maternal diabetes augments the high risk for ODMs of developing obesity and diabetes mellitus later in life (104).

1.6.2.2.1. Obesity

According to the World Health Organization (WHO), obesity is defined as BMI $\geq$30 kg/m^2 (105). In 2005, the WHO reported that at least 400 million adults are obese. The increasing prevalence of overweight and obese children is a major health problem. Gestational and perinatal factors have been shown to influence weight gained in childhood. Silverman et al. (106;107) demonstrated that the offspring of mothers with PGDM or GDM had a high BMI and developed childhood obesity in comparison to their controls. In another study, the offspring of Pima Indian mothers who had diabetes during pregnancy also had significantly higher body weight at 5 to 19 years of age compared to the controls (108;109). These findings strongly suggest that the intrauterine diabetic environment raises the risk of obesity in childhood and early adulthood.

1.6.2.2.2. Diabetes Mellitus

In utero exposure to high maternal glucose concentrations is a risk for the long-term development of diabetes mellitus in ODMs (110), but the underlying mechanisms remain unclear. Both insufficient insulin production and insulin resistance are important factors for long-term diabetes mellitus in ODMs. The insulin secretion response is defective and glucose tolerance is impaired in the adult offspring of mothers with T1DM (111). The offspring of mothers with T2DM or

GDM tend to develop insulin resistance or T2DM. Hunter et al. (112) showed that the offspring of mothers with T2DM were overweight with a trend towards reduced insulin sensitivity compared to normal controls. Pettitt et al. (113) studied the prevalence of T2DM among Pima Indians aged 20-24 years and found that 1.4% of non-ODMs developed T2DM versus 45% of ODMs. Similarly, among Pima Indian youth aged 5-19 years, about 40% of ODMs developed T2DM (114).

1.6.2.2.3. Perinatal Programming

Evidence indicates that many adult diseases originate from events that happen *in utero* (115). Exposure to alterations in the intrauterine and early postnatal nutritional, metabolic, and hormonal environment may predispose to diseases in later life (116). However, exposure of the embryo to either genetic influences or environmental changes during a critical period of development can evoke adaptive responses that have a significant impact on its physiology and metabolism. In the next section, I will discuss perinatal programming, perinatal programming-related hypotheses, clinical and experimental investigations of perinatal programming, and possible mechanisms.

CHAPTER 2: PERINATAL PROGRAMMING

2.1. Perinatal Programming

The concept that intrauterine and perinatal events can influence organ function later in life is called perinatal programming (117). Blood pressure, renal function, and general health later in childhood and adult life may be affected mostly before birth or during the perinatal period by the intrauterine and perinatal environment (118).

2.2. Perinatal Programming-related Hypotheses

2.2.1. Barker's Hypothesis

In the late 1980s, Barker et al. (119) reported the association between poor living standards and ischemic heart disease after a detailed geographic comparison of infant mortality in Britain between 1921 and 1925 and death in adults due to ischemic heart disease and coronary artery disease between 1968 and 1978. This led them to suggest that high infant mortality may be due to poor growth *in utero*, and insufficient fetal growth may be associated with the development of cardiovascular disease in adulthood. They observed that birth weight was inversely correlated with blood pressure and the frequency of cardiovascular diseases in adult life. Their findings became the basis for Barker's hypothesis, also known as the "fetal origins of adult disease" (120). The underdevelopment of organs and tissues affects metabolism in the fetus, and the consequences are reflected in adult homeostasis and health. Epidemiological studies in humans have established correlations between impaired intrauterine growth and an increased incidence of cardiovascular, metabolic, and other diseases in later life (121). Barker et al. (122;123) showed a relationship between LBW or slow growth during infancy and the risk of impaired glucose tolerance or T2DM in adults.

2.2.2. Brenner's Hypothesis

In the late 1980s, Brenner et al. (124) postulated that deficiency in glomerular filtration surface area might be associated with essential hypertension. This hypothesis was based on observations in animal models that surgical ablation of kidney tissue or reductions in filtration surface area as a result of chronic kidney disease can cause hypertension. As illustrated in Figure 2-1, many studies (125;126) support their hyperfiltration hypothesis, in which a decrease in renal mass − either due to low nephron endowment or renal mass diminution because of renal disease or surgery − elicits glomerular hyperfiltration. Lower numbers of glomeruli lead to a higher single nephron GFR. Such hyperfiltering glomeruli spontaneously develop focal glomerulosclerosis, particularly after injury. Numerous studies have reinforced this hypothesis of "low glomerular endowment" (127;128).

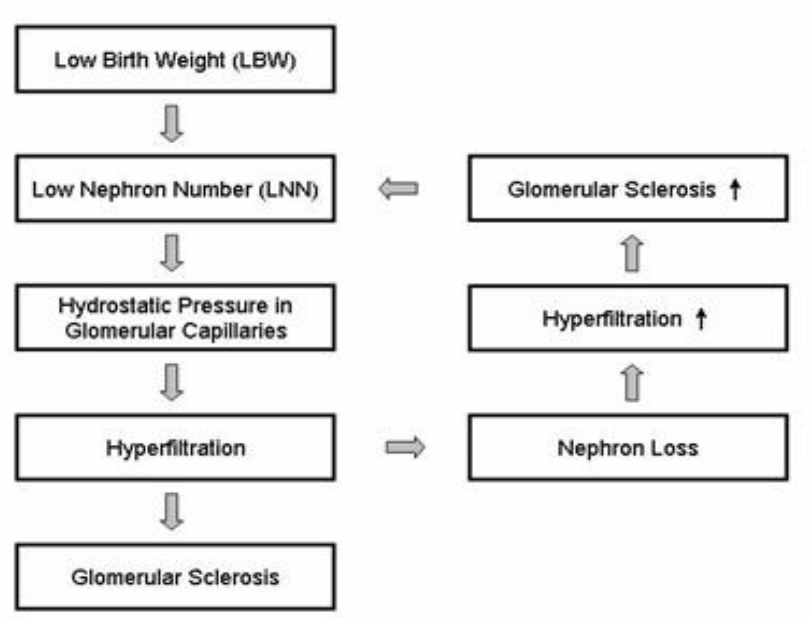

Figure 2-1. Hyperfiltration hypothesis (modified from Schreuder et al.: Kidney Int) (129)

2.2.3 The "Thrifty Phenotype" Hypothesis

The "thrifty phenotype" hypothesis proposed by Hales and Barker (130;131) suggests that the epidemiological associations between poor fetal and early post-natal growth and the increased risk of developing T2DM and metabolic syndrome result from the effects of poor nutrition in early life, which produce permanent changes in glucose-insulin metabolism (Figure 2-2) (131). In adapting, the fetus and infant have to be nutritionally "thrifty", to increase fuel availability. For example, the fetus is programmed to store nutrients such as fat. If poor nutrition continues throughout life, these adaptations are not detrimental. On the other hand, if nutrition is improved in adulthood, the ability of the pancreas to maintain carbohydrate homeostasis is exceeded, causing adult insulin resistance, glucose intolerance, and finally T2DM.

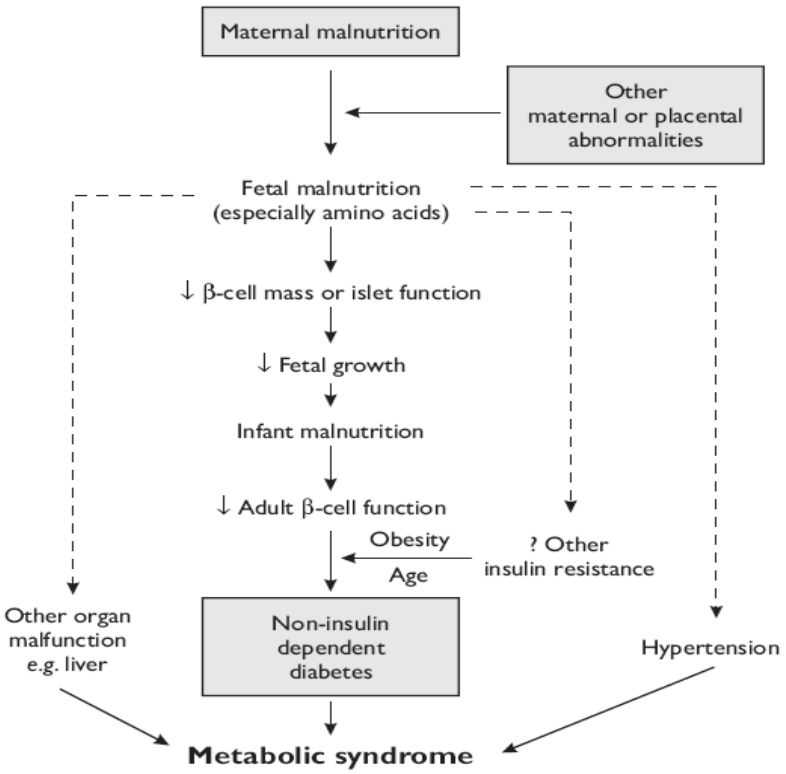

Figure 2-2. Original diagram of the "thrifty phenotype" hypothesis (131)

2.3. Clinical and Experimental Evidence of Perinatal Programming

2.3.1. Clinical Studies of Perinatal Programming

Epidemiological investigations in humans have shown that the offspring of obese mothers are at heightened risk of obesity and diabetes. Fetuses of obese women manifest increased obesity, insulin resistance, and have elevation in markers of inflammation. Catalano et al. (132) demonstrated that the fetuses of obese mothers had greater percent body fat and insulin resistance than those of lean women. Adults with LBW are at risk of chronic diseases, including high blood pressure, cardiovascular disease and chronic kidney disease. The Kidney Early Evaluation Program (USA) with 12,364 participants, whose mean age was 49.1 years, established that men with LBW or high birth weight (HBW) had a significantly greater prevalence of chronic kidney disease, but there was no association among women (133). A Norway cohort study of 2,183,317 participants with a mean age of 21.2 years revealed that men and women with LBW had a high prevalence of ESRD (RR 1.7) and women with HBW had a high risk of ESRD (RR 2.3) (134). The population-based Nord Trøndelag Health Study of 7,457 Norwegian adults aged 20 to 30 years reported a significant association between IUGR and reduced renal function in men with LBW (121). Recently, White et al. (135), in a systematic review of observational studies on birth weight and chronic kidney disease (CKD), determined that LBW is associated with an increased risk of decreased kidney function and kidney failure.

2.3.2. Experimental Studies of Perinatal Programming

Various animal models have been tested to examine the effects of IUGR on chronic adult diseases. The most common animal models of LBW and fetal programming of adult diseases are maternal nutrient restriction, uteroplacental

insufficiency, maternal glucocorticoids and experimentally-induced maternal diabetes. When total maternal food intake was limited to 50% of daily needs during pregnancy and lactation, adult prenatal restriction offspring presented increased microalbuminuria, decreased GFR, and hypertension (136). A low-protein diet is one of the most extensively studied models of early growth restriction. The male offspring of rat dams fed a low-protein diet during pregnancy and lactation developed impaired glucose tolerance at age 15 months and diabetes with insulin resistance at 17 months (137). The female offspring of rat dams fed a low-protein diet during pregnancy and lactation manifested hyperglycemia and impaired glucose tolerance at age 21 months (138). In a model of uteroplacental insufficiency induced by uterine vessel ligation that restricted fetal growth, the offspring were born small and glomeruli number was reduced at age 6 months without glomerular hypertrophy and glomerular hypertrophy was seen at age 18 months (139). Streptozotocin (STZ)-induced maternal diabetes is the most common model for studying the effects of gestational diabetes in the offspring. Low-dose STZ administration causes mild gestational diabetes and fetal macrosomia, whereas high-dose STZ elicits insulin deficiency and diabetes-associated growth restriction. The offspring of STZ-diabetic pregnant rats have low body weight and insulin resistance in adulthood (140).

2.4. Possible Mechanisms of Perinatal Programming

Numerous large studies have confirmed the relationship between LBW and high blood pressure. The number of nephrons is reduced in patients and animals with LBW (141;142). According to Brenner's hypothesis, low nephron numbers may lead to arterial blood pressure elevation. LBW can be the consequence of numerous prenatal events. IUGR is certainly one of the mechanisms of LBW. Recent

experiments have confirmed the effect of IUGR on the development of hypertension in adults (143) .

2.4.1. LBW and Low Nephron Numbers

Infants who weigh 2,500 g or less at birth are classified by the WHO as having LBW. 7.8% of all infants born in the U.S.A. had LBW (144). LBW and IUGR often occur in disadvantaged communities and have been associated with higher risks of adult cardiovascular disease, hypertension, diabetes mellitus, and kidney disease (145). LBW is often accompanied by low glomeruli numbers and is associated with hypertension in adulthood (146-148). Renal disease may also be related to LBW as children less than 1,500 g at birth have an increased risk of renal tubular abnormalities at 7-8 years of age (149). Moderate LBW (<2,500 g) has been linked with renal disease in Australian Aboriginals (150). A study of thirty-five neonates who died within 2 weeks of birth showed that neonates with LBW had lower nephron numbers compared to those with normal birth weight (151). A study of autopsied kidneys revealed that LBW was associated with low nephron numbers in both African-Americans and Caucasians (141).

2.4.2. Low Nephron Numbers and Hypertension

In 1988, Brenner et al. (124) reported an inverse relationship between total renal filtration surface area and the risk of hypertension in rats. Reduced nephron numbers have been advocated as an explanation for the relationship between LBW and hypertension in later life. In an autopsy study specifically designed to examine the association between low nephron numbers and hypertension in West European adults, Keller et al. (152) found that 10 accident victims who were known to be hypertensive had lower glomeruli numbers and larger glomerular volume than

non-hypertensive controls. The development of albuminuria, hypertension, and renal failure in children with oligomeganephronia (OMN) (153) and the low number of nephrons in Australian Aboriginals also support the correlation between low nephron endowment and renal disease (150). On the other hand, in a mostly urban community from Senegal, low glomerular numbers or large glomerular volume was not correlated with hypertension and arteriosclerosis-associated kidney disease (154). In experimental models, congenital nephron deficiency is tied to the development of renal disease and hypertension in postnatal life. In a rat model, maternal iron deficiency led to offspring with reduced nephron numbers and hypertension in adulthood (155). Fibroblast growth factor receptor 2 (FGFR2) mutant mice manifested decreased nephron numbers *in utero* and later developed hypertension and chronic renal failure in adulthood (142).

2.4.3. Possible Mechanisms of Low Nephron Numbers Associated with Intrauterine Alteration

2.4.3.1. Genetic Influence

The genetic factors leading to low nephron endowment associated with hypertension in humans have not been completely identified. Congenital OMN, characterized by low nephron numbers, glomeruli with twice the normal size, and a tendency towards sclerosis, was correlated with heterozygous paired box gene 2 (Pax2) mutations (156). Zeier et al. (157) reported that patients with congenital OMN had hypertension and a modest reduction of glomerular filtration. Suzuki et al. (158) found that body growth was retarded and mean blood pressure elevated in hypoplastic kidney (HPK) rats, a potential model of human OMN. Mice heterozygous for glial cell-line-derived neurotrophic factor (GDNF), that is essential

for kidney development, were born with 30% fewer nephrons than wild type mice and developed high arterial pressure in old age (159;160).

2.4.3.2. Nutrition

It is now widely accepted that maternal nutrition during pregnancy affects fetal growth and results in offspring with hypertension later in life (161;162). Maternal malnutrition is one of the causes of IUGR/LBW. In a Dutch famine cohort, people who were small at birth had high blood pressure in later life (163). Eriksson et al. (164) concluded that the development of hypertension was associated with reduced fetal growth and catch-up growth in early childhood in a Finnish cohort. Restriction of maternal protein intake during pregnancy in the rat produced offspring that developed hypertension in adulthood. Langley-Evans et al. (165) reported a 13% deficit in nephron numbers in rats exposed to a low-protein diet *in utero*, and these animals subsequently incurred elevated mean arterial blood pressure. Wood et al. (166;167) suggested that maternal dietary protein restriction may increase arterial blood pressure in the offspring later in life by decreasing the number of nephrons at birth, resulting in hypertension in adulthood. Similar observations have been made in other animal models. Low maternal caloric intake can reduce nephron numbers, cause glomerular hypertrophy and lead to postnatal glomerular fibrosis. Gilbert et al. (168) found that a 50% diminution of maternal calorie intake lowered nephron numbers by 11%, heightened angiotensin-converting enzyme (ACE) expression in the renal cortex, augmented medullary angiotensin II (Ang II) subtype 2 receptors (AT2R), and evoked metabolic dysregulation in sheep offspring. McGarvey et al. (169) noted that decreased maternal calcium intake during pregnancy can cause preterm delivery and adult hypertension. Crocker (170) reported that low potassium produced decreased ureteral bud branching, failure of nephron induction, and

occasional cystic dilatations of the ureteral bud in human embryonic kidneys organ culture.

2.4.3.3. Vitamin A Deficiency

Vitamin A (retinol) and its analogs (retinoids) are important regulators of cell proliferation, differentiation, immune function, and apoptosis (171). In the early 1950s, Wilson et al. (172) demonstrated that maternal vitamin A deficiency resulted in renal hypoplasia that could be prevented by vitamin A administration to pregnant rats, indicating its direct involvement in kidney development. Studies on retinoic acid receptor (RAR) knockout mice revealed the specific role of vitamin A in renal organogenesis (173). In rats, maternal vitamin A deficiency was associated with the reduction of nephron numbers in the offspring (174). In addition, Goodyer et al. (175) observed a close correlation between nephron numbers and circulating vitamin A levels in humans. Maternal vitamin A deficiency leads to renal hypoplasia in native newborn Indians compared to their Canadian counterparts (175).

2.4.3.4. ROS

Free radicals are atoms or groups of atoms with one or more unpaired electrons and can be formed when oxygen interacts with certain molecules. Thus, oxygen plays a major role as an oxidant in the form of superoxide (O_2^-), hydroxyl (OH^-), peroxyl ($R–OO^-$) radicals and their derivatives, named reactive oxygen species (ROS) (176). Oxidative stress, an imbalance between the production of ROS and the antioxidant defense mechanisms of a cell or tissue, leads to lipid peroxidation, RNA and DNA rupture, and oxidation of proteins including inactivation of many enzymes. The mechanisms by which oxidative stress mediates hypertension and cardiovascular disease are not clear. Animal studies show that oxidative stress

affects blood pressure and renal disease. Clinical trials have established that antioxidant supplementation improves blood pressure and cardiovascular outcomes in humans (177;178). Oxidative stress programming from mother to fetus may be mediated directly by modulation of gene expression or indirectly through the effects of certain oxidized molecules. Epidemiological studies indicate that poor fetal growth is associated with elevated oxidative stress in maternal plasma and erythrocytes (179;180). Gupta et al. (181) demonstrated that intrauterine malnutrition is associated with significant oxidative stress in small-for-gestational-age neonates born at term to undernourished mothers. Indeed, oxidative stress may be the trigger for *in utero* programming of hypertension. Franco et al. (182) showed that intrauterine under-nutrition enhances oxidative stress, resulting in endothelium dysfunction and, consequently, hypertension.

2.4.3.5. Apoptosis

Several studies have determined that apoptosis plays an important role in kidney development and in the pathogenesis of fetal programming. The offspring of pregnant rats fed a low-protein diet had LBW and low kidney weight at birth, low nephron numbers, higher systolic blood pressure, and increased apoptosis in glomeruli, interstitial cells, proximal tubule, and distal tubule cells (183). Rat embryonic day 13 (E13) metanephroi in embryos of mothers given a low-protein diet during pregnancy present increased metanephric apoptosis with a reduced number of progenitor cells during kidney development (184). Uteroplacental insufficiency, which causes IUGR, significantly diminishes nephron numbers while augmenting Bcl-2-associated X protein (Bax) and tumor protein 53 (p53) mRNA expression in IUGR kidneys (185). It has been postulated that the observed increase in apoptosis may be due to downregulation of anti-apoptotic factors, such as Pax2.

In Pax2 promoter-Bax Tg mice, loss of Pax2 anti-apoptotic activity accounts for the reduced ureteric bud (UB) branching seen in renal-coloboma syndrome (RCS), indicating that heightened UB apoptosis may be a key process responsible for a decreased number of nephrons (186).

2.4.3.6. RAS

All components of the RAS, expressed in the developing kidney, play an important role in nephrogenesis (187). RAS suppression by ACE inhibitors leads to renal abnormalities in both structure and function. Ang II receptor blockade also causes renal maldevelopment. In newborn Sprague-Dawley rat pups, administration of the AT1R antagonist losartan from days 1 to 12 of postnatal life reduced the number of nephrons and caused hypertension (188). Several animal model studies of fetal programming have shown that the lowered expression of RAS components during nephrogenesis is associated with low nephron numbers and hypertension in later life (166;167;189). Growth-retarded infants have small kidneys, elevated cord blood renin levels (190) and heightened renin gene expression in the kidneys (191), suggesting that the intrarenal RAS may be elevated after IUGR. In another maternal animal model, ewes were undernourished between days 28 and 77 of gestation, which resulted in increased AT1R expression in the kidneys of lambs at birth (192). In addition, gender may play a role in programming. Wood et al. (167;193) demonstrated that modest maternal dietary protein restriction in rats reduced nephron numbers in both males and females but hypertension developed only in adult male offspring. Renal renin protein and Ang II levels declined by 50-65% in males but not in females.

2.4.3.7. Glucocorticoids

Fetal exposure to maternal glucocorticoids is associated with programming of hypertension in the offspring (194). In human pregnancy, glucocorticoids are used primarily in the management of women at risk of preterm delivery to advance fetal maturation and decrease neonatal morbidity and mortality (195). Ortiz et al. (196;197) reported that dexamethasone administration to pregnant rats during gestation resulted in a 17% reduction of nephron numbers in the offspring, and increased blood pressure in adult life.

2.4.3.8. Social/Behavioral

Excessive alcohol consumption in pregnancy may induce fetal alcohol syndrome (FAS) characterized by craniofacial dysmorphology, growth restriction, and intellectual dysfunction (198). Studies of children diagnosed with FAS have demonstrated that some of them have renal malformations (199). Recently, in a sheep model, fetal exposure to alcohol during the latter half of gestation caused a 11% decrease of nephron numbers (200).

CHAPTER 3: NEPHROGENESIS OR KIDNEY DEVELOPMENT

3. Nephrogenesis or Kidney Development

The kidneys are one of the main excretory and homeostatic organs of the body. These paired, bean-shaped organs regulate the composition and volume of body fluids, and eliminate metabolic waste products. The internal structures of the kidneys include an outer cortex and an inner medulla (Figure 3-1)(201).

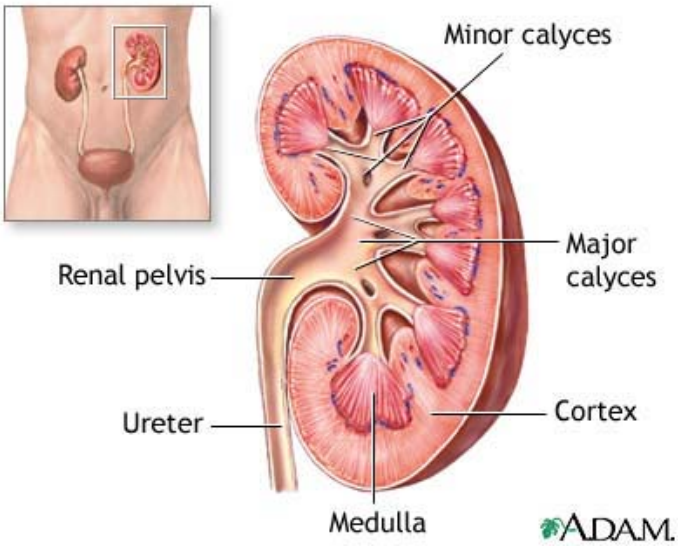

Figure 3-1. Kidney structure (201)

The nephron is the basic structural and main functional unit of the kidney. There are approximately 300,000 to 1 million nephrons per kidney in humans, and 11,000 nephrons per kidney in mice (202). The nephron consists of a renal tubule and a renal corpuscle (Figure 3-2) (203). The renal corpuscle is composed of a glomerulus and Bowman's capsule, which surrounds the glomerulus. The renal tubule is the portion of the nephron containing tubular fluid that is filtered through the glomerulus. After wastes pass through the renal tubule, the filtrate continues to the collecting duct system, which is the end of the nephron.

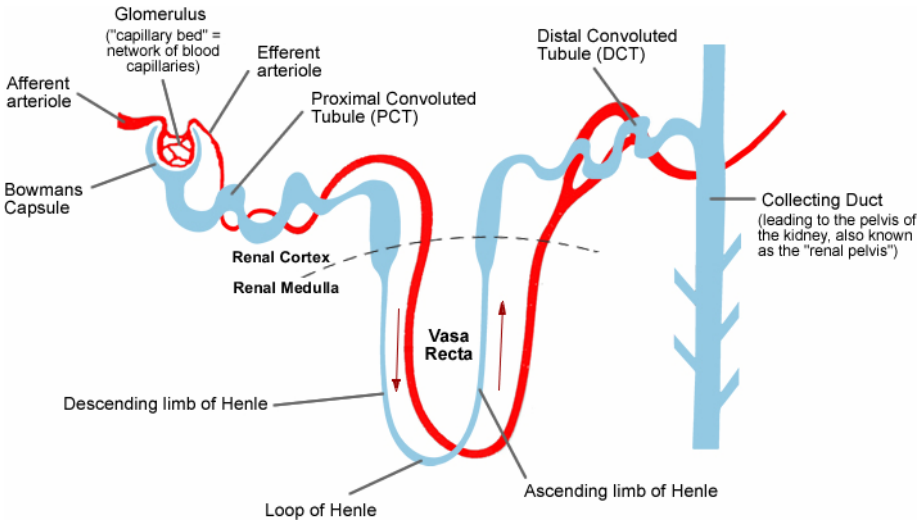

Figure 3-2. Simple diagram of the kidney nephron (203)

In mammals, kidney development, also called nephrogenesis, progresses sequentially in 3 main stages, as seen in Figure 3-3 (204). The first 2 stages of rudimentary kidney development lead to the formation of transient structures, the pronephros and mesonephros, and the third stage results in the formation of the metanephric kidney, which is the permanent kidney. Compared to other organ systems, the excretory system develops quite late in embryogenesis. Metanephric kidney development occurs at E28 in humans and at E10.5 in mice.

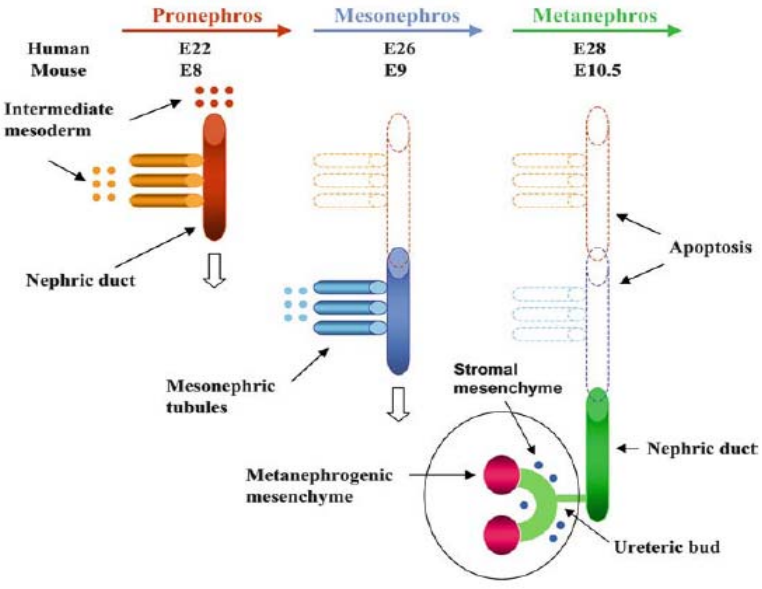

Figure 3-3. Schematic representation of kidney development (204)

3.1. Pronephros

The pronephros, the most basic of the 3 excretory organs that develop in vertebrates, corresponds to the first stage of kidney development. It arises from the portion of the intermediate mesoderm at E22 in humans or at E8.0 in mice, and then completely degenerates through massive apoptosis (205;206). The pronephros is a segmented organ that consists of pronephric tubules originating from the nephrogenic cord and the pronephric duct. Pronephric tubules and the pronephric duct form at the cranial end of the intermediate mesoderm and fuse to form the pronephros. Early regulators have been studied during pronephros formation, including functionally-redundant paired-box homeotic transcription factors, such as Pax2 and Pax8, zinc finger transcription factor GATA-binding protein 3 (GATA3), which belongs to a family of transcription factors that bind to a GATA consensus motif (A/TGATAA/G), and the LIM class homeodomain transcription factor, LIM homeobox protein 1 (Lim1). Pax2 and Pax8, co-expressed in nephric duct precursors at E8.5 in mice, are essential for the initiation of pronephros and mesonephros development (207). GATA3 is expressed in nephric duct precursors, starting at E8.5 in mice and is a putative direct target of Pax2 and Pax8 (208). In mice, Lim1 is initially expressed in the intermediate mesoderm at E7.5 and becomes restricted to the nephric duct at E9.5 (209). Direct targets of Lim1 in the nephric duct are not known, and Pax2 expression is not dependent on Lim1 function (210).

3.2. Mesonephros

The mesonephros is the second type of excretory organ to develop in vertebrates. It is composed of the mesonephric duct (also called the Wolffian duct (WD)), mesonephric tubules, and associated capillary tufts. The mesonephric duct grows caudally, reaches the mesonephric mesenchyme (MM) and induces mesonephros

formation. The mesonephros is the permanent kidney in fish and amphibians, but atrophies in reptiles, birds, and mammals. In humans, the mesonephros begins development at E26. In mice, it starts degenerating at E10.5, and almost all of the tubules undergo apoptosis within 24 hours and disappear in a caudal to cranial direction (211). A number of genes, such as Pax2, Pax8, Wilms' tumor suppressor 1 (WT-1), Forkhead box c 1 (Foxc1), and Sine oculis homeobox homolog 1 (Six1), have been implicated in different aspects of mesonephric development (207;212;213).

3.3. Metanephros

The metanephros is the third, and permanent, excretory organ to develop in vertebrate embryos. The metanephric kidney begins to develop at E28 in humans or at E10.5 to E11 in mice. It develops from 2 major structures, UB and the MM. There are 2 key processes in kidney nephrogenesis: UB branching morphogenesis and mesenchyme-to-epithelial transformation (MET). Kidney development begins when the WD, which is the precursor of UB, grows caudally, and elongates to generate UB at its extreme caudal end. As illustrated in Figure 3-4 (214), UB grow into the loosely-organized mesenchyme. Through secreted signals, the MM induces the UB to elongate further and grow into the blastema, branching and giving rise to the collecting system. At the same time, the UB prompts the adjacent surrounding mesenchymal cells to aggregate and condense around their tips. The condensed mesenchyme proliferates rapidly and differentiates into early-polarized epithelial structures, such as renal vesicles, comma- and S-shaped bodies. Metanephric tubules lead to formation of the glomerulus, proximal tubule, loop of Henle, and distal tubule. The fusion of developing metanephric tubules to a UB branch completes nephron formation.

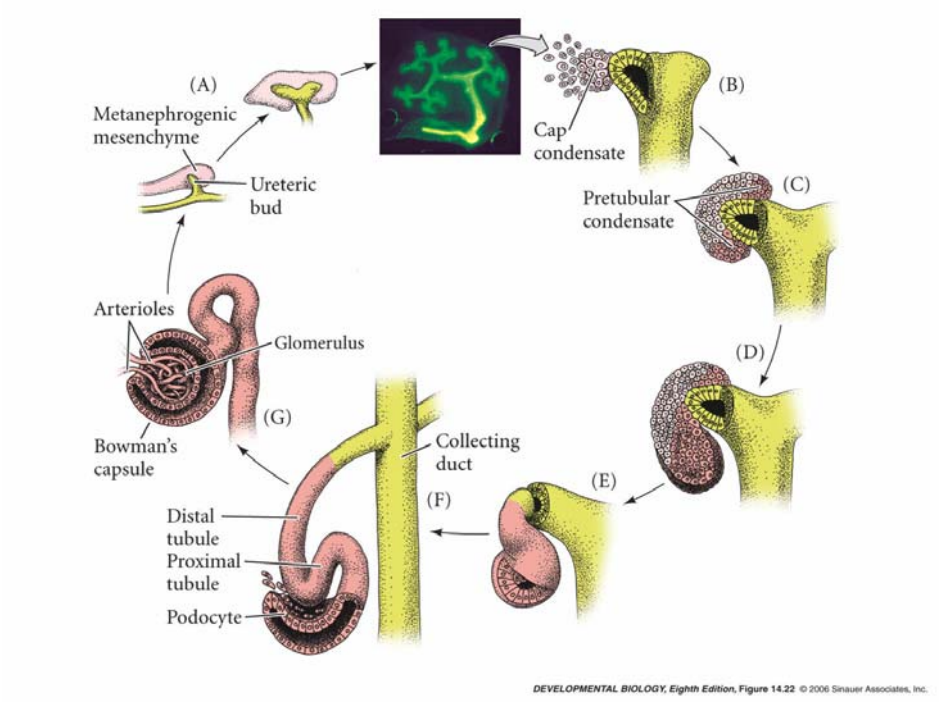

Figure 3-4. Reciprocal induction in mammalian kidney development (214)

3.4. Gene Regulation

At present, the Kidney Development Database website lists more than 300 genes known to be involved in nephrogenesis (URL address http://www.ana.ed.ac.uk/anatomy/database/kidbase/kidhome.html). Although kidney development has been studied for more than half a century, understanding the molecular control of nephrogenesis is still in its infancy. Animal experiments, especially on Tg mouse models and explants of rodents, have identified several genes that are important in kidney development, but their relevance in humans remains to be demonstrated. For example, as shown in Table 3-1 (215), GDNF, rearranged during transfection proto-oncogene (Ret), Pax2, and WT-1 are all crucial in murine renal organogenesis. Genes of the Wnt family, including Wnt4, are also known to be essential in UB branching. Sal-like 1 (Sall1), a mammalian homologue of the *Drosophila* region-specific homeotic gene *spalt* (*sal*), plays an important role in renal development (216). In addition, members of the bone morphogenetic protein

(Bmp) family of secreted factors, such as Bmp4 and Bmp7, have been implicated as anti-apoptotic factors for the MM (217;218). So far, the Pax2/GDNF/c-Ret pathway has been identified as the most critical for kidney development.

Table 3-1. Selection of genes in kidney development (modified from Piscione et al.: Differentiation) (215)

Gene Name Genotype	Tissue Expressed	Phenotype
Transcription Factors		
Eya1	MM	Absence of UB growth and failure of induction of MM
Foxd1	S	Mutant kidneys are small, few nephrons
Pax2	UB, MM	Deficient UB outgrowth, MM uninduced
Sall1	MM	Failure of UB outgrowth
Wt1	MM	MM undergoes unrescuable apoptosis
Growth Factors		
Glial-derived neurotrophic factor (Gdnf)	MM	No kidney as UB bud fails to grow
Wnt4	MM	Failure of kidney-tubule formation
Growth Factors/Receptors		
Gfra1	UB, MM	Agenesis of the kidney as in *Ret* and *Gdnf*
Ret	UB	Failure of UB growth
Proteoglycans & the Biosynthetic Enzymes		
Hs2st	UB, MM	Renal agenesis due to lack of UB branching and mesenchymal condensation

3.4.1. Pax2/Pax8 and Lim1: Earliest Markers of Nephrogenesis

As discussed below (see Section 3.6), Pax2 is a transcription factor that belongs to the family of "paired domain" proteins, and is involved in organogenesis. During kidney development, Pax2 is first detected in the nephrogenic cord in the metanephric region at E9.5 in mice and then in the MM at E10.0. Pax2 is strongly expressed in UB when it emerges from the WD and invades the MM. In Pax2 mutants, the nephric duct does not reach the cloaca, Ret expression is absent, and the duct starts to degenerate by E12.5, resulting in the failure of UB formation and metanephros development (219). Pax2 is detected in early epithelial structures, the

comma- and S-shaped bodies, which differentiate into glomeruli and the proximal portions of emerging nephrons. Indeed, Pax2 is required for mesenchyme-to-epithelium conversion in kidney development. Kidney explant cultures treated with Pax2 antisense oligonucleotides fail to express some epithelial markers, and the tubulogenesis of mesenchymally-derived structures is blocked (220). Pax8 is also present during pro-, meso-, and metanephros development. Pax2 and Pax8 together are essential for pro- and mesonephros formation. Mouse embryos lacking both Pax2 and Pax8 fail to form the pronephros or any later nephric structures and to express the early kidney-specific genes c-Ret and Lim1 (207). In mice, Lim1 is initially expressed in the intermediate mesoderm at E7.5 but becomes restricted to the nephric duct primordium by E9.5 (209). Direct targets of Lim1 in the nephric duct are not known, and Pax2 expression is independent of Lim1 function (221).

3.4.2. UB Outgrowth Requires Signaling: WT-1, Eyes-absent-1 (Eya1) and the GDNF/c-Ret Pathway

WT-1 is one of the most investigated transcriptional regulators in the developing kidney. It encodes a zinc finger-containing nuclear protein that is essential for kidney and urogenital development. During renal development in mice, WT-1 is initially expressed throughout the nephrogenic cord as early as E9.0 and is also detected at a low level in the MM prior to UB outgrowth at E10.5 (222). WT-1 is upregulated when the mesenchyme begins to form epithelial condensates around UB tips. In WT-1 null mice, the UB fail to form and the MM, which appears to be morphologically normal, undergoes apoptosis at E11.0 (223). In adult kidneys, WT-1 expression is restricted to glomerular podocytes, which form the filtration

barrier within the glomerulus. WT-1 is therefore important for normal podocyte function (224).

Eya1, a mammalian homologue of the *Drosophila* eyes-absent gene, encodes a transcriptional co-activator that can complex with other transcription factors to induce the expression of target genes (225). In developing mice kidneys, Eya1 is first detected in the nephrogenic cord at E8.5, and it is only expressed in the MM at E11.5 (226). In mice, Eya1 deficiency ($Eya1^{-/-}$) results in the absence of UB outgrowth and the failure in the formation of a morphologically-distinct population of MM (227). $Eya1^{-/-}$ mice kidneys display an early loss of the GDNF signal in the nephrogenic cord, but Pax2 is present in the remaining blastema cells, indicating that it functions either upstream or independently of Eya1 (226). The first indication that the GDNF/c-Ret developmental pathway is crucial for metanephric development came from an analysis of c-Ret knockout mice (228). GDNF, a distant member of the transforming growth factor-beta (TGF-β) superfamily, is a potent neurotrophic factor for neurons of the central nervous system (CNS) and is essential for renal development (229). During kidney development in mice, GDNF is expressed in the nephrogenic cord at E9.5, but becomes restricted to the MM at E10.5. GDNF family ligands signals through a unique multicomponent receptor complex consisting of glycosyl-phosphatidylinositol (GPI)-anchored coreceptor (Gfrα1–4) as a ligand binding component and RET receptor tyrosine kinase as a signaling component (230). During early kidney development, Ret and Gfrα1 are expressed all along the WD, while GDNF is expressed only in the MM. GDNF binds to the Ret receptor, activates Ret and then induces UB outgrowth. GDNF signaling is important to induce UB outgrowth from the WD and to promote its early growth and branching. GDNF mutations result in renal aplasia. In humans, 5-10% of cases of renal agenesis are found to be linked with GDNF mutations (231). Ret encodes a

transmembrane receptor of the tyrosine kinase (TK) family of proteins and is required for kidney morphogenesis. In the developing kidney, Ret is initially expressed on the mesonephric duct and early UB, and later its expression is restricted to UB branch tips (232). Mice with a c-Ret null mutation exhibit severe renal dysplasia or agenesis as a result of defective UB outgrowth (228). In humans, 30% of cases of renal agenesis are found to be associated with Ret mutations (231).

3.4.3. The Wnt Pathway

Wnts have been implicated in epithelial-mesenchymal interactions (233). The 18 Wnt genes identified in mice encode secreted glycoproteins that bind to the cell surface and the extracellular matrix (ECM) (234). Wnt signaling via the canonical β-catenin pathway has been shown to be involved in UB branching (235). A number of Wnts which signal through the canonical β-catenin pathway are present in the developing metanephros, including Wnt6, Wnt7b, and Wnt9b in the collecting duct system, and Wnt4 in early nephron precursors (236). Wnt-4 plays an essential role in kidney development and is expressed in condensing MM cells very soon after UB induction, persisting in comma- and S-shaped bodies (237). Wnt-4 acts during MET and is required for tubulogenesis in the metanephric kidney. In Wnt4-deficient mice, epithelial renal vesicles fail to form, resulting in small kidneys consisting of undifferentiated MM interspersed with ureter branches. In addition, homozygous Wnt4 mutation pups die of renal failure within 24 hours of birth (237). In Wnt9b mutant embryos, down-regulation of Wnt-11 and GDNF expression prior to morphological appearance of the branching defect indicates a later requirement for Wnt9b in the regulation of secondary branching (238).

3.5. Renal Malformations

Human renal malformations are the main causes of ESRD in children less than 5 years of age (239). They include multiple ureters, renal agenesis (the absence of kidneys), renal hypoplasia (reduced kidney size), and renal dysgenesis (kidneys containing abnormal structures) (240;241). Developmental abnormalities of the kidneys are various, and mutations of genes expressed in the developing kidney cause congenital abnormalities in humans, as shown in Table 3-2 (242).

Table 3-2. Congenital abnormalities and gene defects identified in patients (242)

Renal congenital defects	Symptoms	Syndromes	Gene defects
Duplex (multiple) ureter	Formation of several ureters resulting from defective ureter induction	Usually no symptoms; can however be associated with hydroureter (see below)	
Hydronephrosis	Distention of the pelvis and calices of the kidney. Often the result of ureter obstruction	Ochoa (urofacial) syndrome	On Chr 10q23–q24
Megaureter	Increased ureter size; often coupled with VUR	Ellis van Crefeld (also renal agenesis)	*EVC, EVC2*
Nephrotic syndrome (NS)	Proteinuria resulting from a failure of blood filtration; usually caused by glomerular defects	Frasier, Denys–Drash, WAGR, idiopathic NS	*WT1*
		Finnish type NS	*NPHS1*
		Nail–patella–syndrome	*LMX1B*
		Pierson syndrome	*LAMB2*
		Steroid-resistant NS	*NPHS2*
Oligomeganephronia	Few large nephrons	Oligomeganephronia	*PAX2*
Polycystic kidneys	Formation of cysts affecting either tubules, collecting ducts or both	Renal cysts and diabetes	*HNF1B*
		Polycystic kidney disease (PKD)	*PKD1, PKD2*
		Polycystic kidney and hepatic disease (PKHD)	*PKHD1*
Renal aplasia (agenesis)	Absence of kidney; usually unilateral, but can occur bilaterally	Branchootorenal syndrome(BOR)	*EYA1, SIX1, SIX4, SIX5*
Renal hypoplasia	Reduction of kidney size without abnormal development, probably caused by a reduced number of nephrons	Renal-coloboma (also VUR)	*PAX2*
		Townes–Brocks	*SALL1*
		Pallister–Hall	*GLI3*
Renal dysplasia	Kidneys contain abnormally developed structures; often associated with hypoplasia (renal hypodysplasia)	Fraser (also hypoplasia or aplasia)	*FRAS1, FREM1*
		Campomelic dysplasia (also multicystic)	*SOX9*
		Senior–Loken syndrome (also multicystic)	*NPHP1, NPHP4, NPHP5*
Tubular dysgenesis	Defective proximal tubules formation	Renal tubular dysgenesis	*REN, AGT, ACE, AGTR1*
VUR	Movement of urine from the bladder to the ureter or kidney	VUR, VUR2	*GFRA1←ROBO2*

ACE, angiotensin I converting enzyme; *AGT*, angiotensinogen; *AGTR1*, angiotensin II receptor, type 1; Chr, chromosome; *EVC*, Ellis van Creveld gene; *EYA1*, Eyes-absent homologue 1; *FREM1*, FRAS1-related extracellular matrix protein 1 gene; *GFRA1*, GDNF family receptor α1; GLI3, GLI-Kruppel family member GLI3; *HNF1B*, hepatocyte nuclear factor 1β, also known as *TCF2* or *vHNF*; *LAMB2*, laminin, β2 (laminin S) gene; *LMX1B*, LIM homeobox transcription factor 1β gene; *NPHP*, nephronophthisis gene; *NPHS1*, nephrosis 1, congenital, Finnish type; *NPHS2*, nephrosis 2, idiopathic, steroid-resistant (podocin); PAX, paired-box gene; *REN*, renin; *ROBO2*, roundabout homologue 2; *SALL1*, Sal-like gene 1, SIX, sine oculis homeobox homologue; *SOX9*, SRY (sex determining region Y)-box 9; VUR, vesicoureteral reflux; WAGR, Wilms tumour–aniridia–genitourinary anomalies–mental retardation; WT1, Wilms tumour gene.

3.5.1. Renal Agenesis

Renal agenesis is a medical condition in which one or both kidneys fail to develop in the fetus and are absent at birth. Bilateral renal agenesis, the failure of both kidneys to develop during gestation, has a frequency of 1 in 30,000 newborns (242). Unilateral renal agenesis, the absence of 1 kidney in the fetus, occurs in 1 in 5,000 newborns (242). Children with unilateral renal agenesis have considerably higher chances of developing hypertension (243). Renal agenesis seems to be associated with a failure of GDNF-Ret signaling. GDNF activation in the MM is controlled by several regulators: Eya1, Six1, Six4, and Pax2. A number of recent studies have shown that mice deficient in Eya1, Six1, Six4, or Pax2 lack kidneys (212;227;244;245). Some authors suggest that nephronectin, an ECM protein, is also important for maintaining GDNF expression. Mice lacking nephronectin fail to express GDNF and to develop kidneys (246).

3.5.2. Multiple Ureters

Duplex ureters are the most common malformations of the urinary tract. In a normal urinary tract, each kidney is connected to 1 ureter, and drains urine into the bladder. Patients with a duplex collecting system have 2 ureters for 1 kidney that drain independently into the bladder, rather than 1 ureter for each kidney. Gene targeting experiments have identified several genes, including Foxc1, Slit homologue 2 (Slit2), and its receptor, Roundabout homologue 2 (Robo2), that are required to prevent ectopic UB formation. Mutations in these genes lead to expansion of GDNF expression and cause ectopic ureters (247;248). The receptor tyrosine kinase antagonist sprouty 1 (Spry1) is essential for early kidney development and is also important for limiting branching during kidney

development (249). Spry1$^{-/-}$ embryos have supernumerary UB, resulting in the development of multiple ureters and multiplex kidneys (250).

3.5.3. Renal Dysgenesis

Renal dysgenesis, the consequence of disordered renal tissue differentiation, culminates in a form of dysplasia with or without cysts, hypoplasia or hypodysplasia (251). Renal dysplasia is characterized by the existence of primitive mesenchymal structures, which are remnants of the collecting duct or some other UB surrounded by absent or abnormally-differentiating MM (252). Pax2 or Wnt4 mutations may cause renal dysplasia, originating from the inhibition of mesenchymal-to-epithelial conversion or UB formation occurring at an ectopic site on the WD (253).

3.5.4. Renal Hypoplasia

Renal hypoplasia is a medical condition in which an abnormally small kidney that is morphologically normal has either a reduced number of nephrons or smaller nephrons. In humans, the average number of nephrons ranges from 300,000 to 1 million in each kidney, and there is a correlation between nephron number and the risk of developing hypertension (254). A defect in the rate of UB branching is the most common cause of renal hypoplasia. Studies have shown that RCS, a congenital disease characterized by optic nerve coloboma, renal hypoplasia and diminished nephron number, is due to heterozygous mutations of the Pax2 gene (255). Retinoic acid is important to maintain Ret expression in UB, so deficiency of vitamin A, a precursor of retinoic acid, is well known to elicit renal hypoplasia (174). In rats, early *in utero* exposure of fetuses to hyperglycemia causes a 10-35% decrease in nephron numbers (256).

3.5.5. OMN

Congenital OMN, characterized by a low number of nephrons, glomerular hypertrophy and a tendency towards glomerularsclerosis, is a model of nephron under-endowment (153). OMN has been reported in 2 siblings, indicating a genetic background (257). In addition, Salomon et al. (156) found heterozygous Pax2 mutations in 3 patients with OMN, confirming the critical role of Pax2.

3.5.6. Polycystic Kidneys

Polycystic kidneys are a genetic disorder that is characterized by the presence of multiple cysts. The cysts are numerous and fluid-filled, causing massive kidney enlargement. Mutations in PKD1 (polycystic kidney disease 1), PKD2, and PKHD1 (polycystic kidney and hepatic disease 1) evoke polycystic kidneys (258).

3.6. Pax2 Pathways

Renal malformations account for about 40% of renal failure in childhood (259;260). To date, most human and experimental studies have focused on the phenotype, with only a few investigating the mechanism(s). Pax2 was first cloned and characterized by Peter Gruss's group in 1990 (261). It plays an essential role in nephrogenesis. Pax2 mutations cause increased apoptosis and are associated with renal hypoplasia (186;262). Homozygous null Pax2 ($Pax2^{-/-}$) mice lack kidneys, ureters, and genital tracts (219). In humans and mice, heterozygous Pax2 mutations elicit kidney, eye, and CNS abnormalities, constituting RCS. (263).

3.6.1. Pax Proteins and Gene Structure

The murine Pax gene family was first identified on the basis of sequence homology with the *Drosophila* segmentation gene (264). Pax proteins are a family

of 9 proteins that contain a highly homologous 128 amino acid "paired" domain (265), originally identified in *Drosophila* paired protein (Prd) (266). Pax proteins can be divided into 4 groups based on a paired-box DNA-binding domain, with or without an octapeptide coding region and/or homeodomains, as shown in Figure 3-5 (267). In addition to the "paired" domain, Pax genes have 2 other conserved sequences, the helixed homeodomain, an additional DNA binding motif, and the 8 amino acid octapeptide motif, an additional conserved domain (267). Based on similarities of molecular structure, the 9 Pax genes are divided into 4 groups: Pax1 and Pax9 belong to Group 1; Pax2, Pax5 and Pax8 comprise Group 2; Pax3 and Pax7 are members of Group 3; and Pax4 and Pax6 make up Group 4 (Figure 3-5) (267).

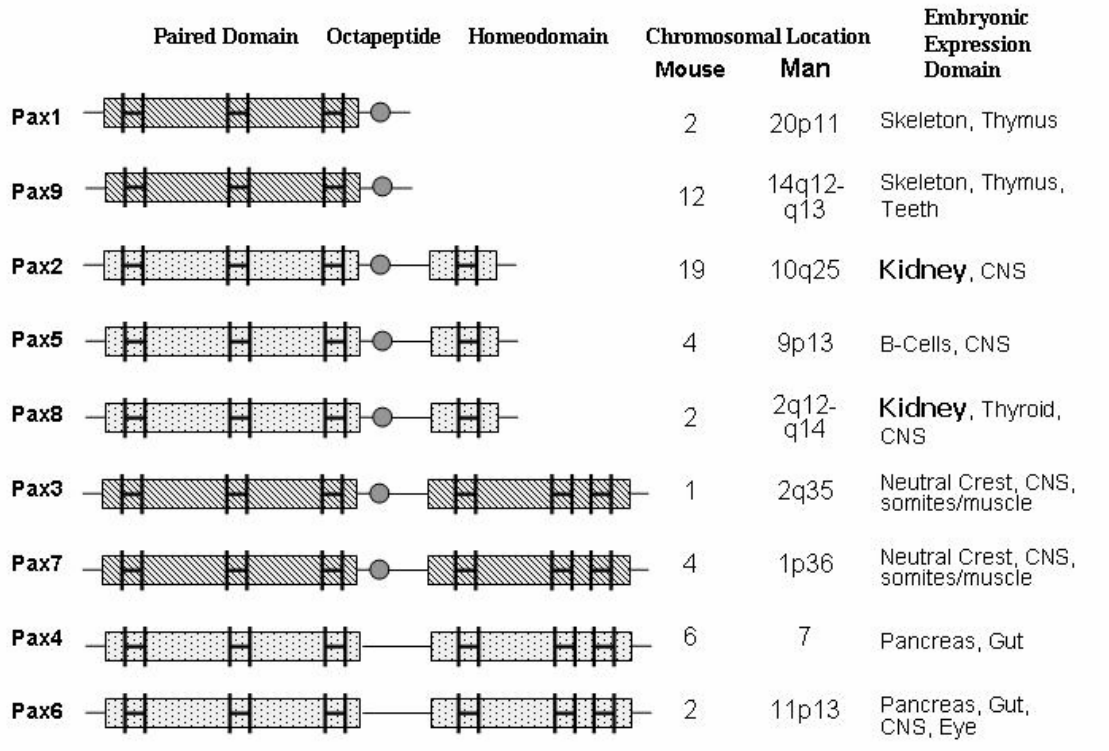

Figure 3-5. The Pax protein family (modified from Mansouri et al.: Cell Differentiation) (267)

3.6.2. Pax Genes in Organogenesis

All Pax proteins are expressed in different tissues during development. Pax1 is present in the thymus and a mutation of Pax1 gene causes reduced thymus size (268). Pax2 is expressed in the eyes, ears, and kidneys during embryonic development; heterozygous Pax2 expression in the mouse ($Pax2^{+/-}$) is associated with kidney hypoplasia (255). Pax3 is found in the limb muscle, neural tube and neural crest. Its mutation leads to Waardenburg syndrome, a rare genetic disorder most often characterized by varying degrees of deafness, minor defects in structures arising from the neural crest, and pigmentation anomalies (269). Pax4 is a master gene for pancreatic development; $Pax4^{-/-}$ mice lack most pancreatic cells (270). Pax5 is expressed in the thymus and is involved in regulating B cell proliferation (271). Pax6, a highly conserved gene that controls eye development, is required for optic cup development (272). Pax7, expressed in somites and muscle, plays an important role in skeletal muscle formation (273;274). Pax8 is detected in the kidneys and thyroid; $Pax8^{-/-}$ mice die from a thyroid defect quickly after weaning (275). Pax9 plays an important role in tooth development; heterozygous mutations in Pax9 have been shown to be associated with human tooth agenesis (276). Nine Pax genes have been described in humans and mice. The expression of Pax genes in many tissues during embryogenesis is associated with their critical roles during development. Mutations in 5 genes, Pax2, Pax3, Pax6, Pax8 and Pax9, cause human congenital abnormalities, as summarized in Table 3-3 (277).

Table 3-3. Abnormalities in humans and mice with heterozygous Pax gene mutations (277)

Gene	Human syndrome	abnormalities	Mouse mutation	Abnormalities
PAX1	-	-	*Undulated* (+/-)	Kinked tail
			Pax1 knockout(-/-)	Skeletal abnormalities in vertebral column and sternum
PAX2	Renal-coloboma syndrome	Optic nerve colobomas or hypoplasia, renal hypoplasia, renal failure, vesicoureteric reflux, sensorineural hearing loss	*Pax2*^{1Neu}, *Krd*, (+/-)	Optic nerve hypolasia, renal hypoplasia
			Pax2 knockout (-/-), *Pax2*^{1Neu} (-/-)	Urogenital tract agenesis, mid/hindbrain deficiency, optic nerve abnormalities, absent chiasma, absent cochlea
PAX3	Waardenburg syndrome	Dystophia canthorum, hypopigmentation sensorineural hearing loss	*Splotch* (+/-)	Hypopigmentation patches, hearing loss
			Splotch (-/-)	Deficient muscle and neuromuscular development, hypopigmentation
PAX4	-	-	*Pax4* knockout (+/-)	No abnormalities
			Pax4 knockout (-/-)	Absence of alpha cells of the pancreas
PAX5	-	-	*Pax5* knockout (+/-)	No abnormalities
			Pax5 knockout (-/-)	Absence of B lymphocyte development
PAX6	Aniridia	Absence of iris, cataracts, Peters' anomaly, foveal hypoplasia, autosomal dominant keratitis	*Small eye* (+/-)	Small eyes
			Small eye (-/-)	Deficient cortical development of brain, absent eyes, absent nasal structures, absent Beta cells of the pancreas
PAX7	-	-	*Pax7* knockout (+/-)	No abnormalities
			Pax7 knockout (-/-)	Absence of myogenic satellite cells
PAX8	Congenital hypothyroidism	Hypothyroidism, thyroid dysgenesis, thyroid hypoplasia	*Pax8* knockout (+/-)	No abnormalities
			Pax8 knockout(-/-)	Absent follicular cells of thyroid
PAX9	Oligodontia	Oligodontia and tooth agenesis	*Pax9* knockout (+/-)	No abnormalities
			Pax9 knockout (-/-)	Absent thymus, parathyroid glands, ultimobranchial bodies, and teeth, supernumary digits, absent hindlimb toe, disturbed craniofacial and visceral skeletogenesis

3.6.3. Pax2 Mutations Cause Human RCS

Pax2 is expressed in the eyes, ears, CNS, and urogenital system during embryogenesis. In the kidneys, Pax2 is expressed in the early stages of

mescenchymal to epithelial differentiation, proceeding to form the glomerulus, and is also detected in UB and at low levels in the collecting duct, renal pelvis and ureter. Pax2 mutations cause a special autosomal dominant human condition, RCS, a multisystem developmental disorder involving optic nerve colobomas and renal hypoplasia/insufficiency (278). Eight different mutations have been identified in the various exons of Pax2 gene. Exon 2, in the N-terminal portion of the paired box domain, is the most frequent site of mutation in RCS patients (263). Patients with Pax2 mutations often have small kidneys with reduced size of the renal pelvis and renal cortex. Renal disease in patients with Pax2 mutations is usually progressive. Renal cortical biopsies from RCS patients show mesangial fibrosis and glomerulosclerosis (Figure 3-6) (278). Fletcher et al. (279) reported that RCS patients seem to display the mild hypertension in adulthood.

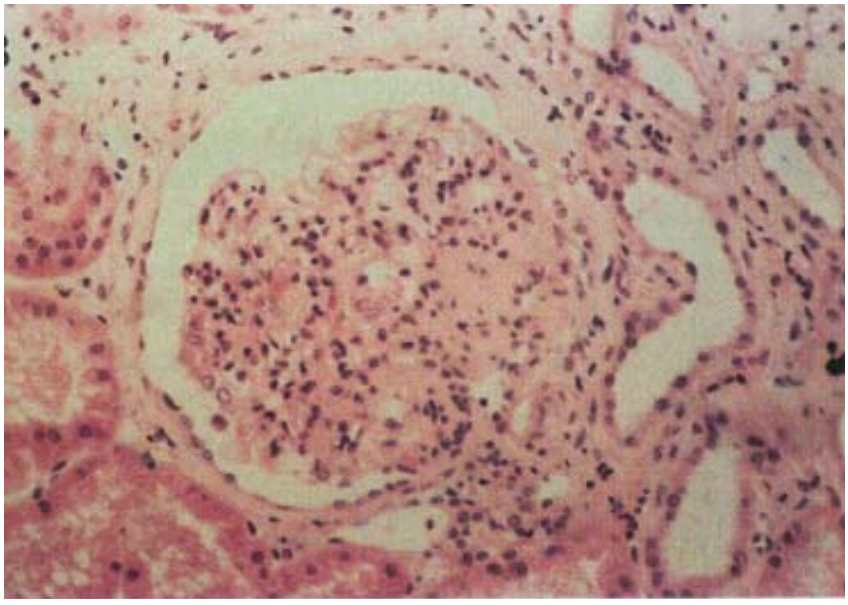

Figure 3-6. Renal cortical biopsy from a RCS patient (278)

3.6.4. Animal Models of Pax2 Mutations

Three different Pax2 mutant mice have been reported: Kidney and retinal defect (Krd) mice (280), $Pax2^{1Neu}$ mice (281), and Pax2 knockout mice (219). The mutation

in Krd mice is transgene-induced with approximately 7 cM chromosomal deletion that includes the Pax2 locus on chromosome 19. $Krd^{/+}$ mice have kidney defects, including aplastic, hypoplasic and cystic kidneys. Homozygosity ($Krd^{-/-}$) results in early embryonic lethality (280). $Pax2^{1Neu}$ mice have a single point mutation, a G insertion with a string of 7 G's in exon 2, in the Pax2 gene. Heterozygous $Pax2^{1Neu}$ mice present reduced kidney size, renal agenesis and cystic kidneys. Homozygous mutant $Pax2^{1Neu}$ mice die at or soon after birth, and lack ureters, kidneys, and other parts of the genital tract (281). Pax2 knockout mice contain a neomycin cassette between exon 1 and exon 2, inducing the deletion of intervening DNA. Pax2 heterozygous mice ($Pax2^{+/-}$) exhibit a renal phenotype similar to that of homozygous mutant $Pax2^{1Neu}$ mice, including smaller kidneys, renal agenesis, delayed ureters and renal cysts. Pax2 homozygous knockout ($Pax2^{-/-}$) newborn mice lack kidneys, ureters and genital tracts (219).

3.6.5. Pax2 in Kidney Development

Experimental studies have demonstrated that Pax2, necessary for kidney development, is highly expressed in the UB as it undergoes branching morphogenesis. There was fewer UB tips and less branching in $Pax2^{1Neu+/-}$ mutant mice (277). Porteous et al. (262) demonstrated that kidney hypoplasia is associated with increased apoptosis of UB cells, with a reduced number of UB branches in the fetal kidneys of $Pax2^{1Neu+/-}$ mutant mice. Similarly, another study revealed a striking decrease in UB branching at E13.5 in metanephric kidneys from heterozygous Pax2 mutant mice (Figure 3-7) (282). Taken together, these experiments indicate that Pax2 is essential for UB branching.

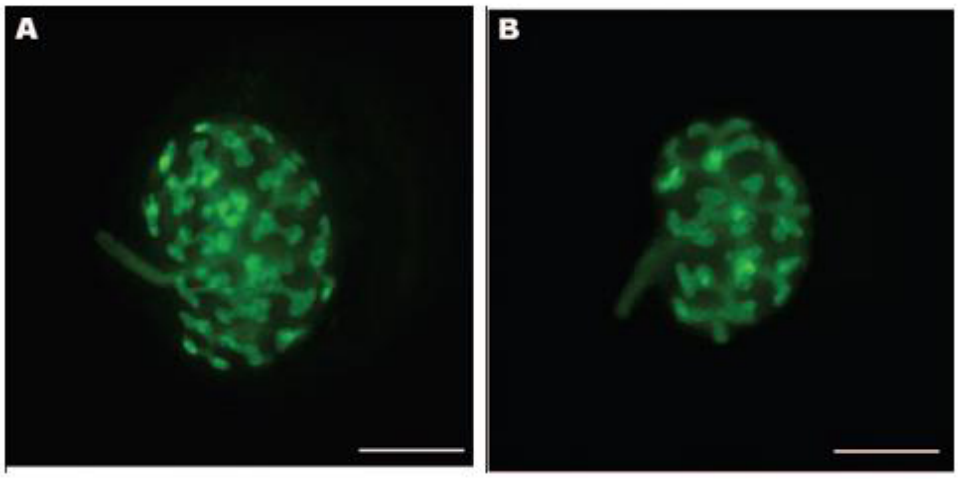

Figure 3-7. (A) Wild type kidney, (B) Pax2$^{1Neu+/-}$ mutant kidney (282)

Torres et al. (219) reported that during mesonephros development, Pax2 mutations cause renal defects, not only in WD and ureter formation, but also in tubule formation. In contrast, in Pax2$^{-/-}$ embryos, metanephric development simply does not occur since the UB is absent. Additionally, Pax2-null mutant mice (Pax2$^{-/-}$) fail to develop any kidneys, ureters and genital tracts (Figure 3-8 C and D), whereas heterozygous Pax2 mutations (Pax2$^{+/-}$) produce smaller kidneys (Figure 3-8 B).

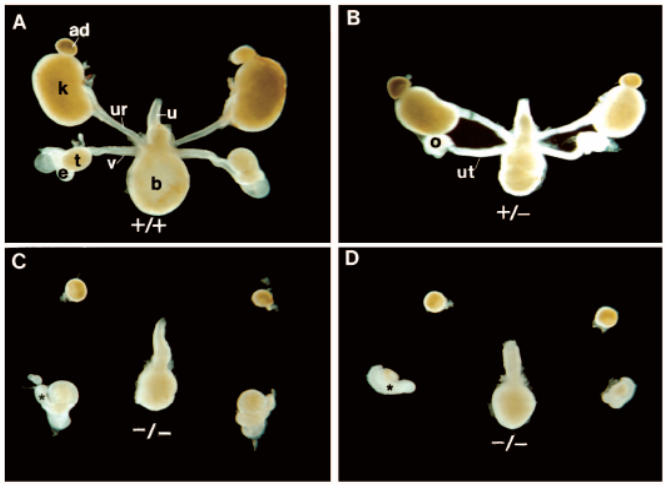

Figure 3-8. Analysis of urogenital system defects in Pax2 mutant mice (A-D). Whole mounts of dissected complete urogenital systems at E17.5 in male (A, C) and female (B, D) homozygous and heterozygous mutants and wild type fetuses (219)

In summary, Pax2 is an essential regulator of genitourinary axis development in mammals. Inappropriate or dysregulated Pax2 expression is associated with a number of malignancies and dysplasia, indicating that Pax2 plays an important role in cell growth and differentiation.

3.7 The GenitoUrinary Development Molecular Anatomy Project (GUDMAP)

The GenitoUrinary Development Molecular Anatomy Project (GUDMAP) is a consortium of laboratories working to provide the scientific and medical community with tools to facilitate research on the kidney and urogenital tract (UGT) (283). The GUDMAP aims to chronicle gene expressions during the development and maturation of the murine genitourinary tract and to create tools for the scientific community to examine the biological function of these genes (283). There are three components to the GUDMAP: (1) providing ontology of the cell types during urogenital tract development and the molecular hallmarks of those cells. Whole-mount *in situ* hybridization (WISH) is being used as a low-resolution approach to map gene expression domains and identify patterns that are diagnostic of specific anatomic regions or individual cell types. The WISH data also provide an excellent prescreen for further high-resolution section *in situ* hybridization (SISH) analysis. SISH and immunohistochemistry (IHC) provide high-resolution approaches that enable gene activity to be mapped at single-cell or near-signal-cell resolution within a tissue. (2) generating novel mouse strain. The generation of novel mouse strains is an important part of GUDMAP initiative. The consortium goal has been to identify cell-type or anatomic regions of interest within the UGT by the expression of a specific gene. The consortium goal is to generate 30 to 40 new mouse lines. (3) The Web-based GUDMAP Database. The Web-based GUDMAP database has been publicly accessible since April 2006. The Web-based GUDMAP

database includes primary WISH, SISH, IHC, microarray-based transcriptional profiling, mouse strain characterization data together with subsequent follow-up analyses of these data set, and information regarding methods, research tools, and community resources relevant to the UGT. The complete ontology for the genitourinary tract has been incorporated into the GUDMAP anatomy database (http://www.gudmap.org) (284). That database interface presents different views of the ontology according to the requirements of the user. The ontology will also be used in the EuReGene Renal Genomics Project (EuReGene) (http://www.euregene.org) and will be used to update the EMAP ontology used by the GXD (http://www.informatics.jax.org/mgihome/GXD/aboutGXD.shtml) (284).

CHAPTER 4: MATERNAL DIABETES MODULATES NEPHROGENESIS

4. Maternal Diabetes Modulates Nephrogenesis

Maternal diabetes has an adverse influence on intrauterine growth of the fetus, which is attributable to exposure of the mammalian embryo to an abnormal metabolic environment. When the fetus is exposed to high, sustained concentrations of peripheral glucose during the first 6 to 8 weeks of gestation in humans, widespread fetal damage may result in diabetic embryopathy, which is characterized by a multitude of congenital birth defects, including neural tube, heart, urogenital system, skeleton and alimentary tract defects and CRS (86). Also referred to as caudal dysplasia and sacral agenesis syndrome, CRS is a rare congenital defect characterized by absence of the sacrum and defects of variable portions of the lumbar spine in association with anomalies of different systems (285). The pathogenesis of RCS is unclear. It is diagnosed in only 0.1 to 0.25 of 10,000 normal pregnancies, but its prevalence is 200-250 times higher in diabetic pregnancies, occurring in up to 1% of pregnancies in diabetic women (286). Up to 22% of CRS cases in women are associated with either T1DM or T2DM, making it the most characteristic fetal abnormality of diabetic embryopathy (287).

4.1. Clinical Phenotypes or Outcome

As described in Section 1.6.2.1, 2 opposite abnormal situations regarding fetal growth are clinically associated with maternal diabetes (Figure 4-1). Fetal macrosomia is a common problem characterized by baby overgrowth, often in mild maternal hyperglycemia characterized by blood glucose values 20% higher than normal. Early pancreatic endocrine cells can be detected during the 7^{th} and 8^{th} weeks of human embryonic development. Pancreatic beta islets mature in the last quarter of gestation and are thus very sensitive to changes in glucose (288;289). High maternal

glucose levels are transmitted to the fetus by placental flow, causing fetal hyperinsulinemia, which, in turn, mediates accelerated fuel utilization, adipose tissue accumulation and excessive growth.

In contrast, maternal diabetes with severe hyperglycemia often leads to microsomic babies. This phenotype is referred to as fetal restriction or IUGR. Severe maternal hyperglycemia during pregnancy creates a rich glucose environment, and fetuses are confronted by very high glucose concentrations, inducing fetal beta cell hypertrophy and/or hyperplasia. These cells then become depleted of insulin and often appear to be disorganized. The degranulated cells are incapable of insulin secretion, and their exhaustion results in fetal hypoinsulinemia. Fetal hypoinsulinemia and reduced numbers of insulin receptors on target cells culminate in decreased fetal glucose uptake (147).

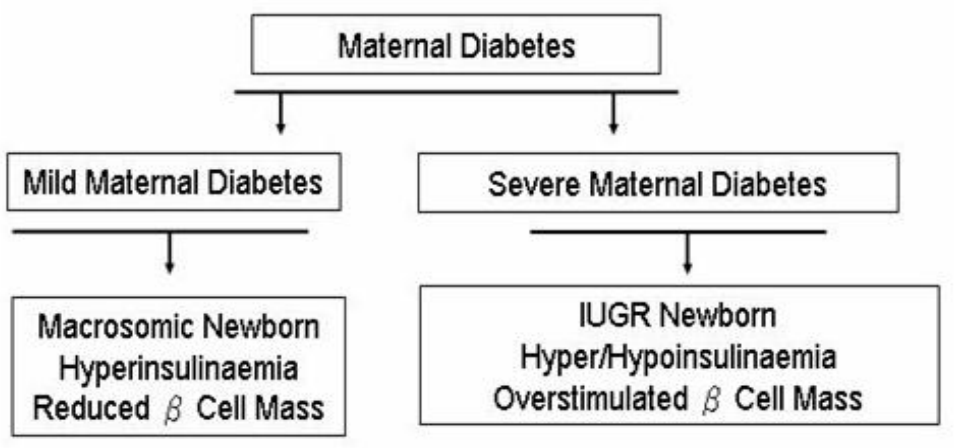

Figure 4-1. Two opposite, abnormal fetal growth situations associated with maternal diabetes

4.2. Possible Mechanisms in Maternal Diabetes Modulating Nephrogenesis

Diabetic mellitus is a major risk factor for congenital malformations. When the fetus is exposed to high, sustained ambient glucose levels, widespread fetal damage

may affect multiple organs, including the kidneys (diabetic embryopathy). The kidneys appear to be one of major organs targeted by diabetic embryopathy. Hyperglycemia constitutes an adverse uterine environment that dynamically and adversely impairs nephrogenesis, resulting in renal agenesis, dysplasia or aplasia (incomplete differentiation, often with cysts) and hypoplasia. However, the molecular mechanisms by which high, ambient glucose levels lead to renal dysmorphogenesis and birth defects have not yet been delineated. There is some experimental evidence, however, that hyperglycemia plays a significant role in this process (Figure 4-2) (95).

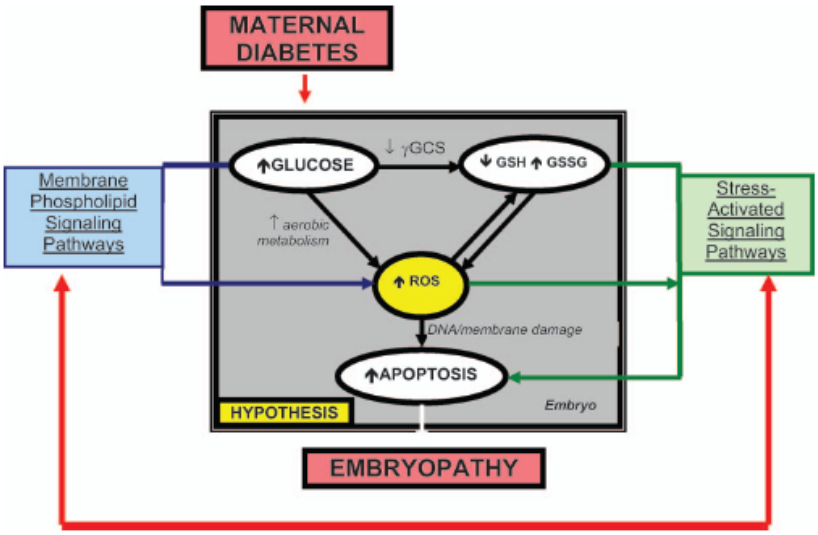

Figure 4-2. Hypothetical model of diabetes-induced embryopathy (95)

4.2.1. Hyperglycemia

Diabetes mellitus is a metabolic disorder in which hyperglycemia damages several organ systems, including blood vessels, nerves, muscles, eyes, and kidneys (290). The 4 main hypotheses associated with diabetic complications caused by hyperglycemia were summarized by Brownlee's group (291). They are: increased polyol pathway flux; augmented advanced glycation end-products (AGEs);

activation of protein kinase C (PKC) isoform; and heightened hexosamine pathway flux (Figure 4-3) (291).

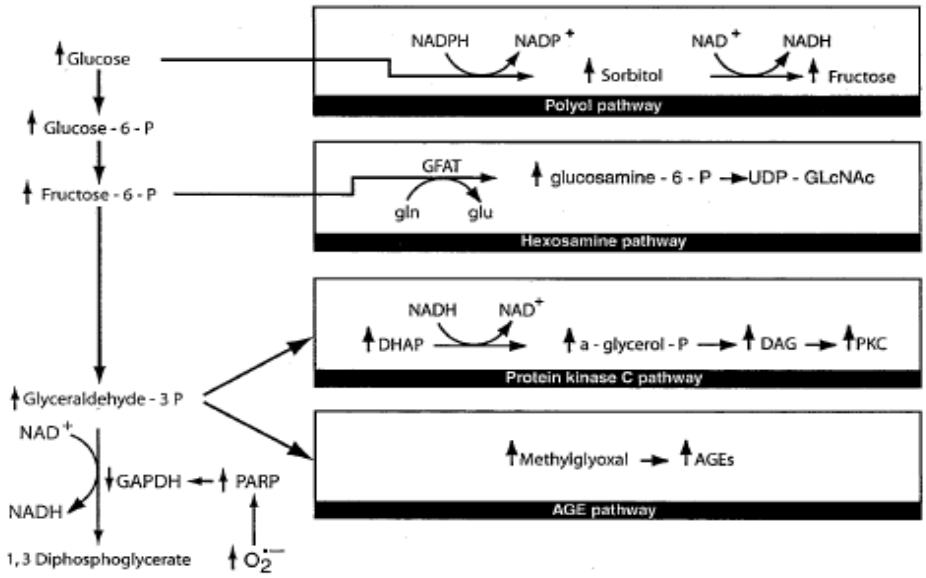

Figure 4-3. Potential mechanisms of hyperglycemic damage (291)

4.2.1.1. Hyperglycemia and Renal Malformations

Metabolic alterations in the diabetic mother have adverse effects on embryogenesis. Congenital malformations of various organs, including the kidneys, occur with increased frequency in the offspring of diabetic mothers (292). Experimental studies suggest that hyperglycemia is a major teratogen in diabetic pregnancies (293). Clinical investigations have demonstrated a positive correlation between hyperglycemia and fetal malformations (87), whereas good blood glucose control of diabetic mothers during this time period decreases the rate of fetal dysmorphogenesis (96). The mechanisms of hyperglycemia-induced renal malformation are not clearly defined. Amri et al. (256) postulated that hyperglycemia *in utero* impairs nephrogenesis in rats, resulting in a reduced number of nephrons in the kidneys of 14-day-old pups. Similarly, E13 embryos exposed to 30 mM D-glucose for 1 week show decreased metanephric size. The UB tips are

swollen, their branches are deformed and thickened, and their tips blunted (Figure 4-4) (294).

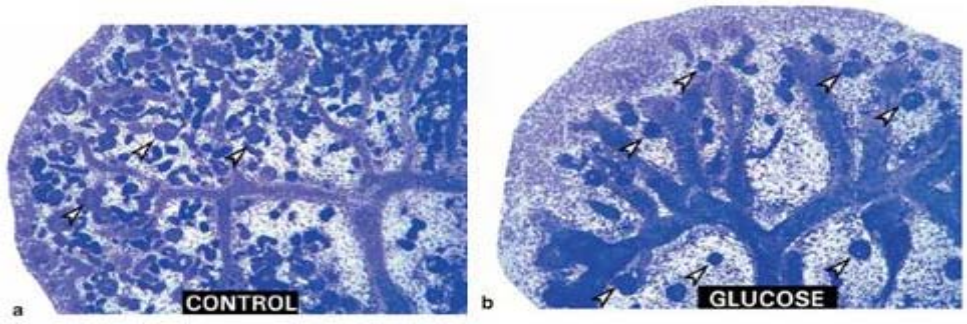

Figure 4-4. Light micrographs depicting the effect of a high glucose enviroment on the morphology of murine embryonic metanephros

Kanwar et al. (295) found that increased apoptosis in E13 metanephroi treated with 30 mmol/L D-glucose when compared to 5mmol/L D-glucose, Moreover, apoptosis was observed in the kidneys of newborn and 1-week-old mice pups from diabetic mothers. Taken together, hyperglycemia impairs kidney development *in utero*.

4.2.2. ROS

ROS include free radicals, such as superoxide ($\cdot O_2^-$), hydroxyl ($\cdot OH^-$), and peroxyl ($\cdot R\text{-}OO^-$), and non-radical species, such as hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) (296). There are a number of enzymatic and non-enzymatic sources of ROS in the diabetic kidney, such as xanthine oxidase (XO), peroxidase, nitric oxide synthase, nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase, AGEs and glucose auto-oxidation (297). Oxidative stress is increased in diabetes, and ROS overproduction is a direct consequence of hyperglycemia. Excessively high ROS levels lead to protein, lipid and DNA damage. ROS can activate transcription factors, such as nuclear factor kappa B (NF-kB) and

activated protein-1 (AP-1), signaling transduction cascades (PKC) and mitogen-activated protein kinases (MAPK), which result in ECM accumulation, glomerular mesangial expansion and tubulointerstitial fibrosis (298). Nam et al. (299) reported that stimulated ROS is significantly higher with NF-kB and AP-1 up-regulation in diabetic patients with DN than in diabetic patients without DN. Ha et al. (300) demonstrated that the antioxidant taurine effectively inhibits membrane translocation of PKC and PKCε in STZ-induced diabetic rat glomeruli, indicating that ROS activate PKC in the diabetic kidney as well. These observations suggest that increased ROS induced by hyperglycemia act as intracellular messengers and integral glucose signaling molecules in DN.

4.2.2.1. Hyperglycemia and ROS

The embryo appropriates both aerobic and anaerobic metabolic pathways during early development (301). Embryos grow under a relatively low oxygen concentration at E7.5-9.5 in mice and E9-11 in rats (302), and high oxygen levels are toxic to them (303). Developing embryos seem to be very sensitive to high ROS levels, especially during early organogenesis. Maternal hyperglycemia causes sustained ROS generation with depletion of antioxidants (304). In diabetic environments or hyperglycemia, increased ROS production leads to embryonic dysmorphogenesis, and ROS scavenging enzymes can reduce the rate of abnormalities in cultured E10.5 rat embryos (305). Hyperglycemia induces embryonic malformations in rats because of glutathione (GSH) depletion, as GSH ester supplementation decreases the formation of free oxygen radical species, virtually normalizing growth retardation and embryonic dysmorphogenesis (306). A hyperglycemic environment *in utero* affects the embryo by increasing ROS, leading

to genetic variations and damage caused by oxidative stress, with excessive apoptosis and impaired organogenesis (307).

4.2.3. Apoptosis

Apoptosis plays an important role in the morphogenesis of various systems. Proper regulation of apoptosis is essential for normal nephrogenesis and to maintain normal renal function in adulthood (308). The kidney mesenchyme is divided into 2 types: the MM and the stromal mesenchyme (SM). The MM gives rise to parts of the nephron from Bowman's capsule to the distal tubule, while the SM does not differentiate into nephrons or the collecting duct system. Most undifferentiated SMs differentiate into interstitial cells. Early in the developing kidney, the uninduced MM is programmed for apoptosis to prevent it from converting to epithelia and then differentiating (308). The undifferentiated SM undergoes apoptosis to free space for the expanding loops of Henle (309). On the other hand, an imbalance of apoptosis in kidney development causes abnormal kidney morphology. Bcl-2, an anti-apoptotic gene, is normally expressed in the UB, metanephric cap tissue, and primitive tubular structures at El2 in mice (310). Sorenson et al. (311) demonstrated that bcl-2-deficient mice develop small kidneys, contain far fewer nephrons and have smaller nephrogenic zones in newborns, resulting from excessive apoptosis within the developing metanephric kidney. Mice carrying a Pax2 (1Neu) mutation show exaggerated apoptosis with reduced UB branching (262). In adult kidneys, apoptosis is normally observed at low levels, but can increase because of hyperglycemia or oxidative stress due to disease.

4.2.3.1. Hyperglycemia and Apoptosis

Both acute and chronic hyperglycemia cause oxidative stress and trigger tubular and glomerular cells into apoptosis, producing DN (296;312). Recent studies have demonstrated that loss of podocytes is an early feature of DN (313). For example, Susztak et al. (314) showed that podocyte apoptosis increases with the onset of hyperglycemia in $Ins2^{Akita}$ mice with T1DM and in $Lepr^{db/db}$ mice with obesity and T2DM. Hyperglycemia can also induce mesangial cell apoptosis, eliciting ECM accumulation. High glucose evokes ROS-dependent apoptosis in mesangial cells via Bax-mediated mitochondrial permeability and subsequent cytochrome c release (315). Functional and structural changes in renal proximal tubular cells (RPTCs) are associated with DN progression. Liu et al. (189) observed that high glucose evokes ROS generation and apoptosis in both rat immortalized renal proximal tubular cells and STZ-induced diabetic mice. Antioxidant administration to animals has protective effects that attenuate DN development (316). The antioxidant taurine inhibits high glucose-induced ROS formation and apoptosis in human renal tubule cells (317).

4.2.4. The NF-kB Pathway

NF-kB was first discovered as a factor bound to the k light-chain immunoglobulin enhancer in nuclei of B lymphoid lineage (318). Emerging evidence demonstrates that the NF-kB signaling pathway is important in the control of cell growth, differentiation, the immune response, inflammation, and the stress response (319). Indeed, NF-kB is a regulator of programmed cell death, either by apoptosis or necrosis, since it possesses both pro- and anti-apoptotic qualities (320). The NF-kB family has 5 proteins: RelA (p65), RelB, Rel, NF-kB1 (p50), and NF-kB2 (p52). Each NF-kB family member may form homo- or heterodimers. As

depicted in Figure 4-5 (321), activated NF-kB is a heterodimer, which usually consists of p65 and p50 or p52 and RelB. Without specific extracellular signal stimulation, NF-kB is sequestered in the cytoplasm as an inactive heterodimer composed of subunits p50 and p65 complexed with the inhibitory protein IkB. Stimuli, such as hyperglycemia, elevated free fatty acids, ROS, and ultraviolet irradiation, trigger a cascade of serine kinase that activate IkB kinase (IKK) phosphorylation leading to IkBa dissociation from the NFkB heterodimers, and degradation in the cytoplasm. The released NF-kB heterodimer is translocated to the nucleus and then regulates the expression of a large number of genes (322).

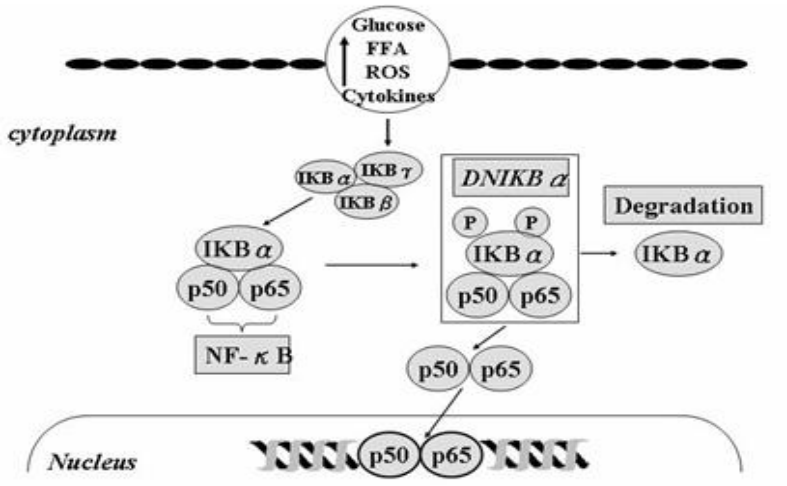

Figure 4-5. The NF-kB pathway (modified from Evans et al.: Endocr Rev) (321)

An important role of NF-kB in epidermal homeostasis and the development of skin appendages has been reported recently. The crucial involvement of NF-kB in embryonic development was demonstrated by Beg et al. (323) in IkBa-deficient mice in 1996. These animals exhibit severe runting and skin defects in the first days after birth, with major skeletal deformities. Mice heterozygous for IKKγ have skin lesions with granulocyte infiltration, keratinocyte apoptosis and abnormal

development of teeth, eyes, and hair (324). Such results underscore the importance of NF-kB in both embryonic development and normal tissue growth.

4.2.4.1. Hyperglycemia and the NF-kB Pathway

The transcription factor NF-kB is a major intracellular target of hyperglycemia (325). Peripheral blood mononuclear cells isolated from patients with DN show a positive correlation with NF-kB activation (326), which is associated with inflammatory processes, and an increase in NF-kB nuclear translocation has been documented in human DN (327). Schmid et al. (328) established that NF-kB-regulated genes are up-regulated in progressive DN, emphasizing the importance of the hyperglycemia-activated NF-kB pathway in human DN. Kuhad et al. (329) found that after 8 weeks of STZ-induced diabetes, rats manifest significant alterations in renal function, and the active p65 subunit of NF-kB can be detected in nuclear lysates of diabetic rat kidneys.

4.2.4.2. Oxidative stress and the NF-kB Pathway

It is well known that NF-kB is critically involved in the oxidative stress response. Oxidative stress is a frequent component of aging, immune disorders, inflammatory diseases, metabolic diseases, and cancers. When ROS are formed intracellularly, NF-kB is up-regulated, leading to the expression of cytokines, chemokines, cellular adhesion molecules, and inflammatory enzymes that activate the immune and inflammatory system. Increased oxidative stress and subsequent NF-kB activation have been linked to the development of late diabetic complications. Hofmann et al. (326;330) found a correlation between NF-kB activity and the severity of albuminuria in DN. Patients with diabetes also show significant suppression of NF-kB activation during treatment with the antioxidant thioctic acid (α-lipoic acid).

These obvervations confirm that hyperglycemia-induced diabetic complications result from the oxidative stress-mediated NF-kB pathway, causing cellular damage. In T1DM, pancreas-specific ROS production is critical in the signal transduction response by activating NF-kB (331;332).

4.2.4.3. Apoptosis and the NF-kB Pathway

NF-kB is a major player that coordinates innate and adaptive immunity, cellular proliferation, apoptosis and development (333). It has been shown that H_2O_2 induces apoptosis through NF-kB activation (334). Ang II elicits podocyte apoptosis via extracellular signal-regulated kinase pathway activation and subsequent NF-kB translocation (335). Schneider et al. (336) observed that NF-kB promotes apoptosis in focal cerebral ischemia. These observations suggest that NF-kB activation regulates apoptosis. The mechanisms associated with NF-kB-mediated apoptosis are not clear, and the role of p53 and c-Myc induction through NF-kB has been studied (337).

4.2.4.4. The NF-kB and Protein 53 or Tumor Protein 53 (p53) Pathways

Both the NF-kB and p53 pathways can be stimulated (338). As discussed above, hyperglycemia activates the NF-kB pathway and induces apoptosis mediated by p53. The mechanisms of hyperglycemia-induced apoptosis mediated by the NF-kB and p53 pathways are poorly defined. Studies have demonstrated that NF-kB-binding sites are present in the promoter regions of p53 (339), and p53 expression can be activated by NF-kB (340). Thus, NF-kB may play a role in p53 regulation in response to certain types of stress (341). Gilli et al. (342) established that NF-kB is an intracellular pathway triggered by glutamate and leads to apoptosis in which p53 induction lies downstream of NF-kB activation. Nakai et al. (337) provided

additional support for the view that NF-kB activation contributes to p53 induction and subsequent apoptosis in an excitotoxic model of Huntington's disease.

4.2.5. The p53 Pathway

Exposure to cellular stress can trigger the tumor suppressor p53, a sequence-specific transcription factor, to elicit cell growth arrest or apoptosis. p53-mediated apoptosis was first reported in 1991 by Yonish-Rouach et al. (343). In mammalian cells, p53 can activate both the extrinsic and intrinsic apoptotic pathways. The extrinsic apoptotic pathway is induced by transmembrane proteins, such as Fas, DR5, and p53 apoptosis effector related to PMP-22, activating caspases, including caspase-8 and caspase-3, which provoke apoptosis. The intrinsic apoptotic pathway is controlled by the Bcl-2 family of proteins, which releases cytochrome c from the mitochondria. p53 stimulates apoptosis through cytochrome c and procaspase-3 (Figure 4-6) (344). Several events can regulate p53 gene expression and activity, including post-translational modification and protein-protein interactions in cells. Phosphorylation of the amino acid serine 15 (Ser15), which increases p53 stability and apoptosis, is one of the important mechanisms that regulates p53 protein level and activity (345). Several kinases, including ataxia telangiectasia mutated kinase, A-T-related kinase, and dsDNA-activated protein kinase, initiate signaling pathways through p53 phosphorylation at Ser15 (346).

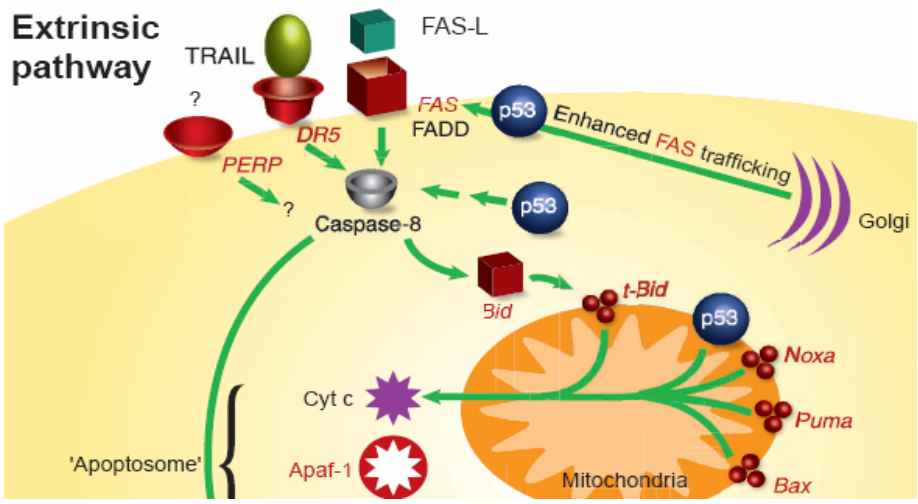

Figure 4-6. A model of p53-mediated apoptosis (344)

4.2.5.1. Hyperglycemia and the p53 Pathway

The mechanisms of apoptosis evoked by hyperglycemia have been investigated. Growing evidence has defined the induction of apoptosis by p53 (347). Studies support the idea that p53 can elicit apoptosis by directly signaling the mitochondria (348) and prompting cytochrome c release (349). It has been shown that p53 provokes apoptosis under conditions of cellular stress, including hyperglycemia and ROS (350;351). Ortega-Camarillo et al. (352) demonstrated that hyperglycemia promotes apoptosis in RINm5F cells, the insulin-secreting cell line, via an increase in ROS production, and is associated with p53 mobilization to the mitochondria. Fiordaliso et al. (353) have reported that hyperglycemia induces myocyte apoptosis by activating p53. In addition, Keim et al. (354) showed that high glucose heightens p53 expression and activates Bax expression, downstream of p53, in mouse blastocysts.

4.2.5.2. The p53 Pathway and Kidney Development

p53 in humans is encoded by the *TP53* gene and expressed during kidney development. Carev et al. (355) observed that p53 is neither expressed in the mesonephros nor in the metanephros, but can be found in the coelomic epithelium

during the 5th and 6th weeks of development. p53-positive cells appear in the mesonephros for the first time at the beginning of the 7th developmental week, except in the WD and Bowman's capsule. During the 8th and 9th weeks of human kidney development, p53 is detected in all structures of the developing metanephros, indicating its importance in the morphogenesis of both the collecting system and nephrons (Table 4-1) (355).

Table 4-1. Immunoreactivity to specific antibodies in the human mesonephros and metanephros during the 7th, 8th and 9th developmental weeks

	Antibodies								Structure
	Ki-67		p53		bcl-2		casp-3		
Weeks of development	7	8 and 9	7	8 and 9	7	8 and 9	7	8 and 9	
Wolffian duct	++	+++	−	−	+	−	++	+	MESONEPHROS
Mesonephric mesenchyme	++	++	+	+	+	+	+	+	
Glomeruli	++	++	+	+	+	+	++	+	
Bowman's capsule	++	++	+	+	+	++	++	+	
Mesonephric tubules	++	++	+	+	+	++	++	+	
Coelomic epithelium	++	++	+	+	−	+	++	−	
Collecting tubules	++	++	−	+	−	−	−	++	METANEPHROS
Ampulla	+++	+++	−	++	−	−	−	++	
Ureter	++	++	−	/	−	/	−	/	
Interstitium	++	++	−	+	+	+	++	++	
Renal vesicle	++	+++	−	+	++	+++	+	+	
S-shaped nephrons	+++	+++	−	+	+	++	+	+++	
Renal corpuscle	/	+++	−	+	/	++	/	+++	

+++ strong reactivity, ++ moderate reactivity, + mild reactivity, − no reactivity, / structure absent in the tissue section

4.2.5.3. The p53 Pathway and Renal Malformation

Apoptosis plays an important role in nephrogenesis, a process that requires structural formation and reformation. p53 is an active component of the apoptosis cascade and appears during kidney development (355). p53 Tg mice present defective differentiation of the UB and hypoplastic kidney due to increased apoptosis in the undifferentiated mesenchyme, resulting in smaller kidneys and about half the normal number of nephrons, with compensatory hypertrophy of the

glomeruli (356). Lichnovsky et al. (357) showed that p53 overexpression can cause defects in human kidney development. As IUGR influences the formation of nephrons, it has been suggested that apoptosis excessively clears progenitor cells during renal development. However, apoptosis is found to be altered by IUGR (185). Baserga et al. (358) demonstrated impaired renal function in the IUGR rat kidney that they attributed to increased p53 phosphorylation at Ser15.

4.2.6. The Intrarenal RAS

The RAS is well-known to play an important role in blood pressure regulation, renal hemodynamics, and fluid and electrolyte homeostasis (359). Angiotensinogen (Agt), the sole substrate and a precursor of Ang, is released from the liver and secreted into the circulation. Circulating plasma renin cleaves Agt to form the decapeptide Ang I, which is converted to the octapeptide Ang II by ACE, a membrance-bound metalloproteinase. Ang II interacts with 2 types of receptors, AT1R and AT2R. Most of its physiological effects are mediated through AT1R. In addition to classical RAS components, several new constituents have been discovered in recent years. ACE2, a homologue of ACE, converts Ang I to Ang 1-9 and Ang II to Ang 1-7 (360). Mas proto-oncogene is a receptor for Ang 1-7 (361). In addition, (pro)renin receptors, which bind and activate renin and prorenin in tissues, has been discovered recently (362).

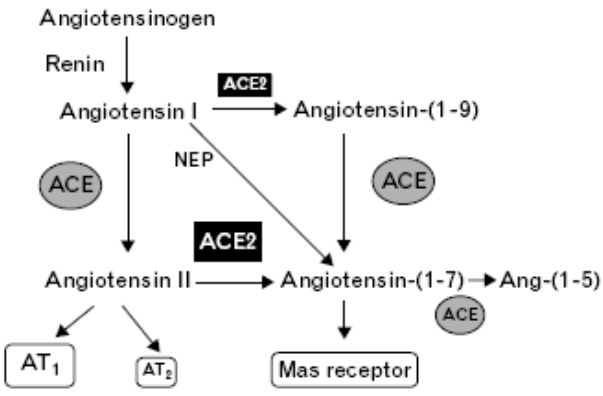

Figure 4-7. The RAS (363)

4.2.6.1. The Intrarenal RAS and Kidney Development

The Intrarenal RAS plays a fundamental role in kidney development. All RAS components are expressed in the metanephric kidney of rodents (Table 4-2) (204). Intrarenal RAS activity is high during fetal and neonatal life and declines during postnatal maturation. Schutz et al. were the first to demonstrate the presence of all RAS components in very early human development (364). In humans, Agt expression is first found in immature tubules as early as E30 and persists throughout embryonic life; renin mRNA expression is detected in the mesonephros and is confined to the juxtaglomerular (JG) apparatus in the metanephros at E25-27. ACE appears at nearly E30 and is expressed in proximal tubules; AT1R mRNA is expressed in glomeruli around E25-27, and AT2R mRNA is evident in the undifferentiated mesenchyme from E23-24 (364). As shown in Table 4-2, RAS components are expressed during metanephric kidney development in rats and in mice.

Table 4-2. Expression of RAS components during metanephric kidney development (204)

	E12	E14	E15	E16	E19
AGT	Mouse: UB, SM	UB, SM, PT	Rat: UB, SM	UB, SM, PT	PT
Renin	Mouse: precursor cells present		M of entire kidney Rat: V	M, close to V and G V	V, G V
ACE				Rat: PT, G, CD	
AT_1	Mouse: UB, M	UB, G	UB, V	PT, UB, SM, G	PT, DT
AT_2	Mouse: MM	MM, SM Rat: MM	Rat: SG, UB, SM	SM Medullary SM, under renal capsule Condensed M	PT, CD, G Medulla, G, V

AGT: angiotensinogen, ACE: angiotensin-converting enzyme, AT_1/AT_2: angiotensin II receptors, UB: ureteric bud, M: mesenchyme, SM: stromal mesenchyme, PT: proximal tubule, G: glomeruli, V: renal vessels, CD: collecting duct

Immunohistochemistry has demonstrated that Agt and AT1 are expressed in the UB and SM in mice at E12 (204). Ang II stimulates UB branching via AT1R activation *in vitro* (365). In *ex vivo* studies, exogenous Ang II increases UB branching but can be blocked by the AT1R antagonist candesartan (366). These findings show that Ang II stimulates UB branching morphogenesis via AT1R. AT2R is important in organogenesis as it is abundantly expressed in fetal tissues of mice (367), rats (368) and primates (369). Zhang et al. documented that Ang II stimulates Pax2 expression via AT2R in the metranephric kidney (370). AT2R mutant mice exhibit ureteral budding and duplicated collecting systems (371). Such observations suggest that AT2R expression may be important in embryological development of the urinary tract. In addition, the major Ang II fraction present in renal tissues, including the proximal tubules, renal interstitium, and renal lymph, is generated from Agt produced locally by RPTCs (372-374). Renin is secreted by JG cells and delivered to the renal interstitium. ACE is localized in proximal tubules, distal tubules, the collecting ducts and renal endothelial cells. Therefore, all components necessary to generate intrarenal Ang II are present along the nephron. Ang II is important in the regulation of renal blood flow, glomerular filtration, and tubular sodium reabsorption. In addition, ACE2, a new member of the RAS, express in the kidney on the luminal surface of tubular epithelial cells (360). Alenina et al. (375) reported that Mas mRNA is abundant in mouse renal cortex. The role of the ACE2-Ang-(1-7) –Mas axis in kidney development and UB branching morphogenesis remains to be determined. However, ACE2 homology collectrin is expressed in the UB branches as early as E13 in the mouse (376).

4.2.6.2. The Intrarenal RAS and Renal Malformation

The genetic interruption of RAS components in mice causes a series of abnormalities in development of the ureters, renal pelvis and papilla (377-382). Agt, renin, ACE or AT1R deficiency in mice results in vascular thickening, interstitial fibrosis, reduced ability to concentrate urine and specific UB/collecting system changes, including atrophy of the papilla and medulla with hydronephrosis (Table 4-3) (204). In addition, AT2R gene deletion in mice causes congenital abnormalities of the kidney and urinary tract (371). Chen et al. (383) studied the effect of AT2R deficiency on renal malformations and showed that a lack of AT2R alters the expression of Pax-2 and N-Myc genes during nephrogenesis.

Table 4-3. Effect of genetic RAS inactivation in mice on renal collecting system development (204)

Gene	Function of gene	Renal phenotype	UB/collecting system phenotype
AGT	Renin substrate	Vascular thickening; interstitial fibrosis; delayed glomerular maturation; reduced ability to concentrate urine	Hypoplastic papilla hydronephrosis
Renin	Enzyme which generates ANG I from AGT	Arterial wall thickening; interstitial fibrosis; glomerulosclerosis	Hypoplastic papilla hydronephrosis
ACE	Enzyme which generates ANG II from AGT	Arterial wall thickening; reduced ability to concentrate urine	Hypoplastic papilla and medulla hydronephrosis
AT_1	ANG II receptor	Decreased kidney weight; delayed glomerular maturation; arterial wall thickening; interstital fibrosis; tubular atrophy; reduced ability to concentrate urine	Hypoplastic papilla and medulla hydronephrosis
AT_2	ANG II receptor		Duplicated ureters; hydronephrosis

The RAS has been reported to be associated with abnormal kidneys and decreased nephron numbers in experimental studies. Woods et al. (167) showed that suppression of RAS components, such as renin and Ang II, in the developing fetus/newborn leads to impaired renal development and lower nephron numbers at birth, culminating in adult hypertension in a maternal protein restriction model.

4.2.6.3. Hyperglycemia and the Intrarenal RAS

There is evidence of an association between the development of diabetes and RAS activation. However, clinical and experimental studies have demonstrated that ACE inhibitors and AT1R blockade may have potent beneficial effects by delaying cardiac disease in diabetes mellitus (384-387). Increased renin expression has been found in proximal tubules from STZ-induced diabetic rats, and renin expression after insulin treatment completely regressed to control values, suggesting intrarenal RAS activation by hyperglycemia (388). Liu et al. (189) have shown that apoptosis in proximal tubules in DN is exacerbated by Agt overexpression. Yoo et al. (389) noted that high glucose induces Agt mRNA and protein expression in cultured podocytes via the PKC pathway, and increased Agt and AT1R expression is also seen in podocytes of diabetic glomeruli. It has been established that the effect of maternal diabetes is associated with consequent hyperglycemia and impaired cardiovascular function in the offspring (390). Wichi et al. (391) determined that hyperglycemia during pregnancy produces long-lasting hypertension in male offspring with enhanced tissue ACE activity.

CHAPTER 5: EXPERIMENTAL APPROACHES

5.1 *In Vitro* Studies

5.1.1 cell line

MK4 cells representing late embryonic MM undergoing epithelial conversion were created from Tg mice with Simian Virus 40 T-antigen gene driven by the Hoxa 11 promoter (392). As illustrated in Figure 6-1 (392), MK4 cells are smaller, more polygonal or epithelial in shape with a cobblestone-like appearance at confluence. Morphologically, they represent a later stage of nephrogenesis when mesenchymal cells are converting to epithelia. MK4 cells express several gene characteristics of MM cells undergoing epithelial conversion, including E-cadherin, Wnt4, Pax8, Pax2, Cadherin-6, collagen IV, and LFB3 (392). In the present studies, mouse MK4 cells were tested as *in vitro* models and cultured in normal glucose or high glucose either with or without inhibitors of signaling pathways. They were obtained from Dr. Steven Potter (Children's Hospital Medical Center, Cincinnati, OH, USA).

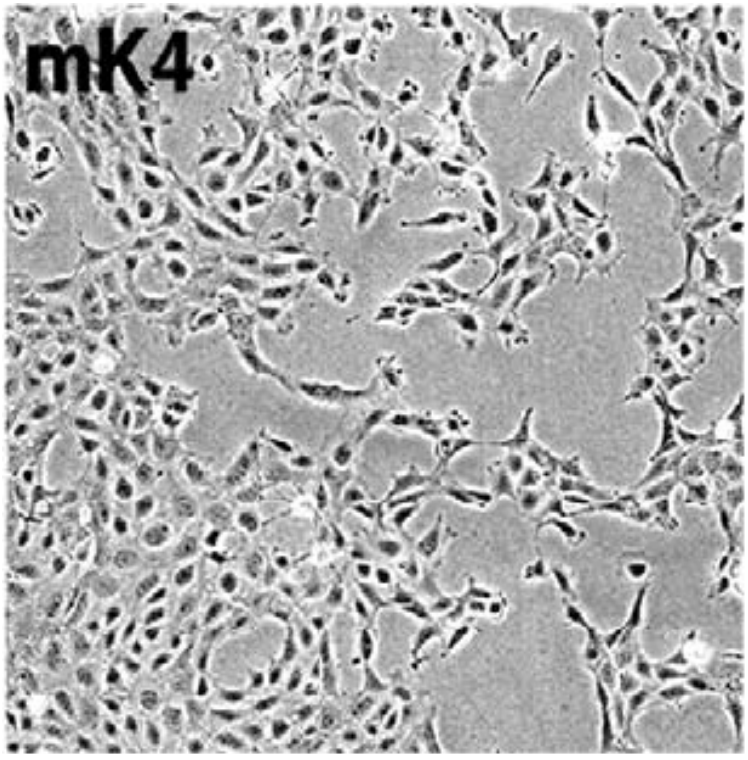

Figure 5-1 MK4 cell morphology (392)

5.1.2 Brief description of different glucose analogues

We have employed the four glucose analogs including D-glucose, L-glucose, D-Mannitol and 2-Deoxy-D-glucose in our studies *in vitro* and *ex vivo*.

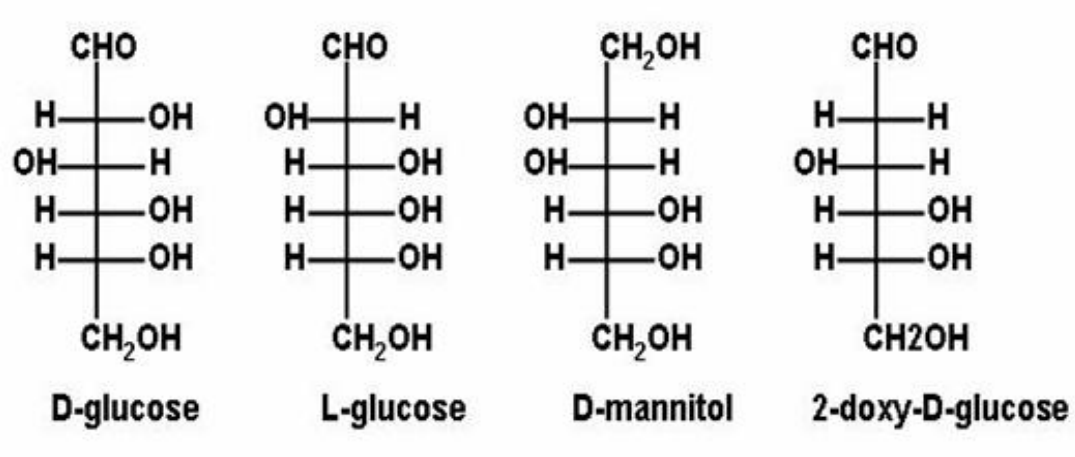

Figure 5-2 The structure of D-gluocse, L-glucose, D-Mannitol and 2-Deoxy-D-glucose

(1) D-glucose is the right-handed form of glucose ($C_6H_{12}O_6$) and plays a major role in biologically active and function as a source of energy and metabolic intermediates. D-glucose is synthesized in the liver and kidneys from non-carbohydrate intermediates, such as pyruvate and glycerol, by a process known as gluconeogenesis; Through glycolysis and later in the reactions of the citric acid cycle (TCAC), glucose is oxidized to eventually form CO_2 and water, yielding energy sources, mostly in the form of ATP. In our studies, we have demonstrated that high D-glucose (25mM) as compared normal glucose (5mM) specifically induced Pax2 gene expression in MK4 cells.

(2) L-glucose is an enantiomer of D-glucose with mirror-image form. It does not occur naturally in higher living organisms and cannot be metabolized in the biochemical process known as glycolysis. In our studies, we have observed that

there is no any stimulatory effect of L-glucose on Pax2 gene expression indicating that the stimulatory effect of D-(+) glucose on Pax2 expression in MK4 is specific.

(3) D-Mannitol: D-Mannitol is a sugar alcohol and derived by reduction of d-fructose or of d-mannose. *D*-Mannitol is widely used in the food and pharmaceutical industries because of its unique functional properties. D-Mannitol is non-cariogenic and has a low caloric content and often used as a sweetner or laxative. D-mannitol is used as an osmotic diuretic agent because that it could be filtered in the glomerulus, but cannot be reabsorbed. Their presence leads to an increase in the osmolarity of the filtrate. In our studies, we have added the supplement of D-mannitol (20 mM) into 5 mM glucose DMEM to maintain constant isotonicity or osmolality as in 25mM glucose DMEM. Our data indicates that the stimulatory effect of high glucose level on Pax2 expression in MK4 is via the specific D-glucose effect, not the osmolality stress effect.

(4) 2-Deoxy-D-glucose: 2-Deoxy-D-glucose is a glucose molecule which has the 2-hydroxyl group replaced by hydrogen, so that it cannot undergo further glycolysis. 2- Deoxy-D-glucose is uptaken by the glucose transporters of the cell. Therefore, cells with higher glucose uptake have also a higher uptake of 2- Deoxy-D-glucose. In our studies, we have observed that there is no any stimulatory effect of 2-Deoxy-D-glucose on Pax2 gene expression.

5.2. *Ex Vivo* Studies

5.2.1. Animals

Hoxb7-GFP mice specifically express GFP in UB driven by the Hoxb7 promoter (393). This model is interesting in that real-time observation of UB development is possible under fluorescence microscopy because GFP permits direct study of the branching process. As seen in Figure 6-2 (393), GFP is expressed throughout the

WD and each branch of the UB, but not in the surrounding MM or its epithelial derivatives throughout kidney development. As GFP can be easily visualized in living tissue, we can follow the dynamic pattern of UB growth and branching morphogenesis in organ culture to directly investigate the UB branching morphogenesis pattern under nondiabetic and diabetic conditions *ex vivo*. In the present studies, murine Hoxb7-GFP mice served as an *in vitro* model. They were obtained from Dr. Frank Costantini (Department of Genetics and Development, Columbia University Medical Center, New York, NY, USA).

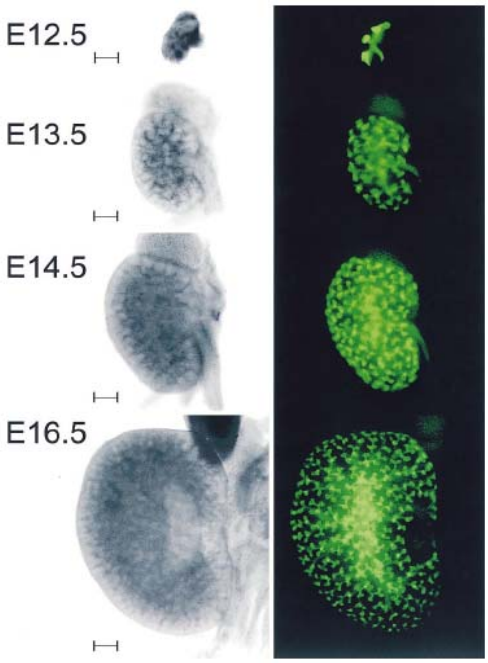

Figure 5-3. GFP expression in UB derivatives of Tg fetuses at E12.5 through E16.5 (393)

5.2.2. Metanephric Organ Culture

Kidney explants (E12 to E18) were microdissected from timed-pregnant mice. GFP-positive metanephroi were photographed immediately after isolation (time 0) and were individually cultured in normal glucose or high glucose either with or without inhibitors of signal pathways at different time points.

5.3. *In Vivo* Studies

5.3.1. Animals

In our *in vivo* experiments, we investigated 2 Tg mice lines: Hoxb7-GFP mice (as described above) and Nephrin-CFP mice. Nephrin-CFP mice were obtained from Dr. Susan Quaggin (University of Toronto, Toronto, ON). As shown in Figure 6-3, Nephrin-CFP mice specifically express Cyan fluorescent protein (CFP) in glomeruli driven by the podocyte-specific nephrin promoter. Nephrin-CFP mice allow us to follow the glomerular morphogenesis pattern during nephrogenesis under nondiabetic and diabetic conditions *ex vivo*.

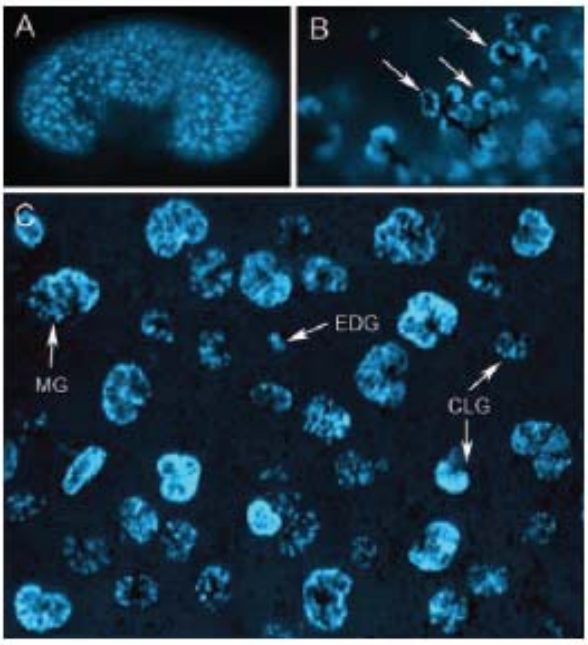

Figure 5-4. Glomerular isolation under dissecting microscopy. EDG: early developing glomeruli (late S shape/early capillary loop); CLG: capillary loop glomeruli; MG, mature glomeruli

5.3.2. Maternal Diabetes Animal Models

5.3.2.1. STZ Injection

STZ has been generally administered to induce T1DM, based on its efficiency in selectively destroying pancreatic beta cells (394). This method has been employed to elucidate the effect of maternal diabetes on fetal growth and development. The phenotype of the offspring is associated with the STZ dose given during pregnancy (395). Low-dose STZ results in mid-maternal diabetes, and fetal macrosomia (396). On the other hand, high-dose STZ induces insulin deficiency diabetes and fetal microsomia (397).

5.3.2.2. BioBreeding (BB) Diabetes-prone Rats

The BB rat is a model of spontaneous T1DM development. Experimental study of spontaneous autoimmune T1DM in BB rats has shown that the effects of severe hyperglycemia on the BB rat pancreas and fetal growth *in utero* are similar to the metabolic conditions induced by STZ injection to create a severely hyperglycemic environment (398). Eriksson et al. (399) demonstrated that the offspring of spontaneously diabetic BB/E rats had smaller kidneys than those of non-diabetic controls.

5.3.2.3. Ins2 Akita Mice

The Ins2 Akita mouse is an autosomal dominant mutant T1DM model. Experimental studies in Ins2 Akita mice have shown that maternal T1DM does not cause cardiac hypertrophy or triglyceride accumulation in the fetal heart, possibly because genes controlling fatty acid uptake are down-regulated (400).

5.3.2.4. Non-obese Diabetic (NOD) Mice

The NOD mouse, with spontaneous development of beta cell failure, may also serve as a model of maternal diabetes with fetal hyperinsulinemia and macrosomia (401;402).

5.3.2.5. Heterozygous $Lepr^{db/+}$ Mice

The db/db mouse is one of the models of T2DM. Heterozygous leptin receptor-deficient ($Leprd^{db/+}$) mice develop spontaneous glucose intolerance during pregnancy, and the offspring of these mothers heterozygous for the leptin receptor ($Lepr^{db/+}$) are macrosomic (403). The offspring of heterozygous $Lepr^{db/+}$ mothers have HBW compared to the controls, as depicted in Table 6-1 (404). Heterozygous $Lepr^{db/+}$ mice are a good model to study the fundamental role of maternal diabetes in HBW and modulation of renal morphogenesis in their offspring.

Table 5-1. Average fetal birth weight and litter size of wild type ($^{+/+}$) and heterozygous $db/^{+}$ mothers (404).

	+/+	*db*/+
Birth weight (g)	1.23 ± 0.02	1.33 ± 0.02*
n	56	66
Litter size (*n*)	5.5 ± 0.4	6.1 ± 0.3

Data are means ± SE. *$P < 0.01$, significantly different from +/+ mice.

5.3.2.6. Rich Diet-induced Rats

A rich diet (cafeteria type with 33% standard food for rats, 33% sweetened condensed milk, 7% sucrose and 27% water) primes the Wistar rat to develop GDM. Rich diet-induced rats are another experimental model used to demonstrate glucose

intolerance in mothers during pregnancy, emphasizing that obesity is one of the most important risk factors for GDM.

5.3.3. Our Maternal Diabetes Animal Models

In our laboratory, we generate maternal diabetes animal models by a single intraperitioneal (IP) STZ injection at a dosage of 150 mg/kg at E13 in Hoxb7-GFP mice. Compared to low-dose STZ, a single high-dose STZ creates a severely high glucose environment *in utero*, resulting in fetal microsomia (146;294). On the other hand, our model of severe maternal diabetes does not represent the phenotypes commonly found in humans in industrialized countries, or gestational diabetes with mild hyperglycemia control and fetal macrosomia.

CHAPTER 6: OBJECTIVES OF THE PRESENT STUDIES

Rationale: Diabetes mellitus is a major risk factor for congenital malformations. When the fetus is exposed to high, sustained, ambient glucose levels, widespread fetal damage may affect multiple organs, including the kidneys. Infants born to women with both PGDM and GDM have a relatively high risk of congenital malformations. Renal malformations account for 40% of childhood renal failure. Hyperglycemia constitutes an adverse *in utero* environment that dynamically impairs nephrogenesis, resulting in absent kidneys, small kidneys, or too few nephrons. Experimental studies have demonstrated that hyperglycemia in diabetic women triggers ROS production which causes oxidative stress. Oxidative stress somehow increases apoptosis and activates the apoptosis-inducing signaling pathway. However, the molecular mechanisms by which high ambient glucose levels lead to renal dysmorphogenesis and birth defects have not yet been delineated. Therefore, the objectives of the present studies are:

(1) To investigate whether high glucose alters Pax2 gene expression in the mouse embryonic MM (MK4 cells *in vitro*)

(2) To demonstrate the influence of a high-glucose milieu on UB branching morphogenesis (*ex vivo)*

(3) To dissect the fundamental role of maternal diabetes in renal morphogenesis impairment in offspring (*in vivo*)

CHAPTER 7 : ARTICLE 1

Reactive Oxygen Species (ROS) and NF-kB Pathway Mediate High Glucose-Induced Pax-2 Gene Expression in Mouse Embryonic Mesenchymal Epithelial Cells and Kidney Explants

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Keywords: High glucose, Pax-2, ROS and NF-kB

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7.1 Abstract

Diabetic mellitus confers a major risk of congenital malformations, and is associated with diabetic embryopathy, affecting multiple organs including the kidney. The DNA paired box-2 (Pax-2) gene is essential in nephrogenesis. We investigated whether high glucose alters Pax-2 gene expression and aimed to delineate its underlying mechanism(s) of action using both *in vitro* (mouse metanephric mesenchymal cells (MK4) and *ex vivo* (kidney explant from Hoxb7-GFP mice) approaches. Pax-2 gene expression was determined by RT-PCR, Western blotting, and immunofluorescent staining. A fusion gene containing the full-length 5'-flanking region of the human Pax-2 promoter linked to a luciferase reporter gene, pGL-2/hPax-2, was transfected into MK4 cells with or without dominant negative IκBα (DN IκBα) co-transfection. Fusion gene expression level was quantified by cellular luciferase activity. Reactive oxygen species (ROS) generation was measured by lucigenin assay. Embryonic kidneys from Hoxb7-GFP mice were cultured *ex vivo*. High D(+) glucose (25 mM), compared to normal glucose (5 mM), specifically induced Pax-2 gene expression in MK4 cells and kidney explants. High glucose-induced Pax-2 gene expression is mediated, at least in part, via ROS generation and activation of the NF-kB signaling pathway, but not via PKC, p38 mitogen activated protein kinase (MAPK) and p44/42 MAPK signaling.

7.2 Introduction

Diabetic mellitus confers a major risk factor for congenital malformations. When the fetus is exposed to sustained high ambient glucose, widespread fetal damage may affect multiple organs including kidney (diabetic embryopathy)[1;2]. Infants

born to women with pre-gestational insulin-dependent diabetes mellitus have a 10-fold relative risk of congenital malformations, and those born to women with gestational diabetes have a five-fold relative risk. Both the diabetic mother and her fetus are at risk for significant morbidity and mortality, even in the 21st century [3;4].

Renal malformations account for approximately 40 percent of childhood renal failure [5;6]. During kidney development, two major events, ureteric bud (UB) branching and mesenchymal-to-epithelial transformation control the main thrust of renal morphogenesis. When the normal pattern of nephrogenesis is interrupted, kidney abnormalities, such as renal agenesis, renal dysplasia or aplasia, may ensue [6-8].

Kanwar et al. [9] recently reviewed the mechanisms that appear to be involved in diabetic embryopathy, pointing out that high glucose increases damage to DNA and the extracellular matrix (ECM) via reactive oxygen species (ROS); high glucose inhibits COX-2, resulting in PGE2 deficiency; and high glucose may induce transcription factors and proto-oncogenes. Kanwar et al. also demonstrated that renal-specific oxidoreductase is closely linked to renal morphogenesis in a high glucose milieu [10]. However, the molecular mechanisms by which high ambient glucose levels lead to renal dysmorphogenesis and birth defects have not yet been delineated [9-11].

ROS have been proposed as a major factor in the pathogenesis of diabetic nephropathy [12;13]. Increased ROS generation by high glucose directly damages DNA and also alters the expression of ECM glycoproteins [14-16]. Additionally, kidney related key proto-oncogenes and transcription factors such as GDNF, cRet and Pax-2 may be altered in diabetic embryopathy [9], although clear experimental

evidence is presently lacking. The paired-box 2 (Pax-2) gene is a "kidney-specific" master gene that is expressed in both UB and mesenchymal cell lineages, normally optimizing UB branching and mesenchymal-to-epithelial transformation in kidney development [17-19]. Mutations in the Pax-2 gene cause increased apoptosis [20-22], associated with renal hypoplasia [20;23;24]. For instance, homozygous null Pax-2 mice fail to form any kidneys, ureters and genital tracts [25]. In humans and mice, heterozygous Pax-2 mutations cause kidney, eye, and central nervous system abnormalities, constituting a syndrome called renal-coloboma syndrome (RCS) [26;27]. The mechanism by which reduced Pax-2 expression leads to decreased UB branching and subsequently to a reduced number of nephrons in patients with RCS seems to be highly related to UB lineage apoptosis [28]. It appears that regulation of UB cell survival by activation of Pax-2 regulated factors such as Naip (neuronal apoptosis inhibitory protein) and Wnt-4 will be powerful determinants of congenital nephron endowment [28-31].

The transcription factor nuclear factor kappa B (NF-kB) is a major intracellular target in hyperglycemia and oxidative stress [32;33]. NF-kB plays a critical role in mediating immune and inflammatory responses and apoptosis. It is also associated with a number of chronic diseases including diabetes and atherosclerosis [34;35].

In the present study, we investigated whether there is a link between hyperglycemia and Pax-2 gene expression that might influence kidney development. We employed *in vitro* and *ex vivo* approaches and observed that high glucose (25 mM D-glucose) as compared to normal glucose (5 mM D-glucose) specifically induced Pax-2 gene expression in MK4 cells and kidney explants, with ROS generation and the NF-kB pathway certainly being involved as underlying mechanisms.

7.3 Materials and Methods

Reagents

Normal glucose (5 mM D-glucose Dulbecco's modified Eagle's medium (DMEM) (Cat. #12320) was purchased from Invitrogen Inc. (Burlington, Ontario, Canada). D(+)-Glucose, L-glucose, D-mannitol, 2-deoxy-d-glucose, diphenylene iodinium (DPI), rotenone, GF109203X, PDTC, xanthine oxidase (XO) and hypoxanthine were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). SB203580, PD98059 were obtained from CalBiochem (San Diego, CA, USA). Mouse anti-β-actin monoclonal antibody (clone AC-15) and rabbit polyclonal anti-Pax-2 antibody were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and Covance (Richmond, CA, USA), respectively. The luciferase activity assay kit was purchased from Promega (Fisher Scientific, Montreal, QC, Canada). Hoxb7-GFP mice were obtained from Dr. Frank Costatini (Department of Genetics and Development, Columbia University Medical Center, NY, NY, USA) [36;37], and the 4.2-kb *Apa*I/*Nco*I fragment of the human PAX2 promoter (AF515729) was a generous gift from Dr. Michael Eccles (Department of Pathology, University of Otago, Dunedin, New Zealand) [20;38]. MK4 cells were from Dr. S. Steven Potter (Division of Developmental Biology, Children's Hospital Medical Center, Cincinnati, OH, USA) [39]. Dominant negative IκBα (DN IκBα) plasmid (pcDNA3.1/DN IkBa) from Dr. John S.D. Chan (CHUM-Hôtel-Dieu, Montreal, QC, Canada), was produced by PCR-based site-directed mutagenesis in Serines 32 and 36 in the N terminal regulatory domain of IκBα (NM_010907) to resist phosphorylation. DN IκBα can bind the p50 and p65 subunits complexed in an inactive form, preventing p50 and p65 from translocating to the nucleus for further action.

Depleted fetal bovine serum (dFBS, depleted of endogenous steroid and thyroid hormones) was prepared by incubation with 1% activated charcoal and 1% AG 1 X 8 ion-exchange resin (Bio-Rad Laboratories, Inc., Richmond, CA, USA) for 16 to 24 hous at room temperature, as described previously [40;41]. Though endogenous steroid and thyroid hormones have been removed from FBS, the purpose of adding 1% dFBS into either 5mM glucose or 25 mM DMEM after rendering them quiescent with serum free medium is to support the cells during the additional 24 hour experimental period until harvested, as demonstrated previously [40;41].

Culture of MK4 Cells

The MK4 cell line is representative of late embryonic mesenchymal mesenchyme (MM) as it undergoes mesenchymal to epithelial conversion [39]. MK4 cells are relatively polygonal or epithelial in shape and express genes typical of late mesenchyme, including Pax-2, Pax-8, Wnt-4, Cadherin-6, Collagen IV, and LFB3 (REF) [39]. In the present study, MK4 cells were cultured in normal glucose DMEM (pH 7.45), supplemented with 5% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin in 95% air and 5% CO_2 at 37ºC.

Ex vivo Embryonic Kidney Culture

Embryonic kidneys were isolated from timed pregnant HoxB7-GFP mice at embryonic stage E16 [36;37] under sterile conditions and cultured either in normal glucose or high glucose DMEM supplied with 1% dFBS. These mice specifically express the green fluorescence protein (GFP) in the UB driven by the Hoxb7 promoter. Each kidney explant was cultured in 1 ml of medium in a separate well of

a 24-well plate for up to 24 hours in the presence or absence of DPI (10^{-6} M), rotenone (10^{-6} M) and H_2O_2 (10^{-5} M).

Immunofluorescence Studies

MK4 cells were grown to 70 to 80% confluence in two-chamber slides, and then synchronized with overnight serum-free medium. After culture in either normal glucose or high glucose DMEM for 24 hours, cells were processed for immunofluorescence investigation as reported previously [40;41]. Immunofluorescence images were recorded with a Olympus 1X71 Microscope (CARSEN, ON, CA). The images are presented at 400 X magnification.

Western Blotting

Western blots were performed as in previous studies [40;41]. Briefly, small aliquots (20-50 μl) of homogenized cell sample were subjected to 10% SDS-PAGE and then transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech, Canada). The membrane was first blotted for anti-Pax-2 and then re-blotted for β-actin. The relative densities of the Pax-2 vs β-actin bands were measured by computerized laser densitometry.

ROS Generation

ROS production was quantified by the lucigenin method [42;43]. After overnight culture in serum-free medium to render them quiescent, cells were incubated in either normal glucose or high glucose DMEM containing 1% depleted FBS for periods of 15 mins to 2 hours. Cells were then trypsinized, collected by centrifugation, and the pellet washed in modified Krebs buffer containing NaCl (130 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.5 mM), K_2HPO_4

(1 mM) and Hepes (20 mM), pH 7.4. After washing, the cells were resuspended in Krebs buffer with 1 mg/ml bovine serum albumin (BSA), and cell concentration was adjusted to 1×10^7 in 900μl buffer. To measure ROS production, the cell suspension was transferred to plastic tubes and assessed in a luminometer (LB 9507, Berthold, Wildbad, Germany). The final value, RLU (related light unit) of ROS generation was normalized by protein concentration of the samples.

RT-PCR for Pax-2 mRNA

Total RNA was prepared from cultured cells according to the manufacturer's protocol using TRIZOL (Invitrogen Inc.) [40;41]. First strand cDNA was synthesized with the Super-Script preamplification system (Invitrogen Inc.). We employed the following forward and reverse primers: forward primer 5' TTT GTG AAC GGC CGG CCC CTA 3', and the reverse primer 5' CAT TGT CAC AGA TGC CCT CGG 3'; these correspond to the nucleotide sequences N+622 to N+642 and N+902 to N+922 of Pax-2 cDNA [40;41]. For internal control, we deployed primers specific for mouse β-actin [44] (forward and reverse primers 5' ATG CCA TCC TGC GTC TGG ACC TGG C 3' and 5' AGC ATT TGC GGT GCA CGA TGG AGG G 3', corresponding to nucleotide sequences N+600 to N+622 and N+1179 to N+1203 of mouse β-actin cDNA (X03672)).

Real Time-Polymerase Chain Reaction (qRT-PCR)

qRT-PCR were performed as in previous studies [40]. In brief, first-strand cDNA was produced from 2 ug of random hexamer primed total RNA using Super-Script pre-amplification system (Invitrogen). Relative quantitation by real-time PCR was carried out using iQ™ SYBR@ Green Supermix Kit (Bio-Rad Laboratories, Mississauga, ON, Canada) and MiniOpticon™ Real-Time PCR

Detection System (Bio-Rad), following the protocol described by the supplier. PCR reactions in triplicate underwent 40 cycles of 95 ^{o}C for 20 s, 60 ^{o}C for 20 s, 72 ^{o}C for 20 s, and 79 ^{o}C for 5 s in the thermal cycler. The parameter CT (threshold cycle) value was measured to determine starting copy number of target genes using the standard curve. Lower value of CT indicates higher amount of PCR products. We employed the following forward and reverse primers for Pax-2: forward primer 5' ACA TCA AAT CAG AAC AGG GGA AC 3', and the reverse primer 5' CAT GTC ACG ACC AGT CAC AAC 3'; these correspond to the nucleotide sequences N+1319 to N+1341 and N+1453 to N+1473 of Pax-2 cDNA (NM_003990). For internal control, we deployed primers specific for mouse β-actin (forward and reverse primers 5' CGT GCG TGA CAT CAA AGA GAA 3' and 5' GCT CGT TGC CAA TAG TGA TGA 3', corresponding to nucleotide sequences N+704 to N+724 and N+820 to N+840 of mouse β-actin cDNA [NM_007393]) [40].

Luciferase Assay for High Glucose Effect on Pax-2 Gene Promoter Activity

We have constructed a fusion gene, pGL-2/hPax-2, containing a full length 5'-promoter of 4.2-kb *Apa*I/*Nco*I fragment of the human PAX2 promoter (AF515729) [20;38] inserted into luciferase reporter pGL-2 basic vector (Promega), and performed transient transfection of this fusion gene into MK4 cells by Lipofectamine 2000 (Invitrogen Inc.), while pGL-2 basic vector serving as control. IκBα is an inhibitor of NF-κB. To study the effect of DN IκBα on high glucose induced Pax-2 promoter activity, we transiently cotransfected both plasmids: pGL-2/hPax-2 and pcDNA3.1/DN IkBa, into MK4 cells, while both pGL-2 and pcDNA3.1 basic vector served as controls. MK4 cells grown in 12-well plates were transfected with 1.5 μg of each plasmid. After a 24 hours stimulation with high glucose medium with or without ROS inhibitors, cells were harvested and the

luciferase activity was quantified by Luciferase assay kit (Promega) according to the protocol from the supplier with *renilla luciferase* as an internal control.

Statistical Analysis

Statistical significance between experimental groups was analyzed initially by student's *t* test or by 1-way ANOVA followed by the Bonferroni test as appropriate. Three to four separate experiments were performed for each protocol. Data are expressed as means ± SD. A probability level of $P \leq 0.05$ was considered statistically significant.

7.4 Results

High Glucose Stimulates Pax-2 Expression in MK4 cells

MK4 cells were incubated in media containing 1% dFBS and 25 mM different glucose analogues such as D-Glucose, D-Mannitol, L-glucose or 2-Deoxy-D-Glucose. After incubation for 24hours, cells were harvested and analyzed for Pax-2 mRNA (RT-PCR) and protein (Western blotting) levels. As shown in Figure 1, high glucose stimulated the Pax-2 mRNA expression (Figure 1A, RT-PCR) and protein (Figure 1B: Western blotting) in a dose-dependent manner from 5mM to 25mM with a maximal effect at 25mM D-glucose. To maintain constant isotonicity or osmolality, 5-mM glucose media was supplemented with D-mannitol (20 mM) (final concentration) in additional studies. Figure 2 indicates that high glucose as compared to normal glucose specifically induced Pax-2 gene expression, while other glucose analogs such as D-Mannitol, L-glucose or 2-Deoxy-D-Glucose in MK4 cells had no effect, suggesting that the effect of high D(+)-glucose medium is specific (Figure 2A: RT-PCR; Figure 2B: Western Blot). Moreover, Pax-2 immunostaining with intranuclear appearance was induced by high

glucose, consistent with the fact that Pax-2 is a nuclear transcription factor (Figure 3) [40;41;45]. Pax-2 expression was normalized by β-actin. The 24-hour incubation period was used for all subsequent studies.

p38 MAPK, p44/42 MEK and PKC Inhibitors Fail to Block the High Glucose Effect on Pax-2 Gene Expression in MK4 Cells

Figure 4A and 4B reveal that inhibitors of p38 MAPK (SB203580), P44/42 MEK (PD98059) and PKC (GFX) could not block the stimulatory effect of high glucose on Pax-2 mRNA and protein expression in MK4 cells, suggesting that p38 MAPK, p44/42 MEK and PKC signaling are not involved in mediating the stimulatory influence of high glucose on Pax-2 gene expression.

ROS Generation and Pax-2 Gene Expression in MK4 Cells

We observed that MK4 cells after 15 minutes incubation in high glucose medium, ROS generation began to increase in MK4 cells, and this elevation lasted 60 minutes (Figure 5A). These data indicate that high glucose induced ROS generation in MK4 cells. In order to confirm that ROS directly regulate Pax-2 gene expression, we performed studies involving the xanthine oxidase (XO) system. Superoxide generated from the XO system directly stimulates Pax-2 gene expression in MK4 cells in a dose-dependent manner (Figure 5C, 5D and 5E).

Inhibitors of NADPH Oxidase, Mitochondrial Electron Transport Chain Complex I, and NF-kB Pathway Block the Stimulatory Effect of High Glucose on Pax-2 Gene Expression in MK4 Cell.

The stimulatory effect of high glucose on ROS generation was inhibited in the presence of DPI and rotenone, but not PDTC (Figure 5B). Figure 6 shows that inhibitors of NADPH oxidase (DPI), mitochondrial electron transport chain complex I (rotenone), and NF-kB pathway (PDTC) block the stimulatory action of high glucose on Pax-2 expression in MK4 cells. These data suggest that the stimulatory effect of high glucose on Pax-2 gene expression is mediated via ROS generation and the activation of NF-kB signaling pathway in MK4 cells.

High Glucose Effect on Pax-2 Gene Expression in Kidney Explants from Hoxb7-GFP mice

In order to confirm our *in vitro* observation, we adapted an *ex vivo* model using embryonic kidney explants. Embryonic kidneys at E16 gestation from Hoxb7-GFP mice were isolated and cultured *ex vivo* as shown in Figure 7A. After culturing the explants for 24 hours in either normal glucose (5mM) or high glucose (25mM) DMEM with or without ROS inhibitors, high glucose stimulated Pax-2 mRNA and protein expression. The high glucose effect was blocked by ROS inhibitors, as illustrated in Figure 7B and 7C. We have also tested H_2O_2, an important source of $O_2^{\bullet -}$, and observed that exogenous H_2O_2 at 10^{-5} M stimulates Pax-2 gene expression modestly; however, in combination with high glucose, the H_2O_2 stimulatory effect is enhanced substantially as shown in Figure 7 E (qRT-PCR) and 7F (Western blot).

High Glucose Effect on Pax-2 Gene Promoter Activity

Transient transfection of pGL-2/hPax-2 in MK4 cells followed by culturing in high glucose medium stimulated Pax-2 gene promoter activity, compared to culturing the cells in normal glucose medium. The stimulatory effect of high glucose

was inhibited in the presence of inhibitors of ROS and NF-kB signaling pathway (Figure 8A). Moreover, after co-transfection with pcDNA3.1/DN IκBα, high glucose-induced Pax-2 promoter activity was abolished, which suggests that NF-κB is involved in Pax-2 transcription stimulated by high glucose (Figure 8B). These data demonstrate that the stimulatory effect of high glucose on Pax-2 gene expression occurs at the transcriptional level via ROS activation of the NF-kB signaling pathway.

7.5 Discussion

The present studies demonstrate that high glucose stimulates Pax-2 gene expression in mouse embryonic metanephric mesenchymal (MM) cells and embryonic kidney explants. The stimulatory effect of high glucose on Pax-2 gene expression is mediated, at least in part, via ROS generation and activation of NF-kB signaling pathway.

During embryogenesis, embryonic stem cells must proliferate dynamically and precisely in order to form functional organs. Additionally, apoptosis and proliferation is involved. Any interruption or error caused by the surrounding environment or misleading signals involved in gene regulation can elicit aberrant organogenesis and may even be lethal. For example, in renal morphogenesis, certain undifferentiated MM must undergo apoptosis to make room for UB branching [7;8]. Diabetes constitutes an adverse *in utero* environment that may impair nephrogenesis. For example, a high glucose milieu can result in an abnormal pattern of UB branching evoking duplex ureters or cystic kidneys; reduced populations of nascent nephrons, resulting in hypoplasia; increased apoptosis in mesenchyme and UB epithelium, resulting in renal agenesis, aplasia or dysplasia [46-49]. Nielsen et al. reported that the prevalence of renal malformations such as renal agenesis and

congenital abnormalities of kidney and urinary tract (CAKUT) have a strong association with pre-gestational maternal diabetes, more than cardiovascular congenital abnormalities or multiple congenital abnormalities [50].

Renal-specific genes are also clearly important for accurate nephrogenesis. The "kidney-specific" master gene Pax-2 is necessary for initial signaling of the Wolffian duct (WD) to optimize UB branching and mesenchymal-to-epithelial transformation [19;26]. Pax-2-null mice fail to form any kidneys, ureters and genital tracts [25]. In humans and mice, heterozygous Pax-2 mutations cause kidney, eye, and central nervous system abnormalities, constituting a syndrome called renal-coloboma syndrome (RCS) [26;27].

To the best of our knowledge, high ambient glucose has not been reported previously to regulate Pax-2 gene expression and its underlying mechanism(s) of action in embryonic kidney cells. In the present study, we employed both *in vitro* (MK4 cells) and *ex vivo* (kidney explants) approaches to explore this potential interaction. As our data indicate, high D(+) glucose (25mM) specifically and dose-dependently stimulated Pax-2 gene expression in MK4 cells, while other glucose analogs such as D-mannitol, L-glucose and 2-deoxy-D- glucose had no effect. A similar stimulatory action of high glucose was also observed in our kidney explant system. Additionally, high glucose ambience increases ROS generation in MK4 cells, and this was blocked by ROS inhibitors, not by NF-kB inhibitors. These data are consistent with our previous observation that high glucose induces ROS generation in immortalized renal proximal tubular cells [42;43]. In order to confirm that ROS directly regulate Pax-2 gene expression, we examined the effect of the xanthine oxidase (XO) system. Indeed, superoxide generated from the XO system directly stimulates Pax-2 gene expression in MK4 cells in dose-dependent manner; another important source of $O_2^{\bullet -}$, H_2O_2 at 10^{-5} M stimulates

Pax-2 gene expression modestly; however, in combination with high glucose, the H_2O_2 stimulatory effect is enhanced substantially in E16 kidney explants.

Brownlee [12;13] has suggested that the underlying mechanisms regarding high glucose as an inducer of kidney damage, indicating that excessive ROS generation and then subsequent PKC and NF-kB activation are the key elements in tissue injury. Other studies have revealed that both p38 MAPK and p44/42 MAPK signaling pathways may also be involved in hyperglycemia-induced ROS generation in proximal tubular cells [42;43] and mesangial cells [51;52]. To determine whether p38 MAPK, p44/42 MAPK and PKC signaling is involved in mediating the high glucose effect on Pax-2 gene expression, inhibitors of these signaling pathways were tested. Our data disclosed that SB203580, PD98059 or GFX could not blocked the high glucose action on Pax-2 gene expressions at both protein and mRNA levels, suggesting that both p38 MAPK, p44/42 MAPK and PKC signaling pathways are not involved. In contrast, we observed that the stimulatory effect of high glucose on Pax-2 gene expression was blocked in the presence of DPI, rotenone and PDTC in both *in vitro* and *ex vivo* studies, indicating that high glucose on Pax-2 gene expression is mediated, at least in part, via ROS generation and activation of the NF-kB signaling pathway.

NF-kB is one of the major intracellular targets of hyperglycemia [13;35]. It is present in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits complexed with IkB and it is activated through a common pathway, which involves the phosphorylation-induced proteasome-mediated degradation of the inhibitory subunit, IkB. Upon stimulation, a serine kinase cascade is activated leading to the phosphorylation of IkB and dissociating from the NF-κB heterodimer. This event primes IkB as a substrate for ubiquitination and subsequent degradation in the cytoplasm. The NF-kB heterodimer is then translocated to the nucleus and

regulates the expression of a large number of genes including growth factors (e.g., vascular endothelial growth factor (VEGF)), pro-inflammatory cytokines (e.g.,TNF-α and IL-1β), RAGE, adhesion molecules (e.g., vascular cell adhesion molecule-1) [34;35]. The enzyme that phosphorylates IκB is IκB kinase (IKK), a heterotrimeric complex consisting of two catalytic subunits, IKKα (also called IKK1) and IKKβ (also called IKK2), and a regulatory subunit, IKKγ [53;54]. In order to further understand the underlying mechanisms of the NF-kB pathway involved in high glucose induced Pax-2 gene expression, we tested DN IκBα which can bind the p50 and p65 subunits complexed in an inactive form, preventing p50 and p65 from translocating to the nucleus. Our data in MK4 cells demonstrate that high glucose stimulates Pax-2 promoter activity at a transcriptional level, which may be blocked by both ROS and NF-kB inhibitors. After cotransfection with DN IκBα, Pax-2 promoter activity induced by high glucose is abolished, suggesting that NF-κB is involved in glucose-induced stimulation of Pax-2 transcription. Using the sequence searching software AliBaba2.1 (www.gene-regulation.com), we have now identified several NF-κB binding motifs including 6 of **GGrmwkyCCC** and 2 of **GGGGmyTyy** located in a full length of 5'-promoter region of Pax-2 (AF515729). Additional studies are ongoing to address the underlying molecular mechanisms.

Taken together, these data demonstrate that the stimulatory effect of high glucose on Pax-2 gene expression is mediated, at least in part, via ROS generation and activation of the NF-κB signaling pathway, but not via the PKC, p38MAPK and p44/42 MAPK signaling pathways. This indicates that modifying these pathways might be important in further understanding diabetic embryopathy.

7.6 Acknoledgements

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ABBREVIATIONS: CAKUT, congenital abnormality of kidney and urinary tract; DMEM, Dulbecco's modified Eagle's medium; DN IkB, Dominant negative IκBα; DPI, Diphenyleneiodonium Chloride; Erk (extracellular signal-regulated kinase); dFBS, depleted fetal bovine serum; FBS, fetal bovine serum; GFP, green florescent protein; MAPK, mitogen activated protein kinase; MK4, mouse late embryonic mesenchymal epithelial cells; MM, metanephric mesenchyme**;** NF-kB, nuclear factor kappa B; PKC, protein kinase C; Pax, paired homeobox gene; ROS, reactive oxygen species; UB, ureteric bud; WD, Wolffian duct.

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7.8 Legends and Figures

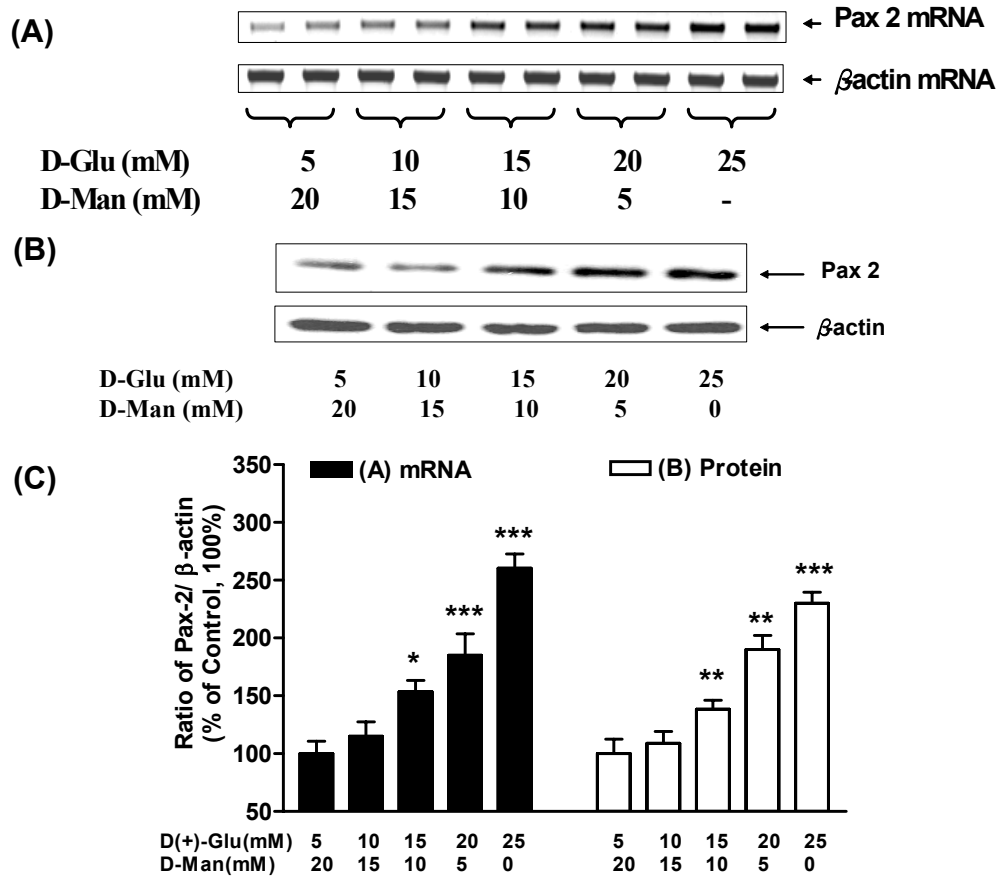

Figure 1

Figure 7-1 High glucose dose-dependent effect. High D(+) glucose up-regulates Pax-2 gene expression in a dose-dependent manner in MK4 cells analyzed by RT-PCR (A) and Western blot (B). After synchronized with serum free medium overnight, the quiescent cells were incubated in DMEM with 1% dFBS containing final glucose concentration from 5mM to 25mM for 24 hours, whereas D-mannitol was supplemented to maintain constant isotonicity or osmolality; (C) Relative densities of Pax-2 were normalized to ß-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered to be the control (100%). Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

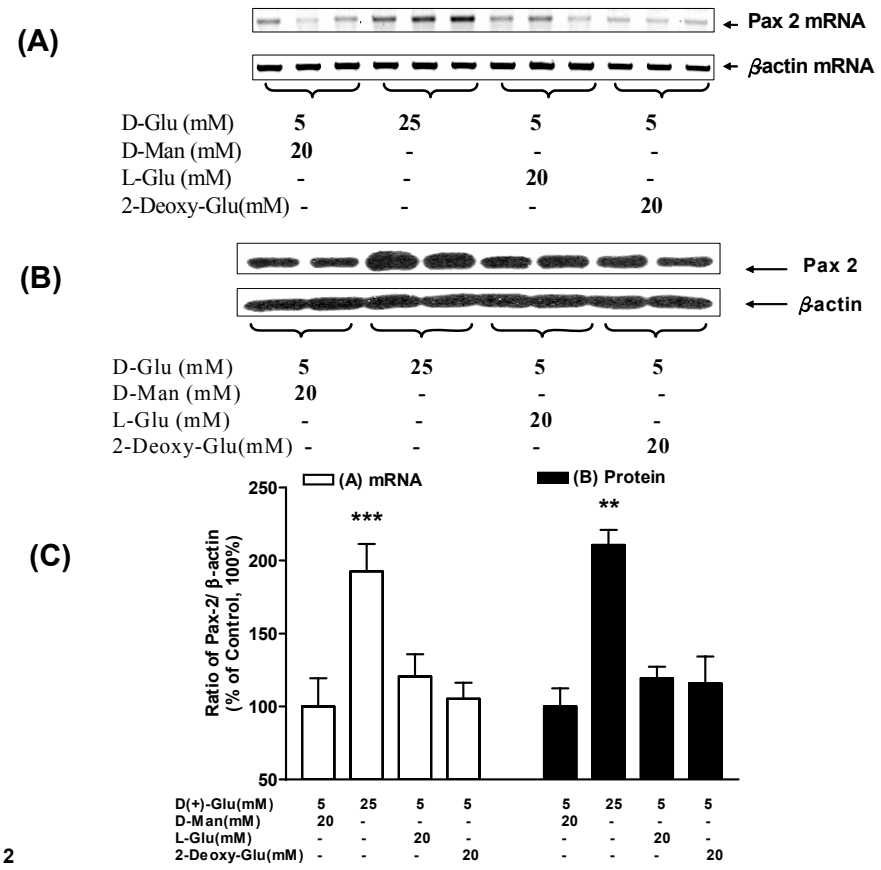

Figure 2

Figure 7-2 High glucose specifical effect. Pax-2 gene expression is specifically stimulated by high D(+) glucose (25mM), not by other glucose analogs such as L-Glucose, D(+)-Mannitol or 2-Deoxy-D-Glucose in MK4 cells analyzed by RT-PCR (A) and Western blot (B). The quiescent cells were incubated in DMEM with 1% dFBS containing different glucose analogs in 25mM as final concentration for 24 hours; (C) The relative densities of Pax-2 were normalized to ß-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

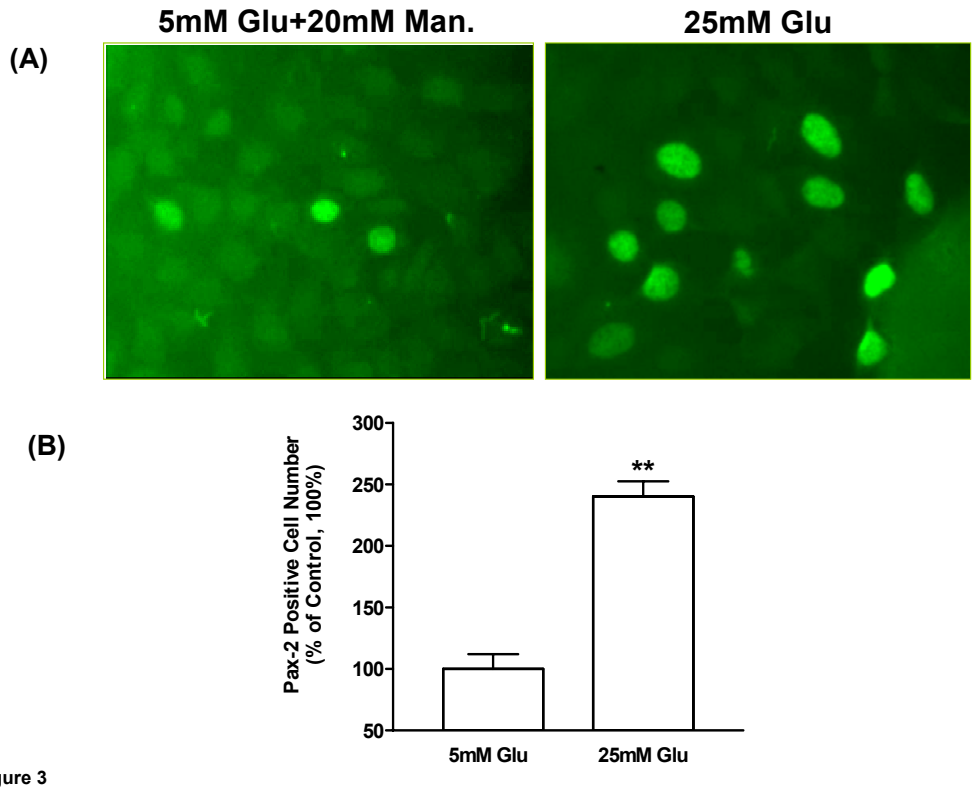

Figure 3

Figure 7-3 Pax-2 immunofluorescence staining. High glucose up-regulatPax-2 expression in MK4 cells as shown by immunofluorescence staining (A) (original magnification x 400). Quiescent cells were incubated in either 5 mM or 25mM D-glucose DMEM containing 1% dFBS for 24 hours; (C) The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

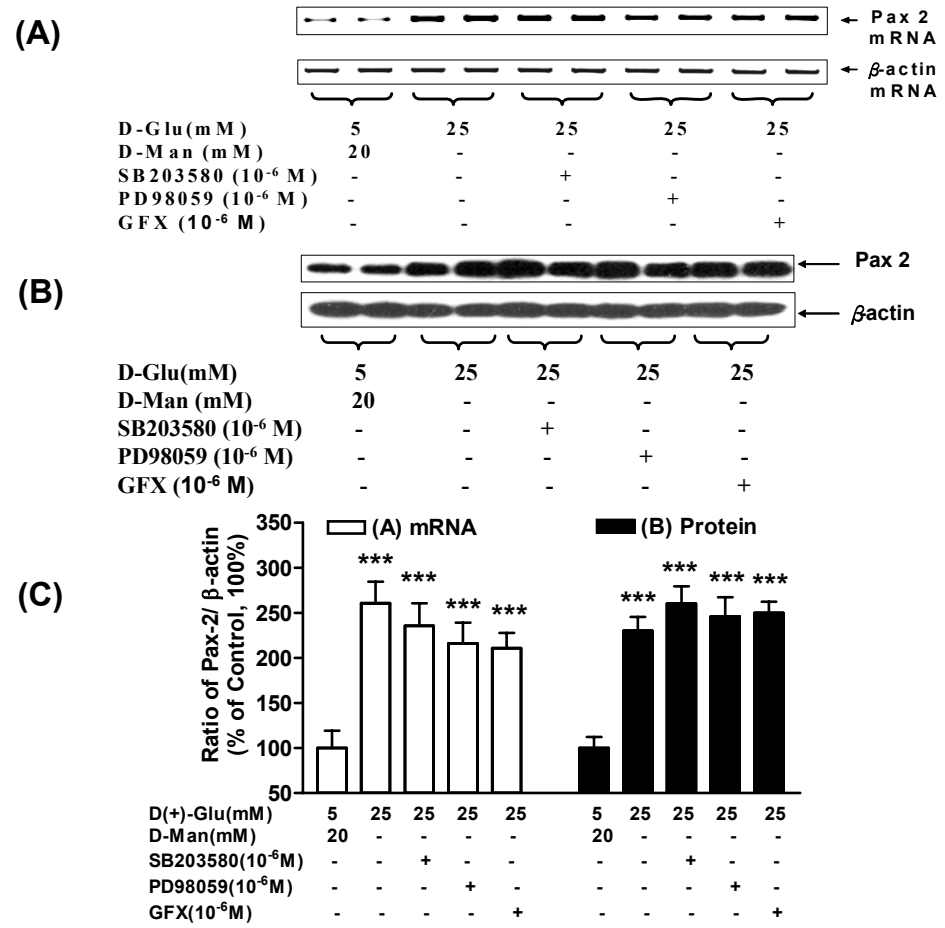

Figure 4

Figure 7-4 p38 MAPK, p44/42 MAPK, and PKC inhibitor effect. Effect of SB203580, PD98059 and GFX on Pax-2 gene expression stimulated by high glucose in MK4 cells as analyzed by RT-PCR (A) and western blot (B); Quiescent cells were incubated in either 5 mM or 25mM D-glucose DMEM containing 1% dFBS for 24 hours with or without inhibitors; (C) The relative densities of the Pax-2 were normalized to the ß-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered as the control (100%). Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

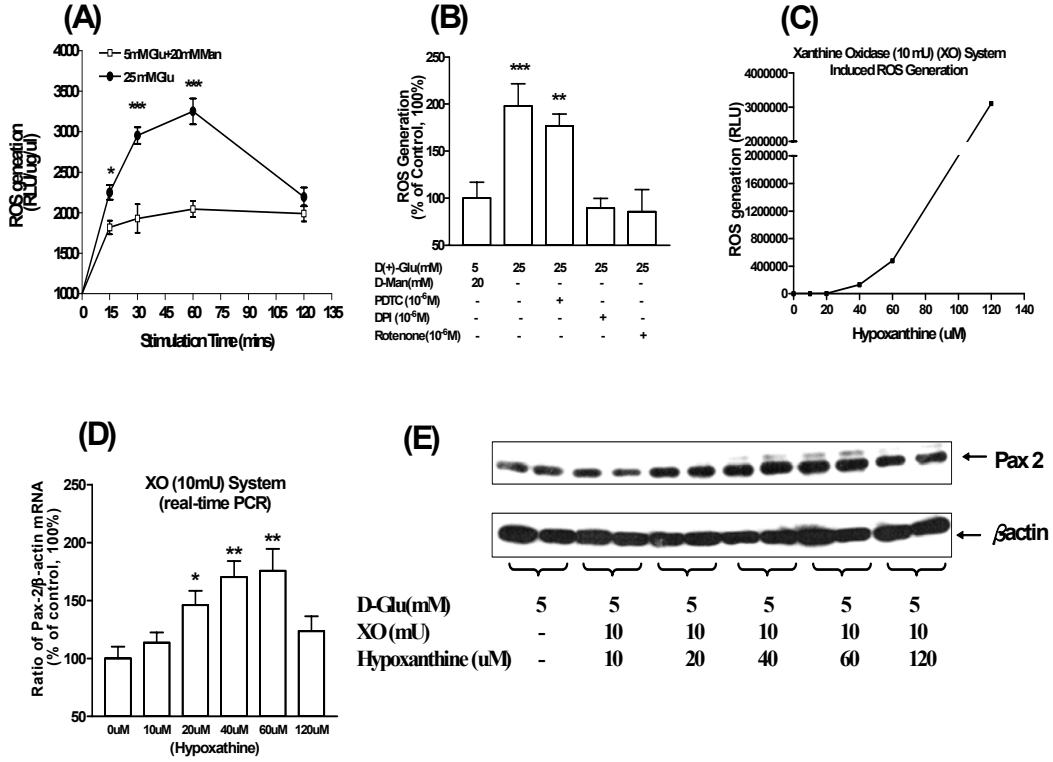

Figure 5

Figure 7-5 High glucose induced ROS on pax-2 gene expression. High glucose time dependently stimulates ROS generation in MK4 cells (A), that is blocked by ROS inhibitors, but not NF-kB inhibitors (B) analyzed by lucigenin assay. Moreover, superoxide generated from xanthine oxidase (XO) system (Figure 5C) directly stimulates Pax-2 gene expression in MK4 cells in a dose-dependent manner, shown here as analyzed by real-time PCR (Figure 5D) and western blot (Figure 5E); (A) Quiescent cells were incubated in either 5 mM glucose DMEM or 25 mM containing 1% dFBS for periods of 15 mins to 2 hours, then trypsinized and assayed. The final value of ROS generation was normalized by the protein concentration of sample. At the 15-min incubation point, basal ROS generation was, RLU/*ug*/*ul* : 1817 ± 84.35 vs 2250.85 ± 90.40 in 5mM and 25 mM glucose medium, respectively; (B) Quiescent cells were incubated in either 5 mM glucose DMEM or 25 mM containing 1% dFBS with or with inhibitors for periods of 30 min, then trypsinized and assayed. The normalized ROS generation in cells incubated in 5 mM glucose was considered the control (100%); (C) superoxide generated from the xanthine oxidase (XO) system was detected by lucigenin assay; (D+E) Quiescent cells were incubated in 5 mM glucose DMEM containing 1% dFBS with or with XO system for 24 hours. Each point represents the mean ± SD of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

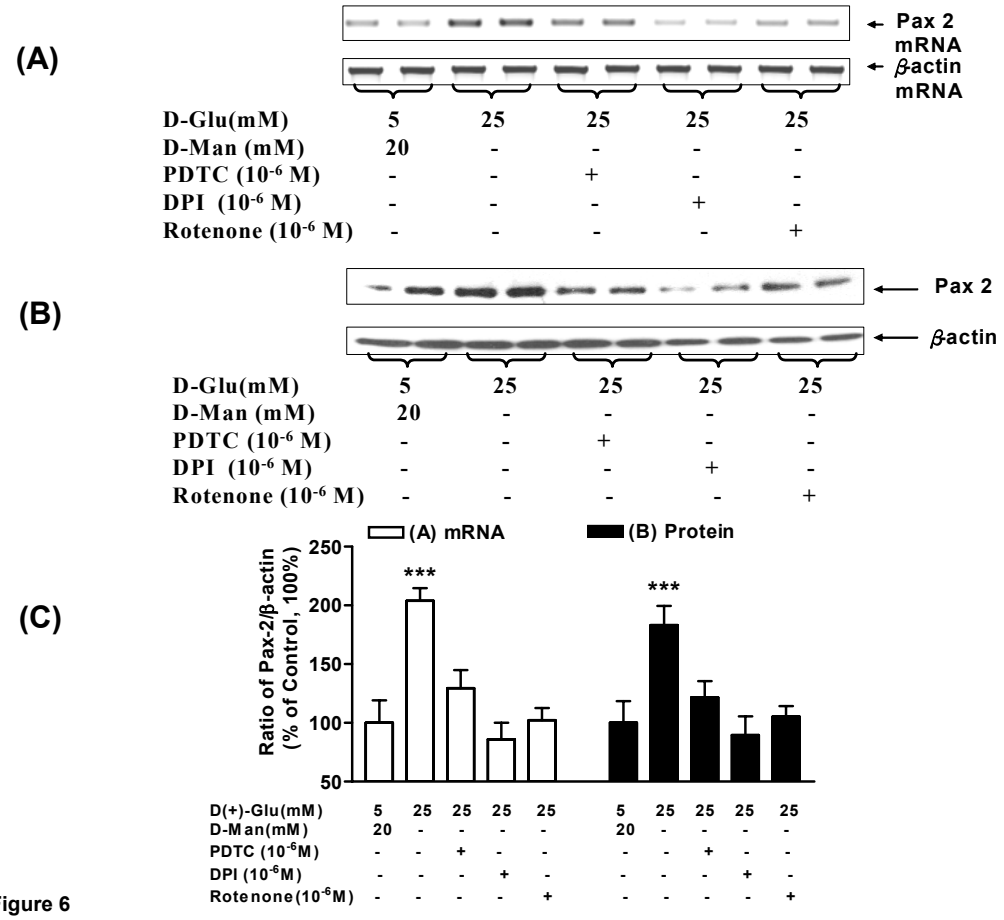

Figure 6

Figure 7-6 Inhibitory effect of ROS and NF-kB inhibitors. The inhibitory effect of PDTC, DPI and rotenone on Pax-2 gene expression upregulated by high glucose in MK4 cells analyzed by RT-PCR (A) and Western Blot (B); Quiescent cells were incubated in either 5 mM or 25mM D-glucose DMEM containing 1% dFBS for 24 hours with or without inhibitors; (C) The relative densities of Pax-2 were compared with ß-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

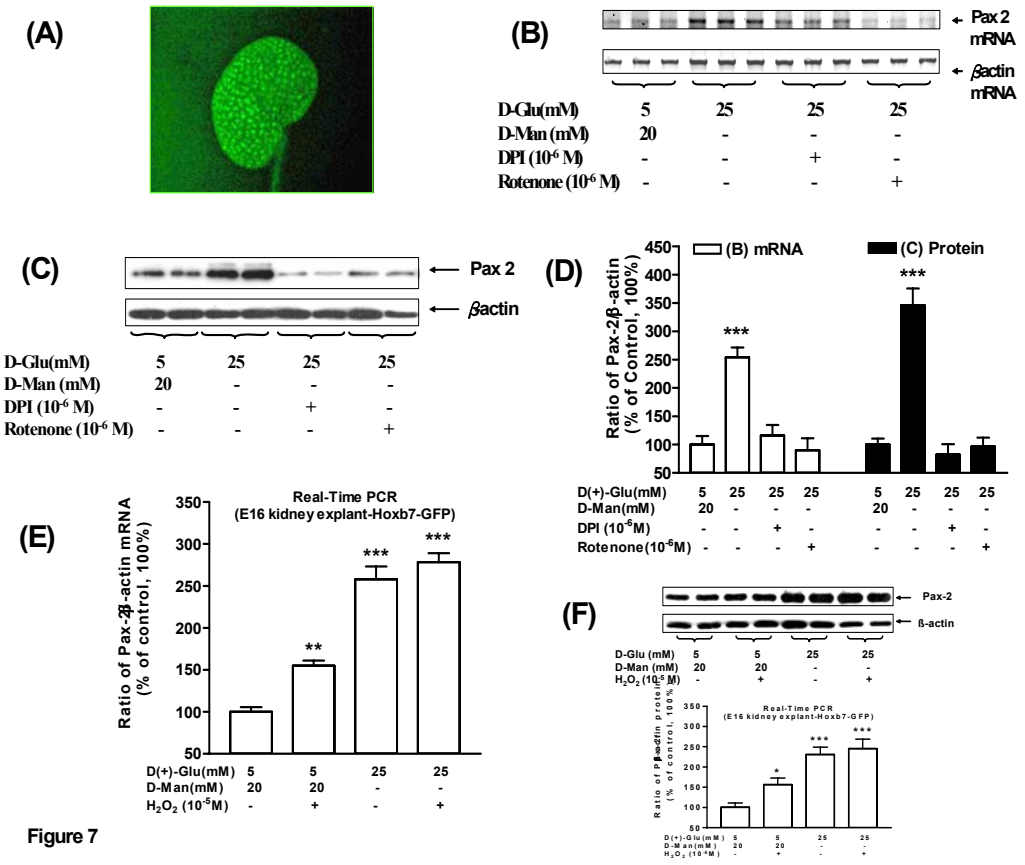

Figure 7

Figure 7-7 High glucose effect on Pax-2 gene expression ex vivo. Ex vivo study. Kidney explant isolated from Hoxb7-GFP mice on embryonic day 16 (A); The inhibitory effect of DPI and rotenone on Pax-2 gene expression was upregulated by high glucose in renal explant analyzed by RT-PCR (B) and Western Blot (C), respectively; (D) The relative densities of Pax-2 were compared with ß-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%); H_2O_2 effect on Pax-2 gene expression analyzed by qRT-PCR (E) and Western blot (F), respectively. Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

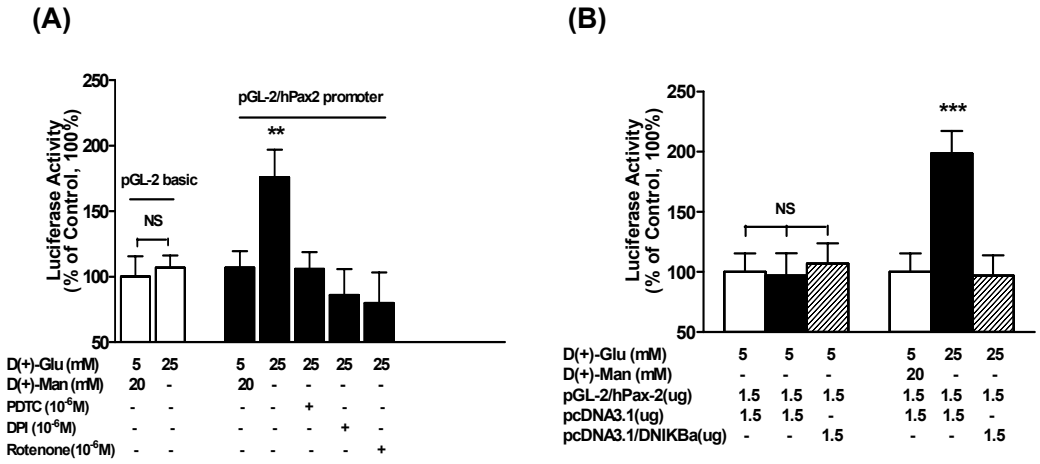

Figure 8

Figure 7-8 High glucose effect on the full length of 5'-franking region of Pax-2 promoter activity in MK4 cells. High glucose effect on the full length of 5'-franking region of Pax-2 promoter activity in MK4 cells. MK4 cells grown in 12-well plates were transiently transfected with 1.5 μg of pGL-2/hPax-2 plasmid by Lipofectamine 2000, with pGL-2 basic vector serving as control (A); DN IKBa effect on high glucose induced Pax-2 promoter activity. MK4 cells were transiently co-transfected with 1.5 μg of each pGL-2/hPax-2 and pcDNA3.1/DNIKBa plasmid by Lipofectamine 2000, while pcDNA3.1 plasmid serving as the control (B). Promoter activity was measured by luciferase assay kit with *renilla luciferase* as an internal control after 24 hours stimulation by high glucose with or without inhibitors. The final value of Pax-2 promoter activity was normalized by the protein concentration of sample. Normalized Pax-2 promoter activity in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

CHAPTER 8: ARTICLE 2

Reactive Oxygen Species in the presence of High Glucose Alter Ureteric Bud Morphogenesis

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8.1 Abstract

Renal malformations are a major cause of childhood renal failure. During the development of the kidney, ureteric bud (UB) branching morphogenesis is critical for normal nephrogenesis. In the present studies, we investigated whether renal UB branching morphogenesis is altered by a high ambient glucose environment and studied underlying mechanism(s). Kidney explants isolated from different periods of gestation (E12 to E18) from Hoxb7- green fluorescent protein (GFP) mice were cultured for 24 hours in either normal D-glucose (5mM) or high D-glucose (25mM) medium with or without various inhibitors. Alterations in renal morphogenesis were assessed by fluorescence microscopy. Paired-homeobox 2 (Pax-2) gene expression was determined by real time-quantitative polymerase chain reaction (RT-qPCR), western blotting and immunohistology. Our results revealed that high D-glucose (25mM) specifically stimulates UB branching morphogenesis via Pax-2 gene expression, while other glucose analogs such as D-mannitol, L-glucose or 2-Deoxy-D-glucose had no effect. The stimulatory effect of high glucose on UB branching was blocked in the presence of catalase and inhibitors of NADPH oxidase, mitochondrial electron transport chain complex I, and Akt signaling. Moreover, in *in vivo* studies, it appears that high glucose induces, via Pax-2 (mainly localized in UB), acceleration of UB branching but not nephron formation. Taken together, our data demonstrate that high glucose alters ureteric bud branching morphogenesis. This occurs, at least in part, via ROS generation, activation of Akt signaling, and upregulation of Pax-2 gene expression.

8.2 Introduction

Maternal diabetes mellitus (DM) constitutes a major risk factor for congenital malformations in the offspring. When the fetus is exposed to sustained levels of high

ambient glucose, widespread fetal damage may develop, affecting multiple systems, including cardiovascular, nervous, skeletal and urogenital systems-- a condition called diabetic embryopathy.[1;2] Infants born to women with pre-gestational diabetes have a 10-fold risk of congenital malformations, and those born to women with gestational diabetes have a 5-fold relative risk of congenital malformations. Both the diabetic mother and her fetus are at risk for significant morbidity and mortality, even in the 21st Century.[3;4] Of those congenital malformations seen in offspring of pre-gestational maternal diabetes, renal malformations such as renal agenesis and congenital abnormalities of kidney and urinary tract (CAKUT) are most prevalent.[5]

Renal morphogenesis involves complex events in which many genes interact to coordinate the formation of the final kidney. Abnormalities occur when the normal pattern of nephrogenesis is interrupted. In humans, the fetal kidneys begin to develop at 5 weeks gestation; renal glomeruli, at 8-9 weeks; and tubular function, after the 14th week. The full complement of nephrons (between 200,000 and well over 1 million) has formed by approximately 36 weeks of gestation; no further nephrons are formed after this time. [6;7] However, rodents, which have a gestation period in the range of 19-21-days, continue nephrogenesis postnatally-- until ~10 days after birth. DM constitutes an adverse *in utero* environment that may impair nephrogenesis in both human and experimental animal models, resulting in renal agenesis, dysplasia or aplasia and hypoplasia.[8-11] We have initiated investigations concerning the interaction between high glucose and paired-homeobox 2 (Pax-2) gene expression in renal development. As a "kidney-specific" master gene, Pax-2 is expressed in both UB and metanephric mesenchyme (MM) lineages, normally optimizing UB branching and mesenchymal-to-epithelial transformation in kidney development. [12-14] Mutations in the Pax-2 gene cause increased apoptosis, [15-17] associated with renal hypoplasia. [15;18;19] We recently reported that high D(+)

glucose (25mM), as compared to normal glucose (5mM), specifically induced Pax-2 gene expression in both *in vitro* (mouse metanephric mesenchymal cells (MK4) and *ex vivo* (kidney explant from Hoxb7-GFP (green fluorescent protein) mice) models.[20;21] High glucose-induced Pax-2 gene expression is mediated, at least in part, via reactive oxygen species (ROS) generation and activation of the NF-kB signaling pathway, but not via protein kinase C (PKC), p38 mitogen activated protein kinase (MAPK) and p44/42 MAPK signaling. [22]

The present work is designed to demonstrate the influence of a high glucose milieu on UB branching morphogenesis and its underlying mechanism(s) using real time in an *ex vivo* model. Additional *in vivo* studies that complement the *ex vivo* studies are also included. Our results indicate that high glucose stimulates UB branching via ROS generation and Pax-2 gene expression. We conclude that the stimulatory effect of high glucose is mediated, at least to some extent, via activation of NADPH oxidase and mitochondrial oxidative metabolism and stimulation of Akt signaling pathway.

8.3 Results

High D-Glucose Stimulates UB Branching in Time-Dependent Manner

Kidney explants isolated from timed-pregnant mice at E13 were cultured either in normal (5 mM) D-glucose DMEM plus 20 mM D-mannitol (left kidney) or high (25mM) D-Glucose DMEM (right kidney) supplemented with 1% dFBS up to 96 hours with fresh medium changed every 24 hours. As seen in fluorescent microscopic sequential images, as comparing to 5 mM Glucose (Figure 1A (A2-A6) and 1B (B2-B6)), 25 mM D-Glucose (Figure 1C (C2-C6) and 1D (D2-D6)) stimulates UB branching morphogenesis in time-dependent manner. Since a stimulatory effect of high glucose is present after 24 hours of incubation, we used 24

hours of stimulation for subsequent studies. By carefully measuring the diameter of metanephroi cultured from 0 to 96 hours (Figure 1E), we have found that high glucose reduced the size of metanephroi in a time-dependent manner.

High D-Glucose Specificity on UB Branching Morphogenesis

E13-kidney explants were incubated in media containing 1% dFBS and 25 mM different glucose analogues such as D-Mannitol, L-glucose, 2-Deoxy-D-glucose or D-glucose. After incubation for 24hours, high D-glucose (Figure 2A-a) specifically stimulates UB branching morphogenesis by fluorescent microscopy, while other glucose analogs such as D-Mannitol (Figure 2A-b), L-glucose (Figure 2A-c) or 2-Deoxy-D-glucose (Figure 2A-d) had no effect. Moreover, high D-glucose stimulates UB branching in a dose-dependent manner (Figure 2B). To maintain constant isotonicity or osmolality, 5-mM glucose media was supplemented with D-mannitol (20 mM final concentration) in additional studies. High glucose stimulated a greater than 2-fold increase of UB tip numbers as compared to normal glucose (Figure 3A+B). This increase in UB branching is induced by high D-glucose (25mM), but not by other glucose analogs.

High Glucose Stimulated Pax-2 Gene Expression

High glucose increased Pax-2 gene and protein expression-- mRNA (RT-qPCR, Figure 4A) and protein (Western Blot, Figure 4B)-- as compared to normal glucose in E18-kidney explants. Immunohistological staining indicated that the upregulation of Pax-2 is localized mainly to the UB (Figure 4C). These data indicate that high glucose stimulation of UB branching morphogenesis is mediated via Pax-2 gene expression.

Inhibitors of NADPH Oxidase and Mitochondrial Electron Transport Chain Complex I Block the Stimulatory Effect of High Glucose on Pax-2 Gene Expression and UB Branching *ex vivo*

It is apparent that inhibitors of NADPH oxidase (DPI, 10^{-6} M) and mitochondrial electron transport chain complex I (rotenone, 10^{-6} M) block the stimulatory action of high glucose on Pax-2 expression (Figure 5) and UB branching morphogenesis (Figure 6) in E14-kidney explants. These data indicate that the stimulatory effect of high glucose is mediated, at least in part, via NADPH oxidase activation and ROS generation,

High Glucose-induced ROS Generation on UB Branching and Pax-2 Gene Expression

We have previously reported that H_2O_2 modestly increases Pax-2 gene expression. However, this stimulatory effect of H_2O_2 was significantly enhanced in the presence of high glucose in E16-kidney explants. [22] The question has been raised about whether H_2O_2 also directly modulates UB branching. Indeed, exogenous H_2O_2 at 10^{-5} M could trigger UB branching morphogenesis, particularly in the presence of high glucose in E13-kidney explants (Figure 7 A-E). Our data also indicate that high glucose activates the total ROS generation in E18 Kidney explants (Figure 7F). Moreover, high glucose induced UB branching in E12-kidney explants could be partially blocked by catalase (250 U) and completely abolished by Akt Inhibitor IV (a cell-permeable benzimidazole compound that inhibits Akt phosphorylation/activation by targeting the ATP binding site of a kinase upstream of Akt, but downstream of PI3K) at concentration of 10^{-6} M (Figure 8). Similar results were also found in Pax-2 mRNA and protein expression (Figure 9). These data indicate that high glucose evokes ROS generation and upregulates Pax-2 gene

expression via Akt signaling, and subsequently stimulates UB branching morphogenesis.

The Effect of Gestational Diabetes on Offspring Neonatal Kidney

We have employed STZ to induce gestational diabetes in the pregnant mother at E13.[23-29] Figure 10A shows our experimental protocol in detail. As may be seen the newborn offspring of STZ-diabetic dams remained significantly smaller and lighter (average 20% less body weight (g)) as compared to offspring of control dams [Figure 10B (control vs STZ (g): 1.414 ± 0.11 vs 1.03 ± 0.07)]. Most impressively, kidneys of the diabetic offspring were significantly smaller and growth retarded as compared to the kidneys of control offspring (Figure 10C).

Pax-2 Expression in Neonatal Kidney *in Vivo*

We have evaluated the Pax-2 gene expression analyzed by immunohistological staining (Figure 11A and B), qRT-PCR (Figure 11C) and western blot (Figure 11D) in neonatal kidney of both control and diabetic dams. Our data indicated that maternal hyperglycemia via Pax-2 (mainly localized in UB) appears to accelerate UB branching but not nephron formation in neonatal kidney of diabetic dams.

8.4 Discussion

In the present study, we observed that high glucose alters UB branching morphogenesis via Pax2 gene expression. The high glucose effect appears to be mediated, at least in part, via ROS generation and activation of the PI3K-AKT pathway.

Maternal diabetes creates a high-risk intrauterine environment that has been directly linked to the development of congenital renal abnormalities, including

caudal regression syndrome, which is highly associated with renal agenesis and abnormalities of the kidney and urinary tract.[8-11] These anomalies have been noted as either isolated events or as part of multiple malformation syndromes that are more common in offspring of maternal diabetes. The incidence appears to be proportional to the degree of maternal hyperglycemia. For example, in the human, a high glucose ambient environment throughout pregnancy (pregestational diabetes) is present prior to embryonic development, which may result in a fetus with markedly teratogenic features that may include the caudal regression syndrome. In experimental animal, Hoxb7-GFP mice in our hand, if the pregnant dams are exposed to STZ before the budding process, in which the wolffian duct becomes UB, the dams are barely able to deliver. We have observed the same consequences-teratogenic embryos including caudal regression syndrome with renal agenesis under those conditions. Based on the timing found in the literature [26;27] and our own experiences, we induced gestational diabetes by STZ injection at E13, and we have successfully managed to obtain small litters of offspring from diabetic dams. Since the budding process is already completed prior to E13, from day E13 to birth, it seems likely that the high glucose ambient environment may impair the nephrogenesis.

To date, most human and experimental studies on gestational diabetes have focused on the phenotype, but only in a few instances, on the mechanism(s). Most maternal diabetes-related kidney explant studies, however, based on long culture time (average 4 to 6 days) revealed that hyperglycemia dose-dependently reduces the size of the metanephros, UB branching dymorphogenesis and the population of nascent nephrons.[26;27;30] This observation has been questioned, since the ideal observation time of the recognized architectural "pattern" of UB tree is between 18-48 hours.[31] In the current project, studies were designed to resolve potential

ambiguities arising from specific protocols or to examine UB morphogenesis under conditions that allow direct comparison to previous studies. [32;33]

In order to understand the effect of high glucose *per se* on renal UB development and its underlying molecular mechanisms, we used Hoxb7-GFP-Tg mice [20;21] as a model with which we would be able to monitor UB branching under normal or high glucose condition *ex vivo*. By employing sequential images, we have observed that E13-kidney explant cultured in high D(+) glucose (25mM) condition display more UB branching as compared to normal glucose medium (5 mM). A stimulatory effect was obvious at 24 hours post-exposure. Moreover, high D(+) glucose specifically triggers UB branching morphogenesis and increased UB tip numbers in a dose-dependent manner, while other glucose analogs such as D-mannitol, L-glucose and 2-deoxy-D- glucose had no effect. We previously reported that high glucose specifically induces Pax-2 gene expression via ROS generation in E16 kidney explants from Hoxb7-GFP mice. [22] In the current study, we have observed that high glucose elevated total ROS generation and triggered UB branching morphogenesis. It appears that the stimulatory effect of high glucose could be blocked by ROS inhibitors such as DPI and rotenone. Although the Pax-2 gene is expressed in both UB and MM lineages,[12;34-39] our immunohistological staining data have clearly revealed that the greatest upregulation of Pax-2 by high glucose is in the area of the UB. Meanwhile, in order to clarify the direct functional impact of ROS on UB branching morphogenesis, we have also tested H_2O_2, an important source of superoxide ($O_2^{\bullet-}$), and observed that exogenous H_2O_2 at 10^{-5} M stimulates UB branching morphogenesis; however, in combination with high glucose, the H_2O_2 stimulatory effect is enhanced substantially, the similar response pattern as Pax-2 gene expression. [22] Taken together, our data suggest that high glucose induced ROS generation has a functional impact on UB lineage *ex vivo*.

Evidence indicates that the high glucose-ROS-PI3K/Akt-NF-κB pathway appears to be a major signaling pathway that almost covers all major renal cell types.[40-45] This scenario has been postulated as that by which high glucose leads to kidney damage.[40-45] Indeed, a study [46] suggests that down-regulation of Pax-2 expression correlates with a decreased Akt phosphorylation and an enhanced sensitivity to renal endothelial cell apoptosis both *in vivo* and *in vitro*, suggesting that Pax-2 promotes angiogenesis, likely via survival, proliferation, invasion, and cell organization via the PI3K/Akt-dependent pathway. Thus, we hypothesize that the pathway, high glucose→ROS→Akt→NF-κB→Pax-2 is involved in impairment in the UB lineage induced by high glucose. Indeed, our data suggest that high glucose action on UB branching morphogenesis as well as on Pax-2 gene expression could be completely abolished by Akt inhibitors, but partially blocked by catalase. The reason for this partial blocking effect may be due to the fact that catalase could only convert H_2O_2 into H_2O, but has no effect on other species such peroxynitrites, hydroxyl radicals, etc. Indeed, more studies are needed to elucidate the action of other ROS.

We used STZ to induce gestational diabetes in an *in vivo* model to generate a high ambient glucose environment during pregnancy. We observed that this impaired UB branching morphogenesis in E13 pregnant Hoxb7-GFP mice. This *in vivo* strategy avoids the limitations imposed by using *ex vivo* studies. Our data indicate that the body weight (in grams) of neonate offspring from diabetic mother remained significantly lower and smaller (average 20% less) than control animals. Renal morphology revealed that kidneys of diabetic offspring showed growth retardation. However, it appears that high glucose via Pax-2 (mainly localized in UB) could accelerate UB branching but not nephron formation, but at the same time, high glucose also triggers cell apoptosis in both UB and nephron, which we believe is the

major mechanism by which renal function is ultimately affected in diabetic offspring over time (our long time follow-up study).

In summary, our data demonstrate that high glucose alters UB branching morphogenesis via Pax-2 gene and protein expression. The stimulatory effect of high glucose appears to be mediated via ROS generation and activation of the Akt signaling pathway.

8.5 Concise methods

Reagents

Normal glucose medium (5 mM D-glucose Dulbecco's modified Eagle's medium (DMEM; Cat. #12320)) was purchased from Invitrogen Inc. (Burlington, Ontario, Canada). D(+)-Glucose, L-glucose, D-mannitol, 2-deoxy-d-glucose, diphenylene iodinium (DPI), rotenone, H_2O_2, catalase and 5-(2-Benzothiazolyl)-3-ethyl-2-[2-(methylphenylamino)ethenyl]-1-phenyl-1H-benzi midazolium iodide (Akt Inhibitor IV), were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Mouse anti-β-actin monoclonal antibody (clone AC-15) and rabbit polyclonal anti-Pax-2 antibody were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and Covance (Richmond, CA, USA), respectively.

Animals

We used the murine Hoxb7-GFP model [20;21] [obtained from Dr. Frank Costantini (Department of Genetics and Development, Columbia University Medical Center, NY, NY, USA)], which is useful for studying alterations in UB branching morphogenesis, as the GFP permits direct observation of the branching process. This is especially useful for studying adverse *in utero* development as in diabetic mellitus. Hoxb7-GFP mice express GFP driven by the Hoxb7 promoter throughout

the Wolffian duct (WD) and UB epithelium but not in the surrounding MM or its epithelial derivatives, allowing UB branching morphogenesis to be visualized in real time during growth of the kidney, either in organ culture or in fixed tissue.[20;21] We used these features for the direct study of UB branching morphogenesis pattern under non-diabetic and diabetic conditions *ex vivo.*

Animal care in these experiments met the standards set forth by the Canadian Council on Animal Care, and the procedures utilized were approved by Institutional Animal Care Committee of the CHUM. Hoxb7-GFP mice were housed under standard humidity and lighting conditions (12 hour light-dark cycles), and were allowed free access to standard mouse chow and water *ad libitum*. Timed-pregnant mice aged 8-10 weeks were used in all experiments. Vaginal wet mounts were made to determine the estrous cycles of the mice. On the evening before estrus, female mice were housed overnight with male mice; the presence of spermatozoa in a vaginal smear the next morning was defined as day 1 of pregnancy.

Metanephric Organ Culture

Embryos (E12 to E18) were dissected aseptically from timed-pregnant mice, and the metanephroi were isolated under sterile conditions.[22] GFP-positive metanephroi were photographed immediately after isolation (time 0) and were individually cultured either in 1 ml of normal glucose (5mM glucose) or high glucose (25mM glucose) DMEM supplied with 1% dFBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin in 95% air and 5% CO_2 at 37ºC in separate wells of a 24-well plate for different time periods, depending on the experiment. For example, we constantly monitored and recorded every 8 hours the sequential images of UB branching in metanephroi cultured either in normal glucose or high glucose DMEM supplied with 1% dFBS condition up to 96 hours with fresh medium changed every 24 hours.

Based on initial results a 24-hour incubation period was subsequently used for the rest of our experiments. The surface area of cultured metanephroi either in 5mM or 25mM glucose DMEM from time 0 to time 96 hours was measured by QCapture Pro 5.1 image analysis program provided in Olympus 1X71 Microscope (CARSEN, ON, CA).

To address the variability in embryonic kidney size and in UB branching patterns among conceptuses, the effect of different treatments on UB branching was studied in kidneys from the same fetus; for example, the left kidney was incubated with normal glucose and the right kidney, with high D-glucose or L-glucose; or the left kidney was incubated with high D-Glucose and right kidney, with high D-Glucose in the presence or absence of DPI (10^{-6} M), rotenone (10^{-6} M), catalase (250 U) and Akt inhibitor (10^{-6} M).

Sequential images of branching UB were recorded with a Olympus 1X71 Microscope (CARSEN, ON, CA). Quantitative assessment of UB branching in each treatment group was performed by manually counting the number of UB tips at time 0 and at 24 hours.

Western Blotting

Western blots were performed as in previous studies. [22;47;48] Briefly, small aliquots (20-50 μl) of homogenized kidney explant sample were subjected to 10% SDS-PAGE and then transferred onto a PVDF membrane (Hybond-P, Amersham Pharmacia Biotech, Canada). The membrane was first blotted for anti-Pax-2 and then re-blotted for β-actin. The relative densities of the Pax-2 vs β-actin bands were measured by computerized laser densitometry.

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

RT-qPCR was performed as reported previously. [22;47] In brief, first-strand cDNA was produced from 2 g of random hexamer primed total RNA using Super-Script preamplification system (Invitrogen). Relative quantitation by real-time PCR was carried out using iQ™ SYBR@ Green Supermix Kit (Bio-Rad Laboratories, Mississauga, ON, Canada) and MiniOpticon™ Real-Time PCR Detection System (Bio-Rad), following the protocol described by the supplier. PCR reactions in triplicate underwent 40 cycles of 95 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s, and 79 °C for 5 s in the thermal cycler. The parameter CT (threshold cycle) value was measured to determine starting copy number of target genes using the standard curve. Lower value of CT indicates a higher amount of PCR products. We employed the following forward and reverse primers: forward primer 5' ACATCAAATCAGAACAGGGGAAC 3', and the reverse primer 5' CATGTCACGACCAGTCACAAC 3'; these correspond to the nucleotide sequences N+1319 to N+1341 and N+1453 to N+1473 of Pax-2 cDNA (NM_003990). For internal control, we deployed primers specific for mouse β-actin (forward and reverse primers 5' CGTGCGTGACATCAAAGAGAA 3' and 5' GCTCGTTGCCAATAGTGATGA 3', corresponding to nucleotide sequences N+704 to N+724 and N+820 to N+840 of mouse β-actin cDNA (NM_007393)). [47]

Immunohistochemistry

Kidney explants were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) (Fisher Scientific, Nepean, ON, Canada) after 24 hours in culture and then paraffin-embedded. Kidney sections of 5 μm were deparaffinized in xylene and rehydrated. Immunohistochemical examination was performed by the standard avidin–biotin–peroxidase complex method (ABC Staining System, Santa Cruz

Biotechnologies, Santa Cruz, CA, USA). Endogenous peroxidase was inhibited in 1% hydrogen peroxide–methanol for 10 min at room temperature and followed by trypsin treatment for 10 min in a moist chamber at 37°C. After serum blocking, the sections were incubated with primary anti-Pax-2 polyclonal antibody diluted 1:100 overnight at 4°C humidity chamber; then, biotinylated secondary antibody was added, followed by the addition of preformed ABC reagents supplied by the ABC kit. The Pax-2 protein was visualized by color development with 3, 3'-diaminobenzidine tetrahydrochloride. All sections were counterstained with hematoxylin, dehydrated, and covered with glass coverslips.

ROS Generation

ROS production was monitored by the lucigenin method with minor modifications. [22;49;50] ROS generated in E18 kidney explant were normalized with protein concentration and expressed as relative light units (RLU) per mg protein.

In vivo Study

We induced gestational diabetes in pregnant Hoxb7-GFP mice with an intraperitoneal injection of streptozotocin (STZ,150 mg/kg body weight (BW)) at E13.[23-29] Maternal glucose concentration (mM) was carefully monitored by Accu-Chek Compact Plus Blood Glucose Meter (Roche Diagnostics, Laval, QC, Canada) (Figure 10 A). Newborn birth weight (BW) was carefully recorded, as shown in Figure 10B. Hematoxylin/eosin (H/E) staining was used to review renal morphology, whereas Dolichos Biflorus Agglutinin-FITC (DBA-FITC, Vector Laboratories) staining for UB identification [15] in 5 μm of paraformaldehyde (4%)-fixed-paraffin-embedded kidney sections under a light microscope (Figure

10C). Pax-2 expression was analyzed by immunohistological staining, qRT-PCR and western blot as mentioned above (Figure 11).

Statistical Analysis

Statistical significance between experimental groups was analyzed initially by Student's *t* test or by 1-way ANOVA followed by the Bonferroni test as appropriate. Three to four separate experiments were performed for each protocol. Data are expressed as means ± SD. A probability level of $P \leq 0.05$ was considered statistically significant.

8.6 Acknowledgements

The authors acknowledge the kind gifts of Hoxb7-GFP mice from Dr. Frank Costantini (Columbia University, New York, NY, USA). Thanks are also due to Dr. John S.D. Chan (CHUM-Hôtel-Dieu, Montreal, QC, Canada) for his unconditional support and discussion for the project. This research was supported by KRESCENT and Kidney Foundation of Canada (KFOC) and a New Investigator Award from KRESCENT of Kidney Foundation of Canada for Dr. Shao-Ling Zhang. The editorial assistance of Ovid Da Silva, Editor, Research Support Office, Research Centre, CHUM, is acknowledged.

ABBREVIATIONS: CAKUT, congenital abnormality of kidney and urinary tract; DMEM, Dulbecco's modified Eagle's medium; dFBS, depleted fetal bovine serum; DPI, diphenylene iodinium, an inhibitor of NADPH oxidase; FBS, fetal bovine serum; GFP, green florescent protein; MAPK, mitogen activated protein kinase; MK4, mouse late embryonic mesenchymal epithelial cells; MM, metanephric mesenchyme**;** NF-kB, nuclear factor kappa B; Pax, paired homeobox gene; PKC,

protein kinase C; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species; UB, ureteric bud; WD, Wolffian duct.

8.7 References

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8.8 Legends and Figures

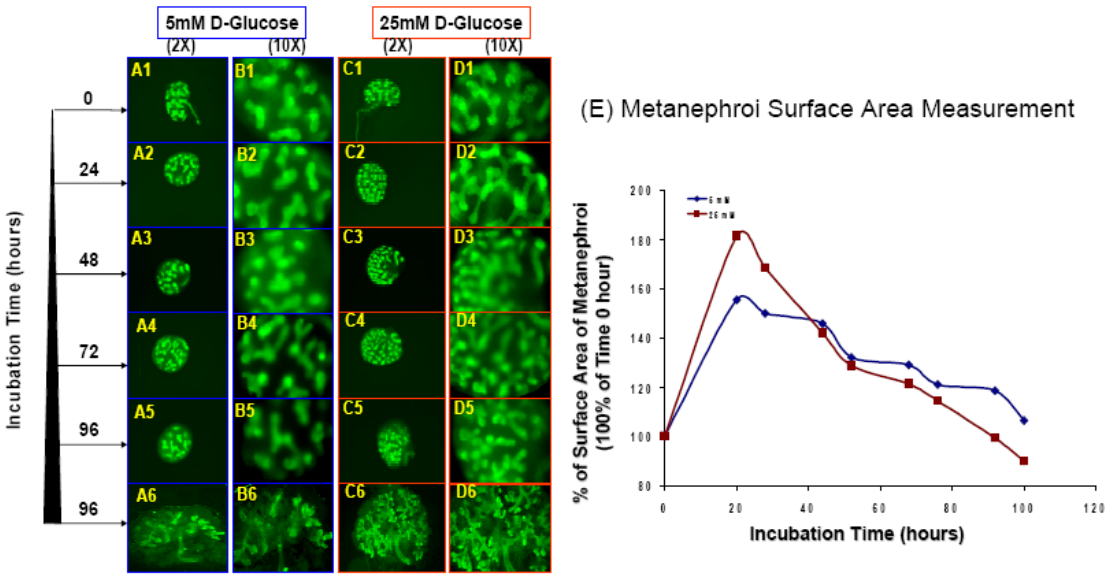

Figure 1

Figure 8-1 High D-Glucose Stimulates UB Branching in Time-Dependent Manner. Kidney explants isolated from time-pregnant mice at E13 stage were cultured either in normal (5mM) D-glucose DMEM (left kidney) or high (25mM) D-Glucose DMEM (right kidney) supplied with 1% dFBS up to 96 hours with fresh medium changed every 24 hours. The UB branching morphogenesis sequential images were recorded by fluorescent microscope. A(1-6) (2X magnification) and B (1-6) (10 magnification) representative the kidney explant were cultured in 5mM Glucose supplemented with 20 mM D-mannitol DMEM to maintain constant isotonicity or osmolality, whereas C (1-6) (2X magnification) and D (1-6) (10 magnification) in 25mM Glucose DMEM. The sequential images were recorded by every 24 hours. E shows that high ambient glucose reduced the surface area of cultured metanephroi in a time-dependent manner.

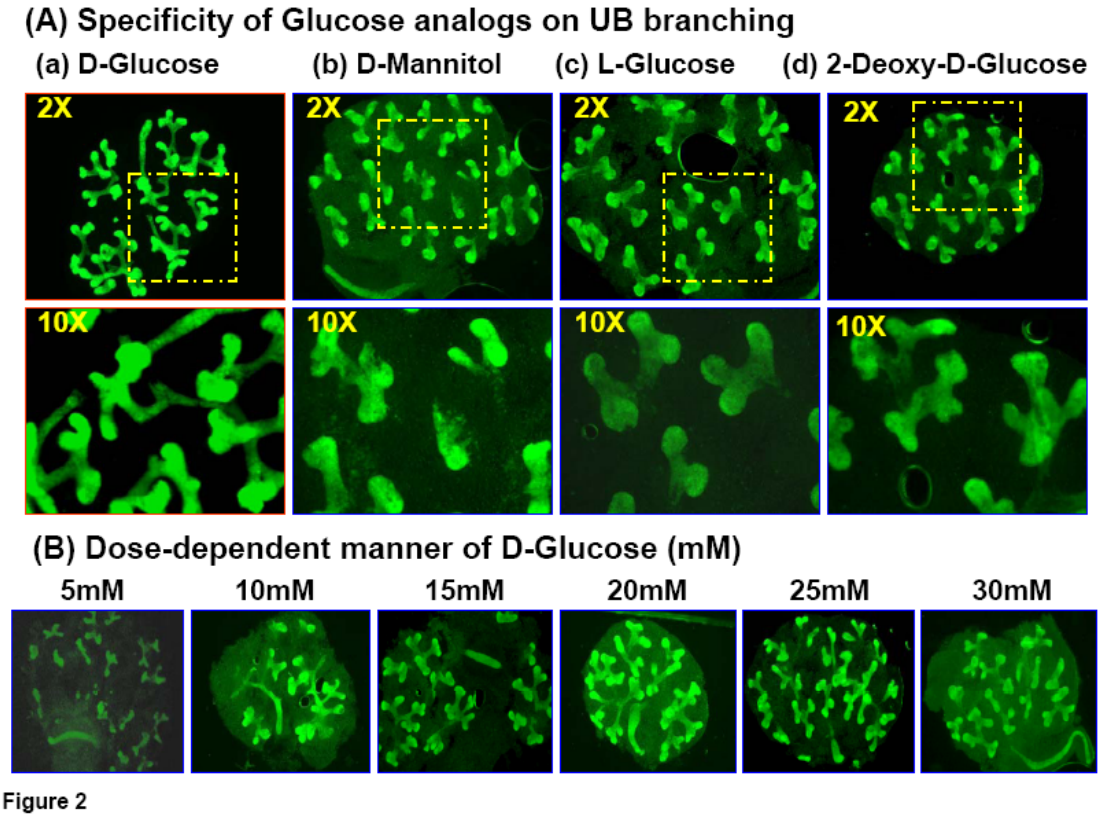

Figure 8-2 High D-Glucose on UB Branching Morphogenesis. Figure 2A shows the specificity of the effect of glucose analogs on UB branching. E13 kidney explants were incubated in media containing 1% dFBS and 25 mM different glucose analogues such as D-Glucose (a), D-Mannitol (b), L-glucose (c) or 2-Deoxy-D-Glucose (d) for 24 hours. The images were recorded by fluorescent microscope (2X and 10X magnification); Figure 2B shows the D-glucose dose-dependent effect on UB branching from 5mM to 30mM in E13 kidney explant (2X magnification).

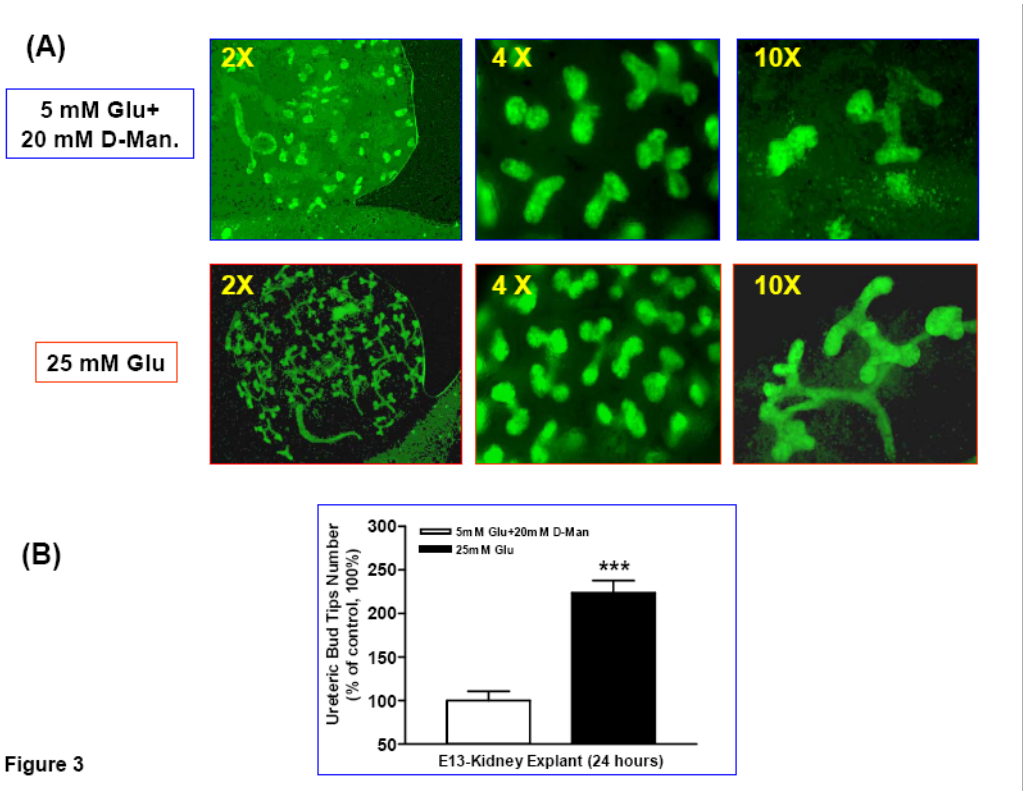

Figure 8-3 High D-Glucose stimulates UB Branching Morphogenesis. (A) E13 kidney explants were incubated either in 5mM glucose (left kidney) or 25 mM glucose DMEM (right kidney) containing 1% dFBS for 24 hours. The images were recorded by fluorescent microscope (2X, 4X and 10X magnifications). (B) Quantification of UB numbers. Kidney explants incubated in 5 mM glucose were considered the control (100%). Each point represents the mean ± SD of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

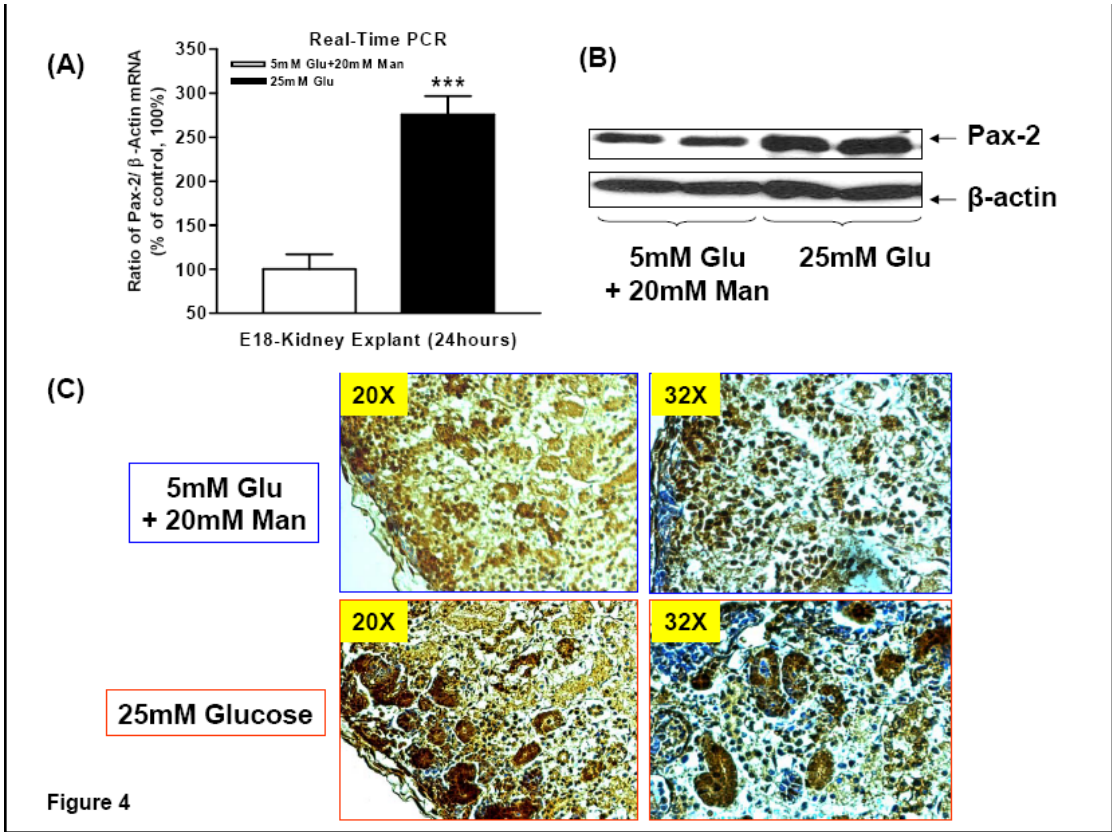

Figure 8-4 High glucose up-regulates Pax-2 expression in E18 kidney explant analyzed by (A) RT-qPCR; (B) western blot; and (C) immunohistological staining (20X and 32X magnification). The normalized Pax-2 level in explant incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

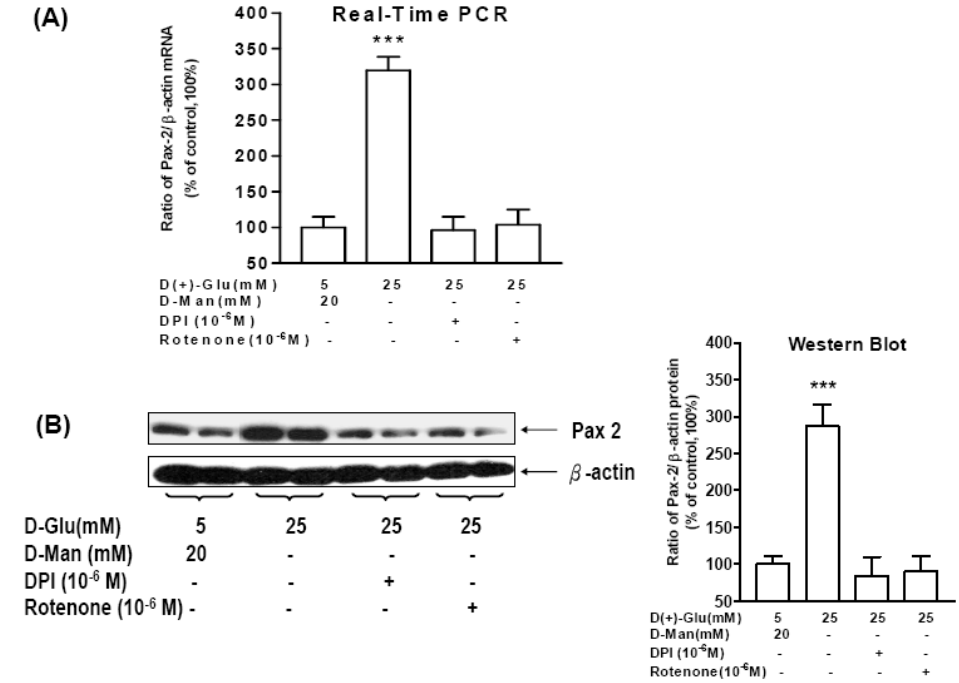

Figure 5

Figure 8-5 The inhibitory effect of DPI and rotenone on Pax-2 gene expression in E16 kidney explants. E16 kidney explants were cultured in either 5 mM glucose or 25 mM glucose DMEM with or without DPI (10^{-6} M) and rotenone (10^{-6} M) for 24 hours. The Pax-2 gene expression was analyzed either by (A) RT-qPCR or (B) western blot, respectively. The relative densities of Pax-2 were compared with ß-actin. The normalized Pax-2 level in kidney explants incubated in 5 mM glucose was considered the control (100%); Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

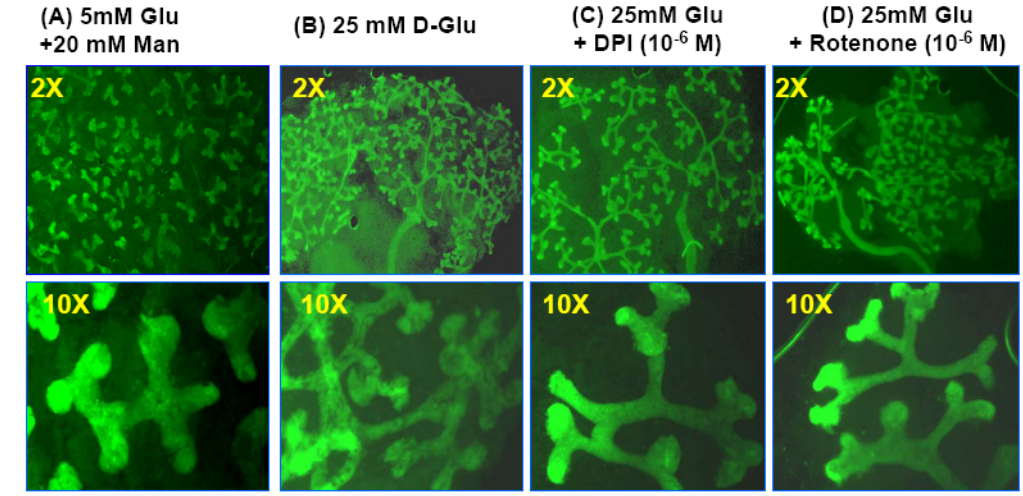

Figure 6

Figure 8-6 The inhibitory effect of DPI and rotenone on UB branching morphogenesis stimulated by high glucose. E14 kidney explants were incubated either in 5mM glucose (A) or 25 mM glucose DMEM (B) in the absent or present of DPI (10^{-6} M) (C) and rotenone (10^{-6} M) (D) for 24 hours. The images were recorded by fluorescent microscope (2X and 10X magnifications). (E) Quantification of UB numbers. Kidney explants incubated in 5 mM glucose were considered to be controls (100%). Each point represents the mean ± SD of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

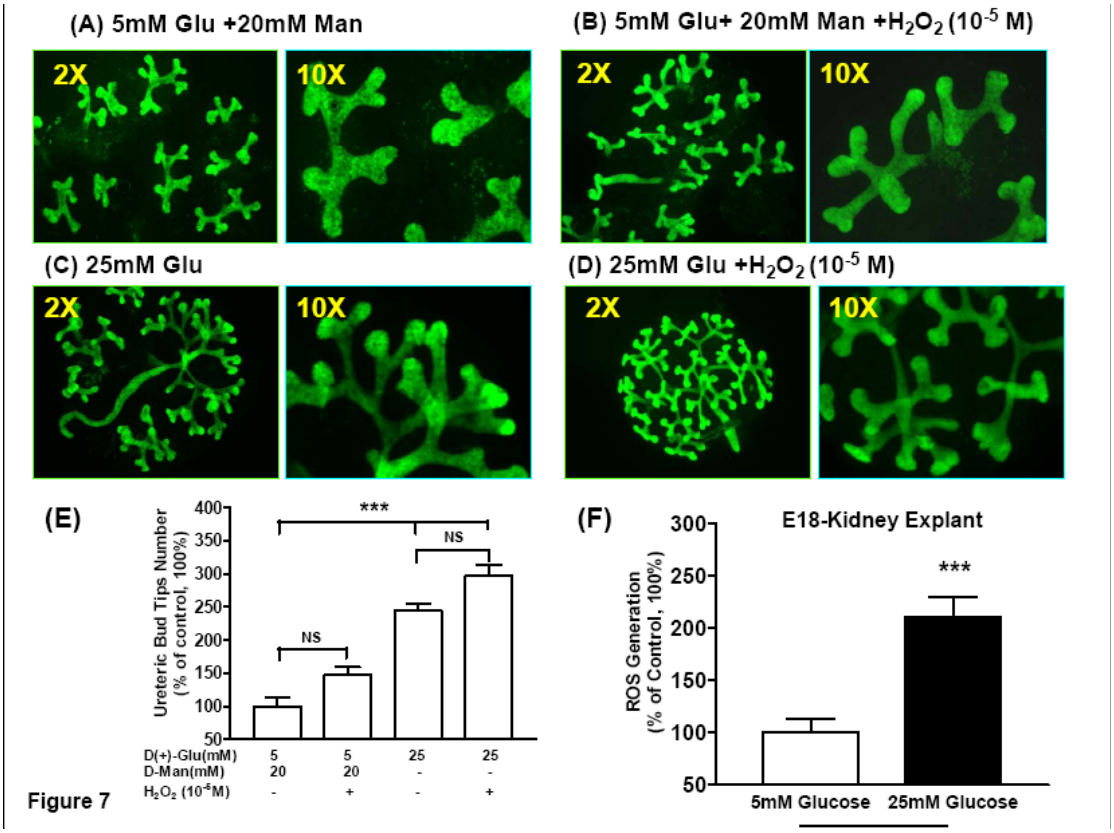

Figure 7

Figure 8-7 H_2O_2 effect on UB branching morphogenesis in E13 kidney explants. E13 kidney explants were incubated either in 5mM glucose (A and B) or 25 mM glucose DMEM (C and D) with or without H_2O_2 (10^{-5} M) for 24 hours. The images were recorded by fluorescent microscope (10X magnifications). (E) Quantification of UB numbers. Kidney explants incubated in 5 mM glucose were considered as controls (100%). (F) ROS generation was assessed by lucigenin method, and the final value of ROS generation was normalized by the protein concentration of sample. The normalized ROS generation in cells incubated in 5 mM glucose was considered as the control (100%). Each point represents the mean ± SD of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

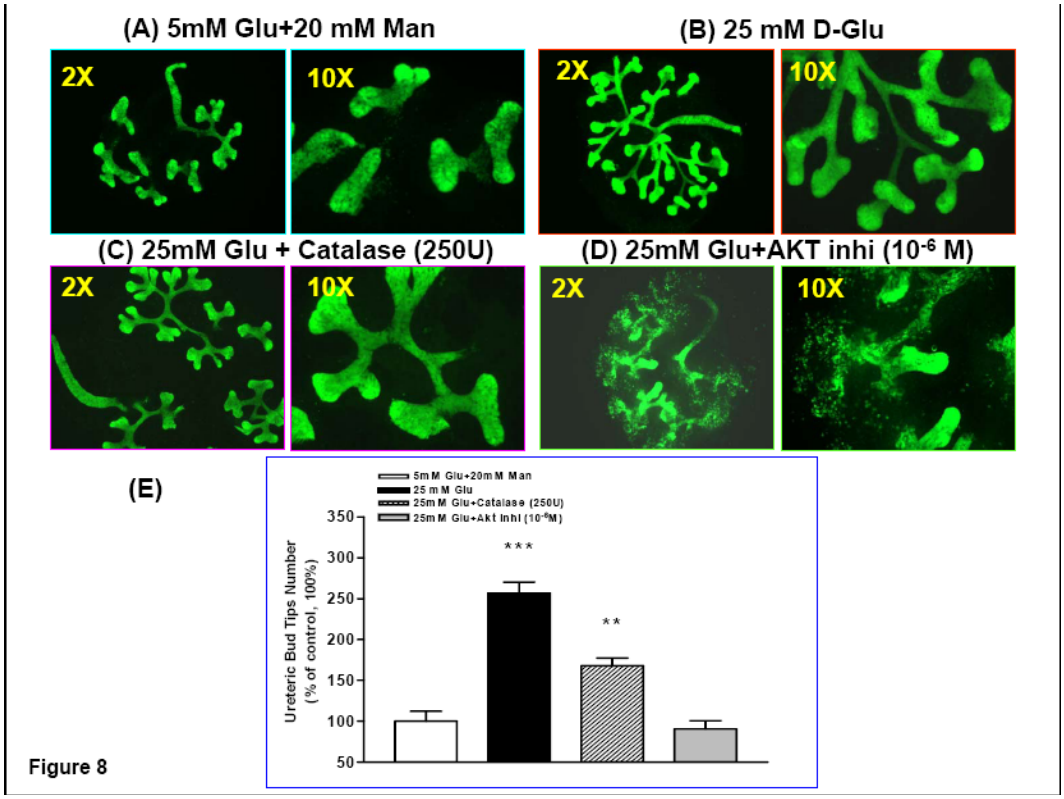

Figure 8-8. The inhibitory effect of catalase and AKT inhibitor on UB branching morphogenesis in E12 kidney explants. E12 kidney explants were incubated either in 5mM glucose (A) or 25 mM glucose DMEM (B) in the absent or present of Catalase (250 U) (C) and AKT inhibitor (10^{-6} M) (D) for 24 hours. The images were recorded by fluorescent microscope (2X and 10X magnifications). (E) Quantification of UB numbers. Kidney explants incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± SD of three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

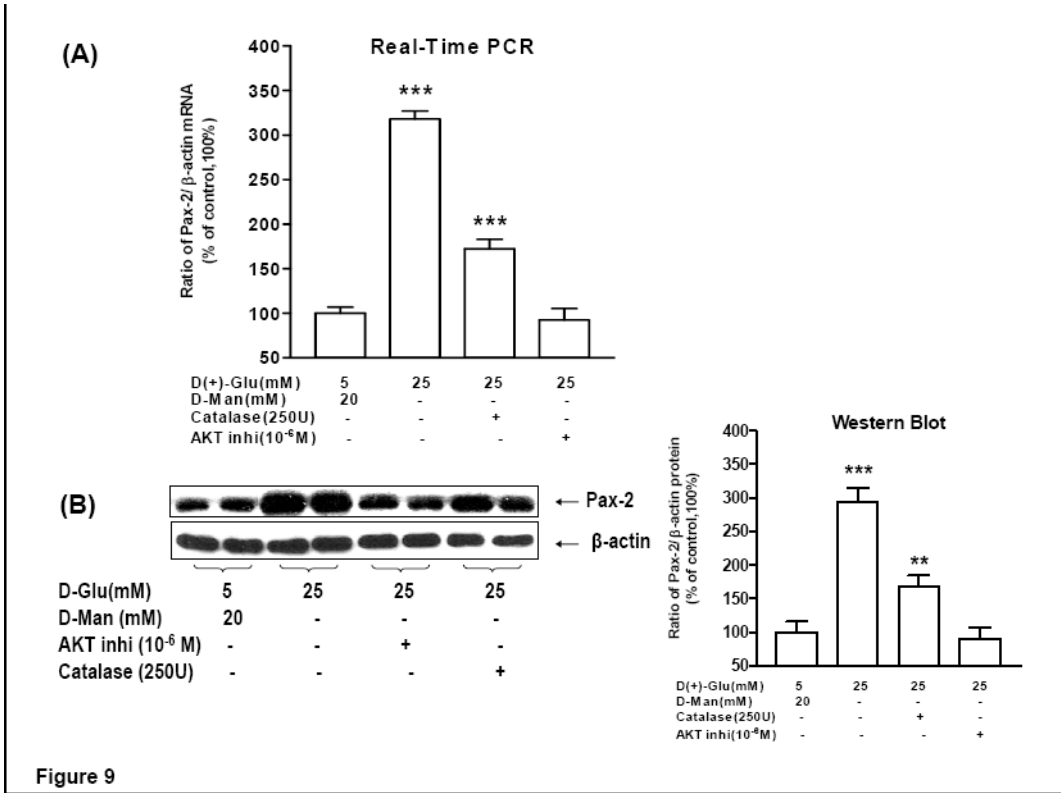

Figure 9

Figure 8-9. The inhibitory effect of catalase and AKT inhibitor on Pax-2 gene expression in E17 kidney explants. E17 kidney explants were cultured in either 5 mM glucose or 25 mM glucose DMEM with or without Catalase (250 U) and AKT inhibitor (10^{-6} M) for 24 hours. The Pax-2 gene expression was analyzed either by (A) RT-qPCR or (B) western blot, respectively. The relative densities of Pax-2 were compared with ß-actin. The normalized Pax-2 level in kidney explants incubated in 5 mM glucose was considered the control (100%); Each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

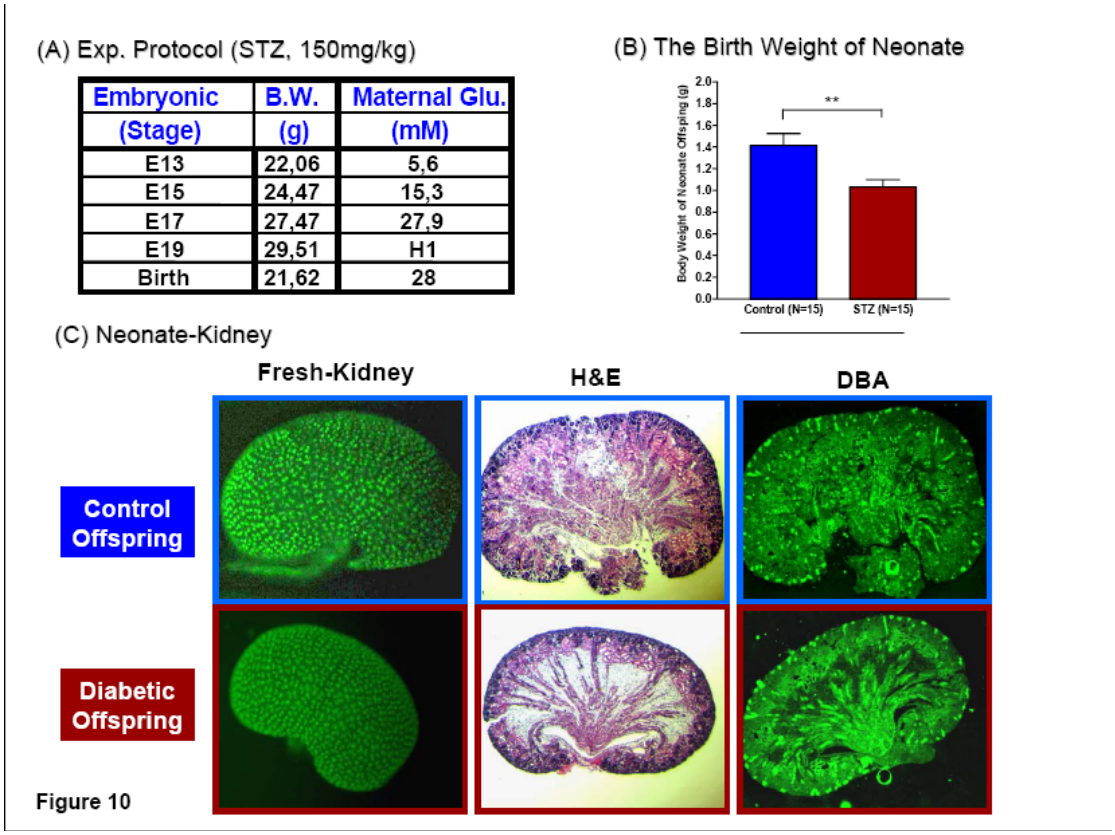

Figure 8-10. ***In vivo*** **studies on the effect of maternal diabetes on the neonatal kidney of the offspring.** Figure 10 A shows a representative mouse in our experimental protocol in detail. Diabetic-STZ newborn offspring remained significantly smaller and lighter (average 20% less (g)) as compared to control animals, as shown in Figure 10B (control vs STZ (g): 1.414 ± 0.11 vs 1.03 ± 0.07). Kidney of diabetic offspring was significantly smaller and growth retarded (H&E staining and DBA staining) compared to control (Figure 10C).

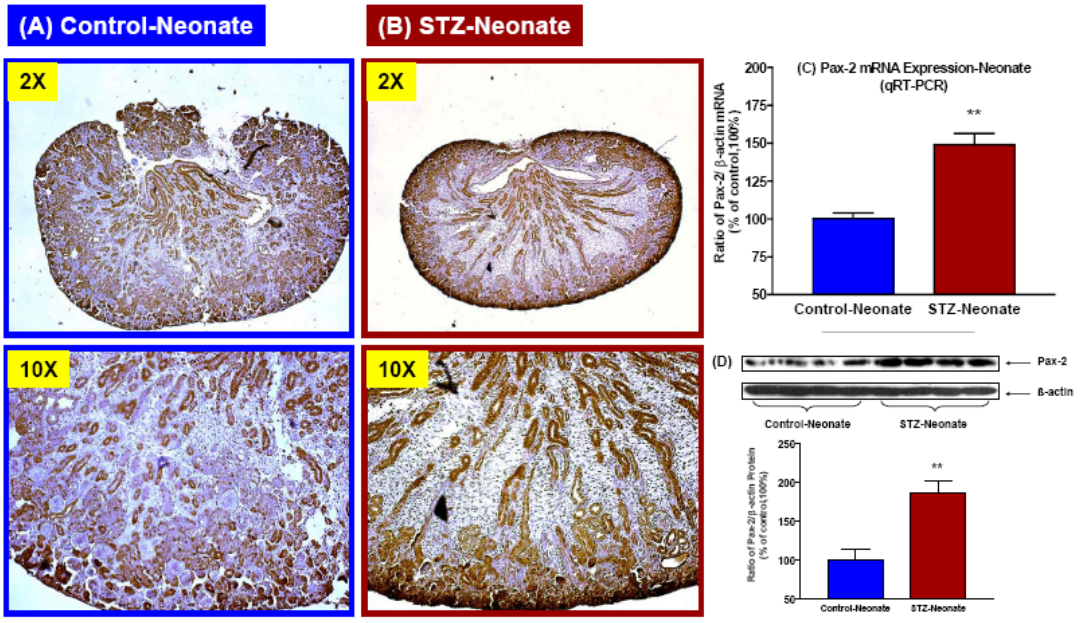

Figure 11

Figure 8-11. ***In vivo*** **Pax-2 expression in neonate kidneys.** Pax-2 immunohistological staining in newborn kidney of offspring: Control (A, 2X and 10X magnification) and STZ (B, 2X and 10X magnification). Pax-2 gene expression in neonatal kidney was analyzed by qRT-PCR (C) and western blotting (D). The normalized Pax-2 level in control-neonate-kidney was considered as 100%; each point represents the mean ± SD of 3 independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.005$.

CHAPTER 9 : ARTICLE 3

Maternal Diabetes Modulates Renal Morphogenesis in Offspring

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9.1 Abstract

Maternal diabetes leads to an adverse *in utero* environment but whether maternal diabetes impairs nephrogenesis is unknown. Diabetes was induced with streptozotocin in pregnant diabetic Hoxb7-green fluorescent protein mice at embryonic day 13, and the offspring were euthanized at several time points after birth. Compared with offspring of non-diabetic controls, offspring had lower body weight, body size and kidney weight, and nephron number. The observed renal dysmorphogenesis may be the result of increased apoptosis, because immunohistochemical analysis revealed significantly more apoptotic podocytes, as well as increased active caspase-3 immunostaining in the renal tubules compared with control mice. Regarding potential mediators of these differences, offspring of diabetic mice had increased expression of intrerenal angiotensinogen and renin mRNA, upregulation of NF-kB isoforms p50 and p65 and activation of the NF-kB pathway. In conclusion, maternal diabetes impairs nephrogenesis, possibly *via* enhanced intrarenal RAS activation and NF-kB signaling.

9.2 Introduction

Diseases such as maternal diabetes create an adverse *in utero* environment that may impair the process of embryogenesis, thus predisposing infants of low birth weight (LBW) to subsequent increased risk of future disease [1-5]. The developing kidney appears particularly sensitive to a high glucose milieu, exposure to which may result in congenital renal malformations, i.e., renal agenesis, dysplasia or hypoplasia. [6-9]

Intrauterine growth retardation, defined as birth weight below the 10th percentile for gestational age, is associated with a reduction in nephron number. [10] Although the so-called "thrifty phenotype" hypothesis suggesting that LBW is linked to

perinatal programming, [10;11] the underlying mechanisms whereby nephron number may be affected and/or nephron function altered, are not yet completely delineated.

The NF-kB pathway has been reported to be a major intracellular target in hyperglycemia and oxidative stress, [12;13] and its two functional pathways (canonical-classical and noncanonical) have been studied in the diabetic kidney. [14-16] Five members of the NF-kB family have been identified: NF-kB1 (p50/p105), NF-kB2 (p52/p100), RelA (p65), RelB, and c-Rel. It appears that RelA (p65) and p50, in particular, can contribute to p53-, tumor necrosis factor-a (TNF-a)- and reactive oxygen species (ROS)-mediated cell apoptosis [17-21] There is a growing consensus that a high glucose milieu and diabetes-induced activation of the NF-kB pathway has a functional impact on the course of diabetic nephropathy. [15;16;22;23] However, the underlying mechanisms of NF-kB signaling in impairing renal morphogenesis are not well understood.

Deficiency, mutation or abnormal expression of genes of the intrarenal renin angiotensin system (RAS) during organogenesis in experimental animal models often leads to abnormal kidneys, [12;24-28] with a decrease in ultimate nephron numbers. [29-36] Studies including ours have demonstrated that intrarenal RAS activation plays a key role in the development of hypertension and renal injury in diabetes. [37-40] In a model of experimental diabetes in the rat Lee et al reported a functional interaction between NF-kB (in particular, the p65 subunit) and the Ang II type I receptor (AT_1-R).[23] Given these data, we hypothesize that, in diabetes, enhanced intrarenal RAS activation and NF-kB signaling are two key elements intimately involved in the process of apoptosis in nascent nephrons, ultimately leading to nephron deficiency.

9.3 Results

Neonatal Offspring from Control- and Diabetic -Hoxb7-GFP-Tg Mothers

The body length of neonatal offspring of STZ-induced diabetic mothers was significantly smaller as compared to those from control mothers (control vs diabetic neonate offspring in length (cm): 2.54 ± 0.27 vs 2.03 ± 0.25), as shown in Figure 1(A+B).

Biological Parameters

Offspring from diabetic mice remained significantly smaller and lighter (average 20% less) than control offspring during the entire suckling period. Figure 2A shows body weight (BW) for control vs STZ offspring (1.414 ± 0.11 vs 1.03±0.07 g ($p \leq 0.001$) in neonate; 3.744 ±0.90 vs 3.00±1.01 g ($p \leq 0.05$) in 1 week; 6.93 ±1.32 vs 5.71±1.11 ($p \leq 0.001$) 2 week old; 12.12 ±1.16 vs 9.79 ±2.07 ($p \leq 0.001$) 3 week old animals), and Figure 2B indicates the ratio of kidney weight (KW) to BW, suggesting that kidneys of diabetic offspring are relatively large for their BW.

Renal Morphology, Nephron Number and Glomerular Volume in Neonatal Kidneys

H&E stained sections of whole-mount neonatal kidneys indicated that offspring from diabetic pregnant mice had smaller kidneys with smaller glomeruli size as compared to the kidneys of control offspring (Figure 3A). Meanwhile, we have found that the glomerular volume (V_G) of young diabetic offspring from neonate to 2 week-old is persistently less than one of control animals (Figure 3B). By carefully counting the number of nephrons, we have observed that neonatal nephron number in diabetic offspring is significantly lower than in control animals

(average 40% less) [control vs diabetic neonate offspring in number: 3,038 ± 175.52 (N=6) vs 1,862 ± 128.74 (N=5) ($p \leq 0.001$)] (Figure 3C)..

STZ Toxicity Effect

STZ is an unstable product with a biological half-life in cell culture medium of approximately 19 minutes (www.sigmaaldrich.com). Since STZ administration does not induce diabetes 100% of the time, we had the opportunity to examine nephrogenesis in fetuses of STZ-exposed animals with or without diabetes. We performed additional experiments to determine whether STZ could affect nephrogenesis in Nephrin-CFP-Tg mice *in vivo*. We have observed that renal damage (kidney size and number of glomeruli forming) appeared to be dependent on the level of maternal hyperglycemia (Figure 4A) but independent of STZ administration (Figure 4B) or the length of exposure to STZ (from 0 to 5 days (Figure 4C)). Thus, we feel that it is unlikely that a small amount of STZ (if it crosses the placenta) exerts toxicity in the fetus *in utero* in our model.

Apoptosis in Kidneys of Diabetic Offspring

TUNEL assay revealed that apoptotic cells appear to be increased in the collapsed nephron region in neonates and 1 week-old offspring of diabetic mothers as compared to control offspring at the same ages (Figure 5A). Double immunostaining with anti-Wilms tumor-1 (WT-1) and anti-active cleaved caspase-3 antibody indicates that glomerular podocytes undergo apoptosis, which ultimately results in nephron collapse (Figure 5B). Similarly, we have observed augmented cleaved caspase-3 immunostaining in renal tubule of offspring of diabetic mothers from neonate to 3-weeks of age as shown in Figure 6. Taken together, these data suggest that high glucose milieu creates an adverse *in utero* environment that

dynamically triggers nascent nephron apoptosis during nephrogenesis, consequently resulting in dysmorphogenesis with small kidneys.

Activation of the Intrarenal RAS and NF-kB pathways in Diabetic Offspring

RT-qPCR assays revealed that high glucose milieu *in utero* is capable of affecting intrarenal RAS gene expression in kidneys of offspring of diabetic dams; in particular, ANG and renin mRNA expression was persistently upregulated from the neonatal period to 3 weeks of age (Figure 7). Immunohistochemical examination of renal sections confirmed that augmented ANG protein expression is generally localized to the proximal tubule region in kidneys of offspring of diabetic mother (Figure 8), whereas cells positive for renin appear in a glomerular or tubular position in offspring of diabetic mothers rather than in the juxtaglomerular apparatus as in normal offspring (Figure 9). Moreover, we have observed that the p50 and p65 subunits of NF-κB were up-regulated and translocated from the cytosol to the nucleus in the proximal tubules of offspring from diabetic mother (Figure 10A). In gel mobility shift assays (GMSA), we have found that NF-kB activation is more elevated in the neonatal kidneys of offspring from diabetic mothers compared to the offspring of control dams (Figure 10B). Taken together, our data indicate that a hyperglycemic environment *in utero* reduces kidney size and triggers apoptosis of nascent nephrons, possibly via the activation of the intrarenal RAS and NF-kB pathways.

9.4 Discussion

In the present work we aimed to delineate the functional role of maternal diabetes in modulating renal morphogenesis in their offspring and to study their underlying mechanisms. Our data indicate that a hyperglycemic environment *in*

utero reduces kidney size and triggers nascent nephron apoptosis via intrarenal RAS activation and NF-Kβ signaling.

Maternal diabetes presents an environmental challenge *in utero* and may fundamentally and dynamically impair the process of embryogenesis, thus predisposing to low birth weight (LBW). [1-5] By comparing the global phenotypes displayed in young offspring of control and diabetic mothers, we have observed that LBW pups with small kidneys are frequent in the offspring of diabetic dams. In the kidneys, we observed that glomeruli are smaller and that there are a relatively low number of nephrons; there is evidence of nephron collapse in these kidneys. These findings may constitute the genesis of low glomerular endowment.

In the 1980s Brenner and associates hypothesized that "low glomerular endowment" or "fewer numbers of nephrons" are a risk factor for hypertension and ESRD in adulthood. [41-44] In principle, decreased nephron number leads to renal hyperfiltration (higher filtration pressure and an increased glomerular filtration rate (GFR) per glomerulus). Consequently, later in life, pressure natriuresis curves shift, leading to increase in blood pressure (BP), thereby enhancing the risk of injury due to hypertension and ESRD. Although outcomes such as LBW, small kidneys, fewer nephron numbers resulting from an adverse intrauterine environment that might predispose to future hypertension are known, the mechanisms by which this occurs remain incompletely delineated.

Increased apoptosis in the blastocyst, and later, in embryonic kidnesy, has been reported in rodent embryos developing in diabetic dams.[45-50] We would suggest that apoptosis, in particular differential apoptosis of specific renal lineages during nephrogenesis, induced by a high glucose milieu, is the major mechanism by which renal function is ultimately affected in diabetic offspring over time. The activation

of intrarenal RAS and NF-kB pathways are two key mechanisms that appear critical in the apoptosis induced by an intrauterine high glucose milieu.

In normal nephrogenesis, apoptotic events occur normally throughout renal organogenesis until the formation of the final kidney is complete. For example, the undifferentiated stromal mesenchyme either becomes interstitial cells or is destined undergo apoptosis to make space for the expanding loops of Henle; in contrast the differentiated metanephric mesenchyme (MM) normally undergoes epithelialization as a result of mesenchymal-to-epithelial transformation and becomes the proximal portion of the nephron [51;52]. However, under certain circumstances, for example, in maternal diabetes, if the resultant high glucose milieu triggers apoptotic events in cells that do not normally undergo apoptosis (e.g., differentiated mesenchymal mesenchyme), nephron formation may be altered and result in nephron collapse. Indeed, our data suggest that a high glucose milieu *in utero* retards renal morphogenesis by inducing a significantly higher number of apoptotic podocytes in the developing glomeruli and inducing a high level of capase-3 activity in the renal tubule, perhaps via activation of NF-kB pathways and the intrarenal RAS.

Based on our observations and those of others, we propose that high glucose-induced cell apoptosis resulting in nephron collapse in diabetic offspring may be due to several factors. First, although the NF-kB pathway has been reported as a major intracellular target in hyperglycemia and oxidative stress, [12;13;53] the expression pattern of NF-kB in the kidneys of diabetic adults is still controversial, [14-16] Regarding the offspring of diabetic mothers, the functional impact of NF-kB pathways on apoptosis is unknown. We observed that NF-kB pathway is upregulated in kidneys of diabetic offspring. Further, p50 and p65 subunits of NF-κB were markedly upregulated, and these subunits were translocated from the cytosol to the nucleus in proximal tubular cells of diabetic offspring. Linking this

observation to apoptosis, a first possibility might be that NF-kB activation evokes several pro- and anti-apoptotic genes including Fas (CD95), TRAIL receptors (DR4, 5 and 6), the death-inducing ligands FasL, TNFa and TRAIL, tumor suppressor p53, Bcl-xL and Bcl-xS, [17;19] which could lead to apoptosis of proximal tubular cells, consequently resulting in nephron collapse and, ultimately, in nephron deficiency. Secondly, the intrarenal RAS, which has been extensively linked to diabetes-induced apoptosis in both human [54] and experimental animal models, [55] may play a role. ANG and renin are major contributors to the production of Ang II, the most physiologically active peptide of the RAS. We have observed that the expression of ANG and renin are dramatically activated and correlate with apoptotic events in kidneys of offspring of diabetic mother. Additionally, the cells that express renin, mainly in the renal juxtaglomerular apparatus [56-58] in the kidneys of the normal offspring, are found in the glomerular or tubular region in kidneys of offspring from diabetic mother. This shift in renin expression together with increased mANG expression might be capable of stimulating increased local Ang II formation, which could contribute to the observed increase in glomerular or tubular apoptosis; Finally, the cross-talk between NF-kB pathways and the intrarenal RAS [23;59] may be fundamentally associated with nephron deficiency.

In conclusion, our present results demonstrate that maternal diabetes impairs renal development and induces nascent nephron cell apoptosis via enhanced intrarenal RAS activation and NF-κB signaling

9.5 Material and Methods

Animals

For these present *in vivo* studies we employed two fertile transgenic mouse (Tg) lines that have normal phenotype: Hoxb7- green fluorescent protein (GFP)-Tg

(Hoxb7-GFP-Tg**)** and Nephrin- cyan fluorescent protein (CFP)-Tg (Nephrin-CFP-Tg). Hoxb7-GFP-Tg mice were kindly provided by Dr. Frank Costantini (Columbia University Medical Center, NY, NY, USA), [60;61] which have been used to study ureteral bud branching in nephrogenesis. [53] Nephrin-CFP-Tg mice, which have CFP expression driven by the podocyte-specific nephrin promoter in glomeruli, were obtained from Dr. Susan Quaggin (University of Toronto, Toronto, ON, Canada). [62]; these mice permit us to follow glomerular development during nephrogenesis.

Animal care in this set of studies met the standards set forth by the Canadian Council on Animal Care, and the procedures utilized were approved by the Institutional Animal Care Committee of the CHUM. Mice were housed under standard humidity and lighting conditions (12 hour light-dark cycles) and were allowed free access to standard mouse chow and water *ad libitum*. Timed-pregnant hoxb7-GFP mice aged 8-10 weeks were used in all experiments. Vaginal wet mounts were made to determine the estrous cycles of the mice. On the evening before estrus, female mice were housed overnight with male mice; the presence of spermatozoa in a vaginal smear the next morning was defined as day 1 of pregnancy.

Animal Model and Experimental Design

Based on our previous report [63] as well as those of others, [64-70] maternal diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, Sigma) at a dose of 150 mg/kg of body weight (BW) at E13 gestation age in Hoxb7-GFP mice. Meanwhile, we have performed pilot studies regarding STZ potential toxicity effect on nephron formation in Nephrin-CFP mice.

Offspring from diabetic Hoxb7-GFP pregnant mice were euthanized at 4 time periods after birth (N=24 at each time point): neonates, 1-week, 2-weeks and 3-weeks; Offspring from non-diabetic pregnant mice at same time point were used as controls.

Isolation of Metanephroi

Post-STZ embryos were micro-dissected aseptically from timed-pregnant Nephrin-CFP mice (E16 and E18), and the metanephroi were isolated under sterile conditions as previous report.[53;63] Glomerular images and quantification in nephrin-CFP-Tg were analyzed by fluorescence microscopy (Nikon Eclipse TE 2000-S Microscope, Montreal, QC, CA).

Biological Parameters, Renal Morphology Review & Renal Endowment Measurement (Mean Glomerular Volume (V_G) and N:

Biological parameters such as kidney weight, body weight and body length were carefully monitored during the entire suckling period. Hematoxylin/eosin (H/E) staining was used to review renal morphology; [63] We measured glomerular size using an estimate of mean glomerular volume (V_G) and also quantitated nephron number. V_G was determined by the method of Weibel and Gomez [71] with the aid of an image analysis software system (Motics Images Plus 2.0, Motic, Richmond, BC, Canada). The V_G was estimated by the mean glomerular tuft area (A_T) derived from the light microscopic measurement of 30 random sectional profiles of glomeruli from each group (n=6 animals per group) using the formula: $V_g = \beta / k \; x \; A_T^{1.5}$, where $\beta = 1.382$ (shape coefficient for spheres) and $k = 1.1$ (size distribution coefficient). Quantification of nephron number was adapted from J.F. Bertram's method using serial sections. [72]

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

RT-qPCR [iQ™ SYBR@ Green Supermix Kit and MiniOpticon™ Real-Time PCR machine (Bio-Rad Laboratories, Mississauga, ON, Canada)] was performed as reported previously [36;53]. The forward and reverse primers corresponding to mouse (m) angiotensinogen (mANG), mouse renin (mRen) and β-actin cDNA [36;53] in RT-qPCR assays were as follows: mANG forward primer (5'- CCA CGC TCT CTG GAT TTA TC-3') and reverse primer (5'- ACA GAC ACC GAG ATG CTG TT-3') (NM_007428); mRen forward primer (5'- CTG GCC AAG TTT GAC GGT GTT-3') and reverse primer (5'- GTG TCC ACC ACT ACC GCA CAG -3') (BC061053); β-actin forward primer (5'- CGT GCG TGA CAT CAA AGA GAA -3') and reverse primer (5'- GCT CGT TGC CAA TAG TGA TGA -3') (NM_007393).

Terminal Transferase-Mediated Deoxyuridine Triphosphate (dUTP) Nick End-labeling (TUNEL) Assay

Paraffin-embedded kidney sections (5 μm) fixed in 4% paraformaldehyde were deparaffinized in xylene and rehydrated. Apoptosis quantified with a TUNEL kit (La Roche Biochemicals, Laval, QC, Canada) according to the supplier's instructions.

Immunohistochemistry and Immunofluorescent staining

Paraffin-embedded kidney sections (5 μm) fixed in 4% paraformaldehyde were deparaffinized in xylene and rehydrated. Immunohistochemical examination for ANG, renin, caspase-3 and NF-kB pathway (p50 and p65) was performed by the standard avidin-biotin-peroxidase complex method (ABC Staining System, Santa Cruz Biotechnologies, CA, USA).[39;73] The primary antibodies used include a polyclonal anti-ANG antibody [39;73] (gift from Dr. John S.D. Chan, CHUM-Hôtel

Dieu Hospital, Montreal, QC, Canada) in a 1:100 dilution; anti-Wilms tumor-1 (WT-1) (clone 6F-H2, Dako Cytomation, Carpinteria, CA, USA) in 1:100 dilution; anti-cleaved caspase-3 polyclonal antibody (Cell Signaling, USA) in a 1:100 dilution; a polyclonal NF-kB pathway antibody (p50/p65, Santa Cruz Biotechnologies, CA, USA) in 1:100 dilution; and a polyclonal anti-renin antibody (Cat.#: RDI-rtreninabm) (Research Diagnostics Inc., Concord, MA, USA) in a 1:500 dilution.

Gel Mobility Shift Assay (GMSA)

Nuclear protein extracts were prepared from neonatal kidneys of control and diabetic offspring. GMSAs were performed as described previously, [74;75] employing ^{32}P-labeled NF-κB probes [NF-κB consensus oligonucleotide: C1 (5' – AGT TGA GGG GAC TTT CCC AGG C – 3') and C2 (5' –GCC TGG GAA AGT CCC CTC AAC T– 3'); NF-κB mutant oligonucleotide: M1 (5' – AGT TGA GG**C** GAC TTT CCC AGG C – 3') and M2 (5' –GCC TGG GAA AGT CGC CTC AAC T– 3')] (Cat.# SC-2505 and SC-2511, Santa Cruz Biotechnologies, CA, USA).

Statistical Analysis

Statistical significance between experimental groups was analyzed initially by Student's *t* test or by 1-way ANOVA followed by the Bonferroni test as appropriate. Three to four separate experiments were performed for each protocol. Data are expressed as means ± SD. A probability level of $P \leq 0.05$ was considered statistically significant.

9.6 Acknowledgements

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ABBREVIATIONS: CFP, cyan fluorescent protein; GFP, green fluorescent protein; GFR, glomerular filtration rate; H/E, hematoxylin/eosin; LBW, low birth weight; mANG, mouse angiotensinogen; MM, metanephric mesenchyme**;** mRen, mouse rennin; NF-kB, nuclear factor kappa B; RAS, renin-angiotensin system; ROS, reactive oxygen species; RT-qPCR, real-time-quantitative polymerase chain reaction; STZ, streptozotocin; Tg, transgenic mouse; TNF-a, tumor necrosis factor-a; TUNEL, terminal Transferase-mediated deoxyuridine triphosphate (dUTP) nick end-labeling.

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9.8 Legends and Figures

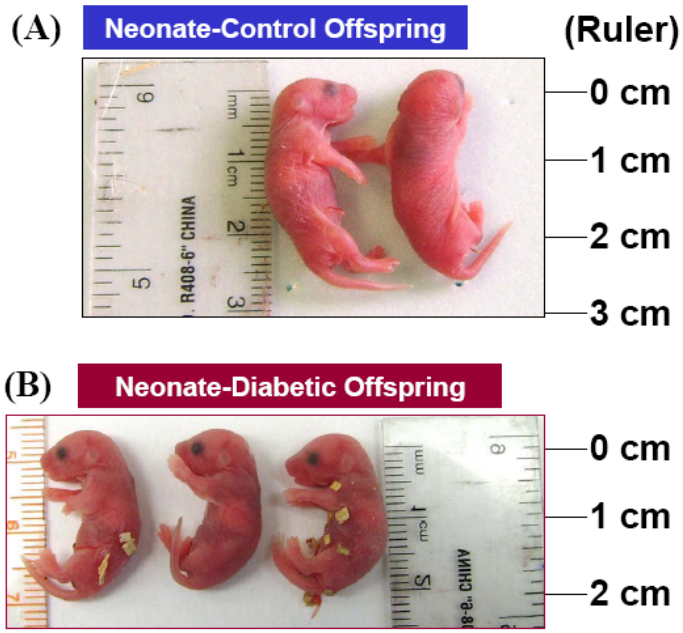

Figure1

Figure 9-1 Image of representative newborn Hoxb7-GFP mice from non-diabetic and diabetic mother. The offspring of diabetic mother are significantly smaller than one of non-diabetic mother (control).

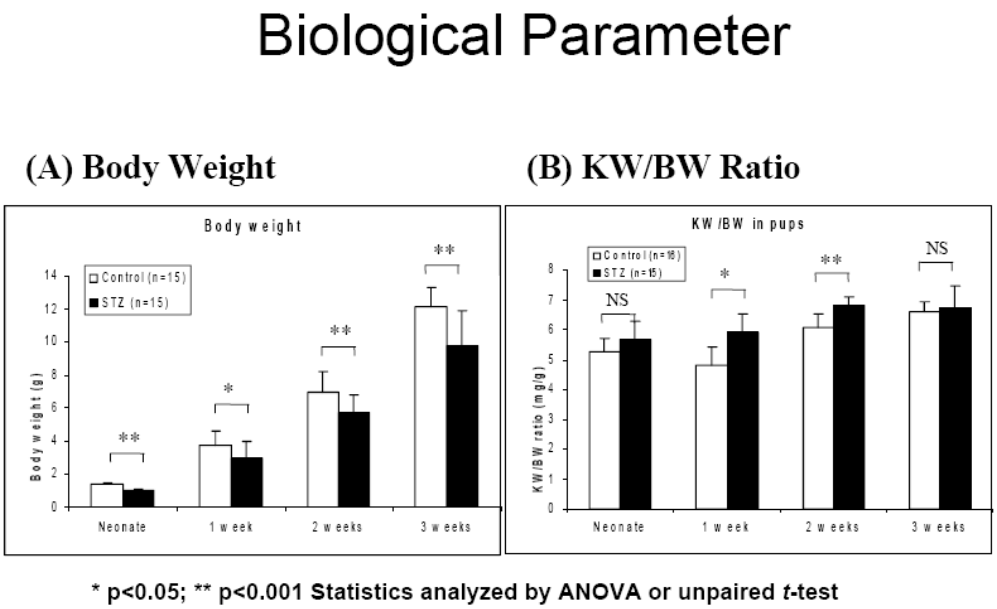

Figure 2

Figure 9-2 Physical parameters in offspring of Hoxb7-GFP-Tg Mice. Offspring from diabetic mother had significantly lower body weight (20% less on average) than control offspring during the entire suckling period (Neonate to 3 weeks of age) (A); The ratios of kidney weight (KW) to BW in offspring from diabetic mother were also significantly higher than control offspring (B). (Blue bar: control offspring; purple bar: offspring from diabetic mother). *, $P \leq 0.05$; **, $P \leq 0.01$.

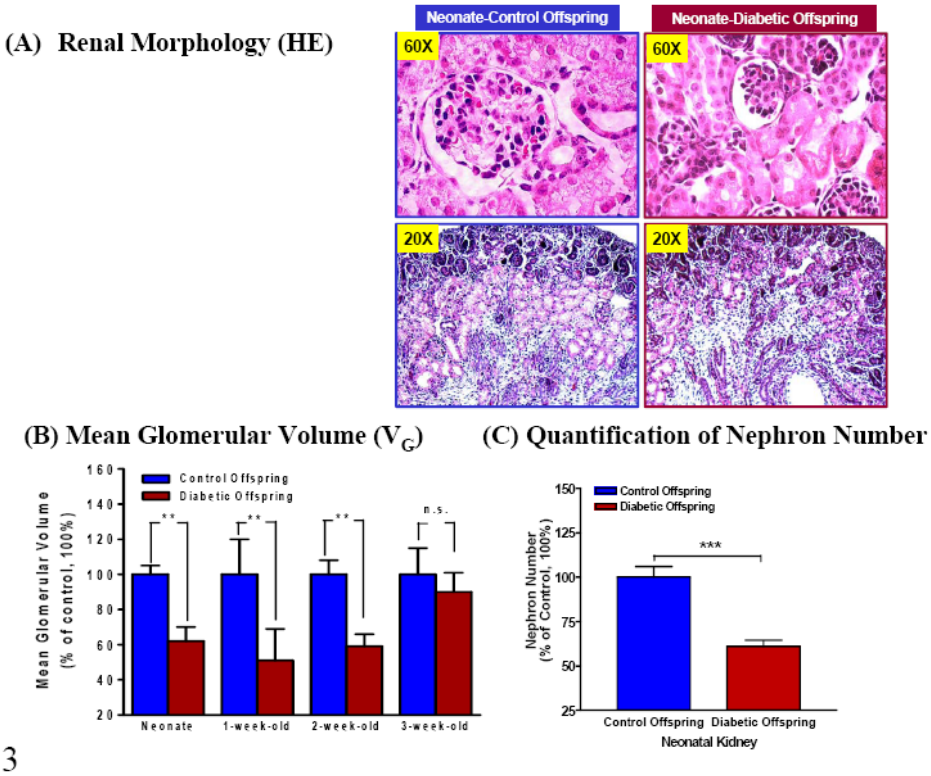

Figure 3

Figure 9-3 Renal Morphology and V_G Measurement. (A) Hematoxylin & Eosin (HE) staining indicates that kidney and glomerular size of neonatal offspring from diabetic mother are apparently smaller as compared control offspring. (control neonate in blue frame; neonate from diabetic mother in red frame; Magnification, 20X and 60X). (B) Quantification of V_G value in control and diabetic offspring from neonate to 3 week-old. The y axis shows the percentage of V_G value compared to control animal (100%). [Blue bar: control offspring (Neonate: N=9; 1week-old: N=12; 2 week-old: N=9; 3week-old: N=8); Red bar: Diabetic offspring (Neonate: N=8; 1week-old: N=8; 2 week-old: N=7; 3week-old: N=8)]. **, $P \leq 0.01$; (C) Quantification of neonatal nephron number. The y axis shows the percentage of nephron number compared to control animal (100%). [Blue bar: control offspring (Neonate: N=6); Red bar: Diabetic offspring (Neonate: N=5). ***, $p \leq 0.001$)].

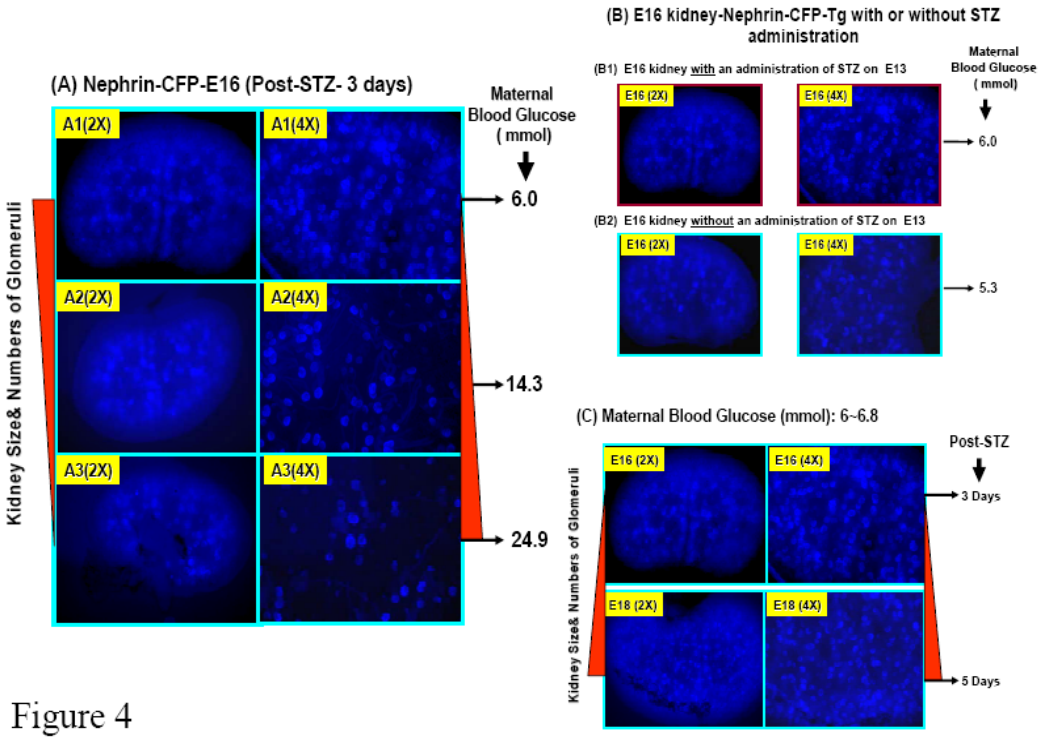

Figure 4

Figure 9-4 STZ toxicity studies in Nephrin-CFP-Tg mice. Figure 3A show that E16 embryonic kidney isolated from pregnant mice with three different maternal hyperglycemic levels: normal (6.0 mmol), mild (14.3 mmol) and severe (24.9 mmol) after 3 days of STZ administration (150 mg/kg, ip, at E13). Figure 4B depicts E16 embryonic kidneys isolated from pregnant mice in normal maternal glucose range with or without administration of STZ at E13. Figure 4C shows E16 and E18 embryonic kidneys isolated from pregnant mice in normal maternal glucose level with STZ administration at E13 (Magnification, 2X and 4X).

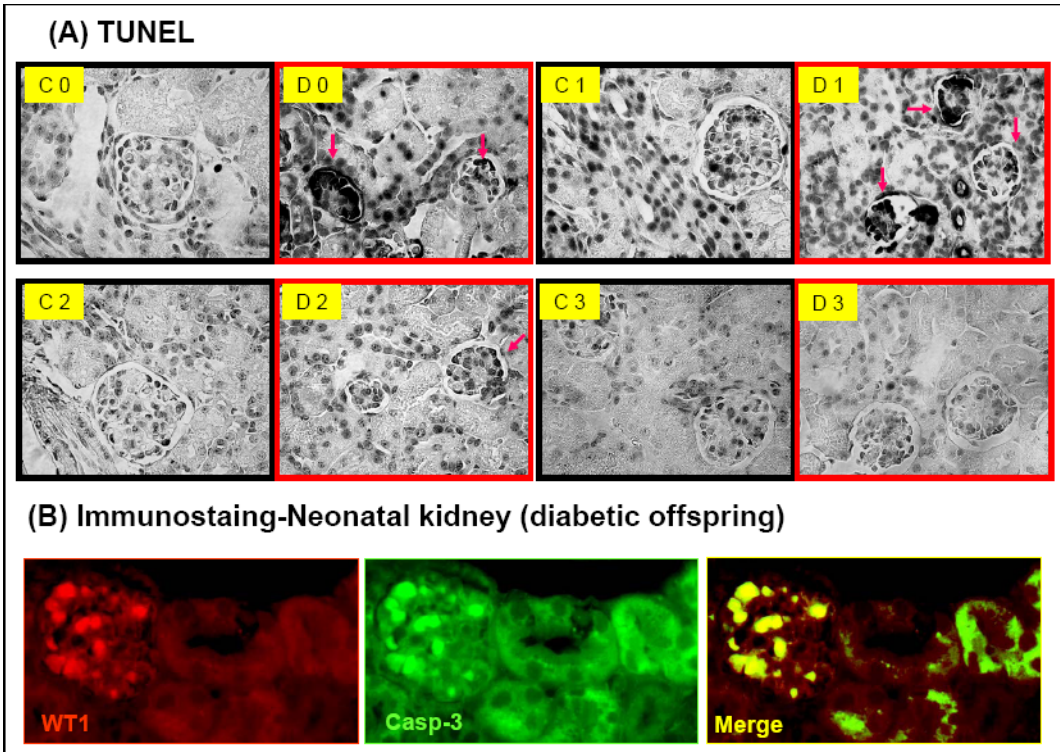

Figure 5

Figure 9-5 Apoptotic assay (TUNEL, Figure 5A) in kidneys of offspring from non-diabetic and diabetic mothers. Neonate (C0 and D0); offspring at 1-week old (C1 and D1); offspring at 2-weeks old (C2 and D2); and offspring at 3 week-old (C3 and D3) (Magnification 60X); Figure 5B shows the double immunostaining of WT-1 (a) and active caspase-3 expression (b) as well as a merged image (c) in neonatal kidneys of offspring from non-diabetic and diabetic mothers. (Magnification 60X).

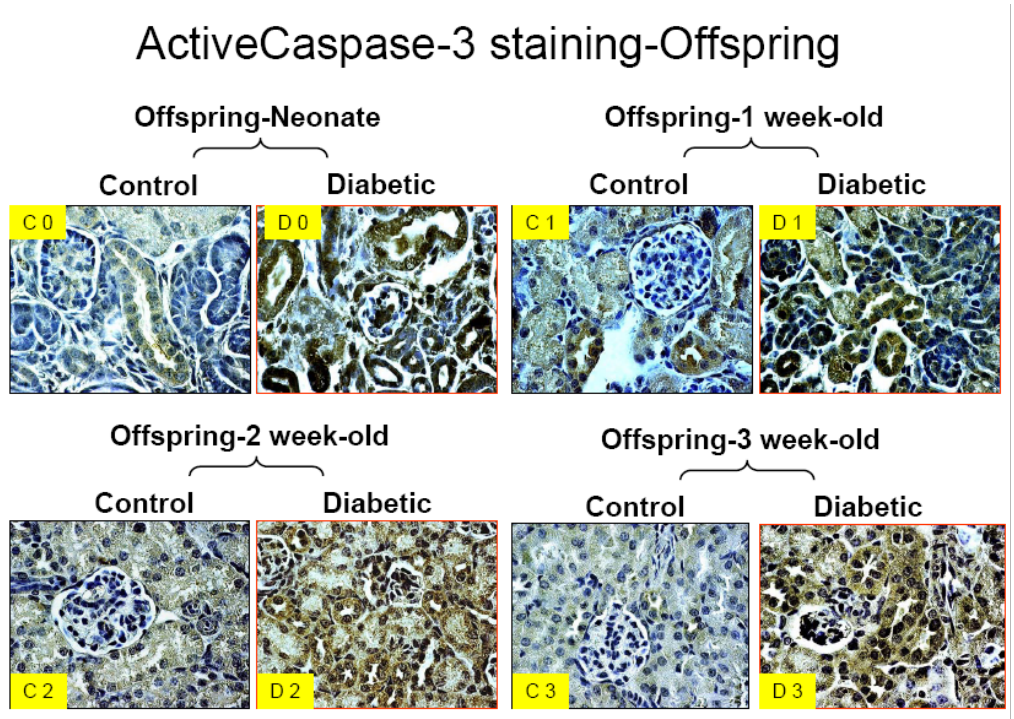

Figure 6

Figure 9-6 Active caspase-3 expression in kidneys of offspring from non-diabetic (control) and diabetic mothers: neonate (C0 and D0); offspring at 1-week old (C1 and D1); offspring at 2-weeks old (C2 and D2); and offspring at 3 week-old (C3 and D3) (Magnification 60X).

qRT-PCR

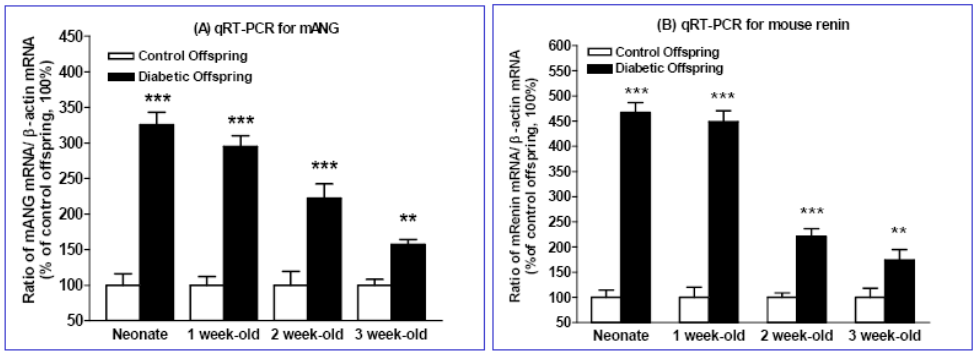

Figure 7

Figure 9-7 Mouse ANG and renin mRNA expression assayed by RT-qPCR. Figure 7A and Figure 7B show that the expression levels of ANG and renin mRNA mRNA, respectively, in kidneys of offspring from non-diabetic and diabetic mothers aged from neonate to 3 week-old. **, $P \leq 0.01$; ***, $P \leq 0.001$.

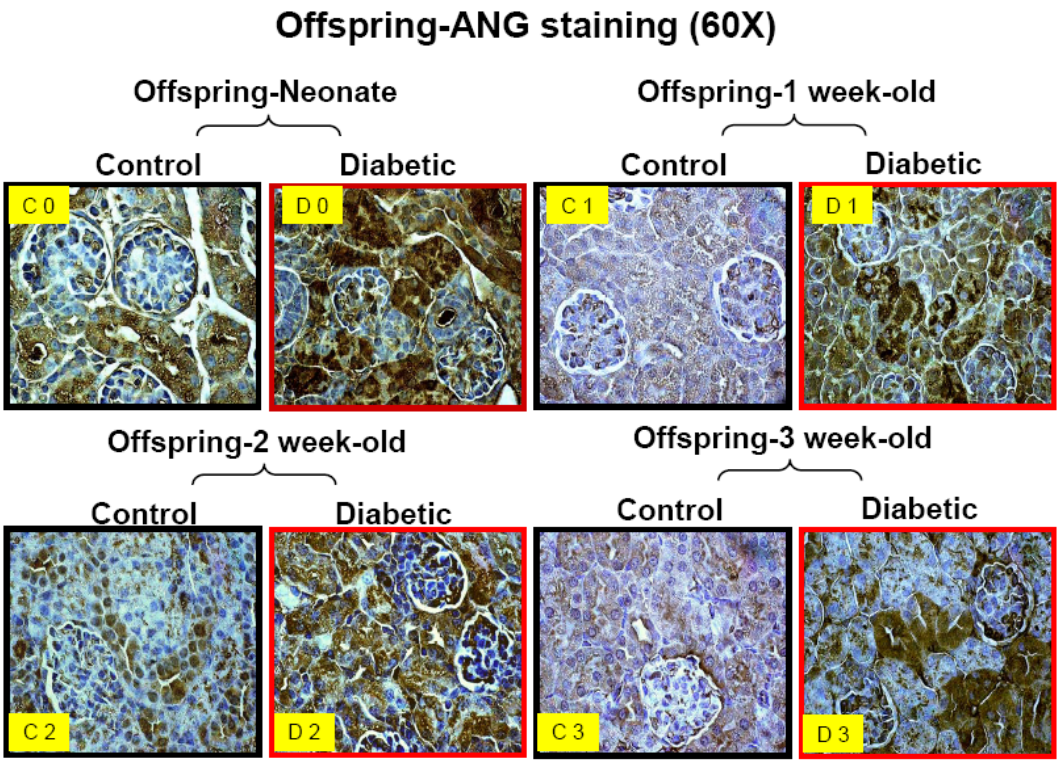

Figure 8

Figure 9-8 ANG protein expression in kidneys of offspring from non-diabetic (control) and diabetic mothers: neonate (C0 and D0); offspring at 1-week old (C1 and D1); offspring at 2-weeks old (C2 and D2); and offspring at 3 week-old (C3 and D3) (Magnification 60X)

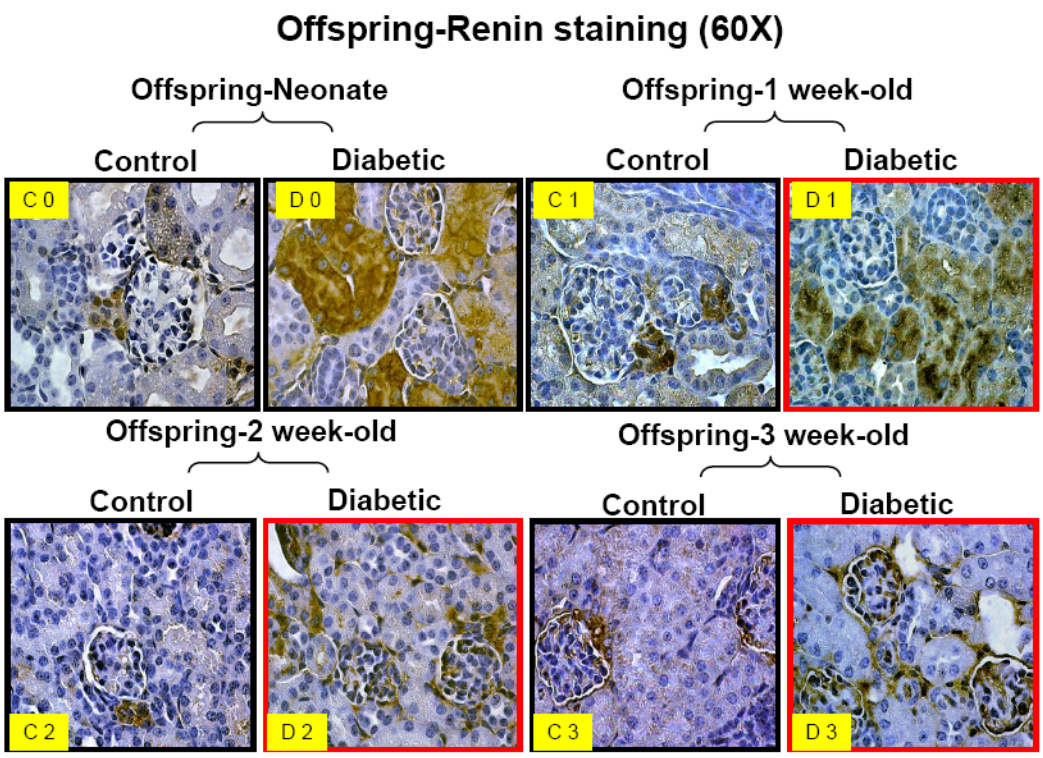

Figure 9

Figure 9-9 Renin protein expression in kidneys of offspring from non-diabetic (control) and diabetic mothers: neonate (C0 and D0); offspring at 1-week old (C1 and D1); offspring at 2-weeks old (C2 and D2); and offspring at 3 week-old (C3 and D3) (Magnification 60X).

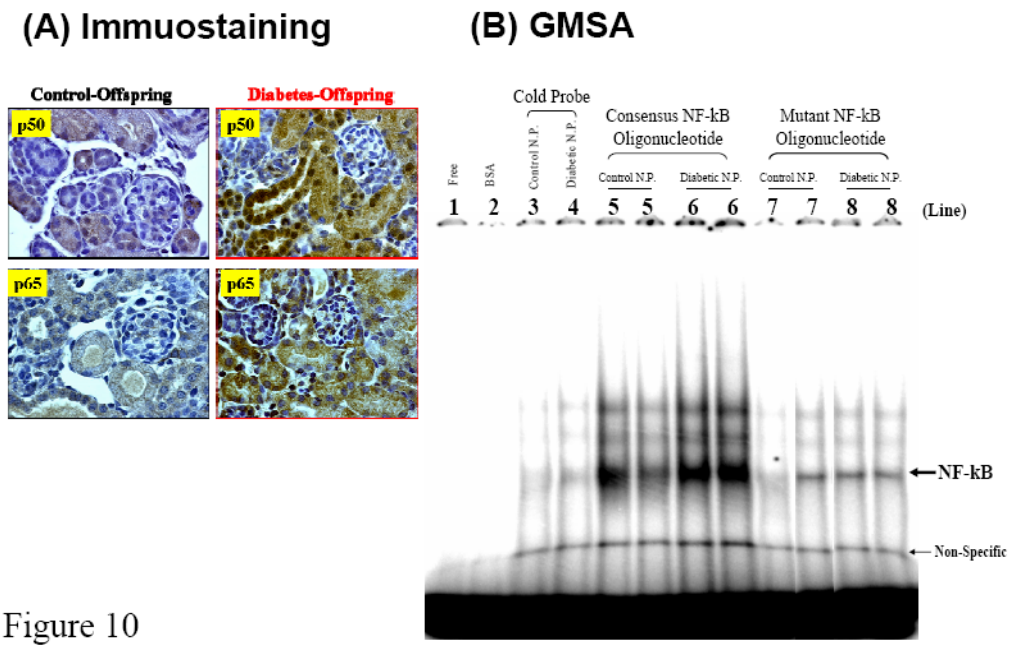

Figure 10

Figure 9-10 NF-kB expression and localization as well as activation in neonate kidneys from control and diabetic mothers. (A) Expression and localization of two isoforms of NF-kB, p50 and p65, were displayed by immunostaining (Magnification 60X); (B) GMSA assay. The labeled DNA probe (0.1 pmol) was incubated without (lane 1) or with BSA (10 μg) (lane 2) or renal nuclear protein(s) (N.P., 10 μg each) of neonatal kidney (lanes 3-8) in the presence of 0.3 units of poly dI-dC. Renal N.P. from neonatal control (line 3, 5 and 7) and diabetic offspring (line 4, 6, 8) is incubated with consensus NF-κB DNA cold probe (line 3 and 4), consensus NF-κB DNA probe (line 5 and 6) and mutant NF-κB DNA probe (line 7 and 8), respectively.

CHAPTER 10: DISCUSSION

Maternal diabetes creates a high-risk intrauterine environment that has direct implications in congenital malformations, including congenital renal abnormalities. Hyperglycemia constitutes an adverse *in utero* environment that dynamically impairs nephrogenesis, resulting in an abnormal pattern of UB branching and reduced nephron numbers. As a "kidney-specific" master gene, Pax2 is absolutely required for initial signaling of the WD to optimize UB branching and mesenchymal-to-epithelial transformation. Pax-2-null mice fail to form any kidneys, ureters and genital tracts. In humans and mice, heterozygous Pax2 mutations cause kidney, eye, and CNS abnormalities constituting RCS. During my PhD studies, I have focused on the influence of hyperglycemia on nephrogenesis and delineated the fundamental role of maternal diabetes in modulating renal morphogenesis and gene expression in the offspring.

In studies *in virto*, our data demonstrated that high glucose induced Pax2 gene and protein expression in MK4 cells. The stimulatory effect of high glucose on Pax2 expression is mediated, at least in part, via ROS generation and activation of the NF-kB signal pathway, but not via the PKC, p38MAPK, and p44/42 MAPK signaling pathway,

In studies *ex vivo*, our data indicated that high glucose alters UB branching morphogenesis via Pax2 gene and protein expression. The stimulatory effect of high glucose seems to be mediated via ROS generation and activation of the Akt signal pathway.

In studies *in vivo*, our data indicated that the offspring of diabetic dams had lower body weight, body size, kidney weight, nephron number and smaller size of glomerular volume as compared with the offspring of nondiabetic controls. Our

results demonstrate that maternal diabetes impairs renal development and induces nascent nephron cell apoptosis via enhanced intrarenal RAS activation and NFkB signaling.

10.1. To Investigate Whether High Glucose Alters Pax2 Gene Expression in the Mouse Embryonic MM (MK4 Cells)

Our laboratory is particularly interested in Pax2, a transcription factor that acts as a "kidney-specific" master gene. As noted in Section 3.6, Pax2 is essential for kidney development. Mice deficient in Pax2 fail to develop an urogenital system (219), whereas Tg mice overexpressing Pax2 develop multicystic dysplastic kidneys (405). In the present studies, we investigated whether high glucose alters Pax2 gene expression and aimed to delineate its mechanism of action in mouse embryonic mesenchymal epithelial cells (*in vitro)* and kidney explants from Hoxb7-GFP mice *(ex vivo*).

10.1.1. Hyperglycemia Induces Pax2 Gene Expression

To the best of our knowledge, high glucose has not been demonstrated to regulate Pax2 gene expression and its underlying mechanism in embryonic kidney cells. In the present studies, we employed both MK4 cells and kidney explants to examine the effect of high glucose on Pax2 gene expression. MK4 cells were incubated for 24 hours in medium that contained 25 mM of different glucose analogues, such as D-mannitol, L-glucose, 2-deoxy-glucose and D-glucose. As our data indicated, high D-glucose (25 mM) specifically and dose-dependently stimulated Pax2 gene expression in MK4 cells (Figure 7-1, page 104) but other glucose analogs, such as D-mannitol, L-glucose and 2-deoxy-glucose, had no effect

(Figure 7-2, page 105), suggesting that the action of high D-glucose (25 mM) is specific. Moreover, immunofluorescence staining showed that high glucose induces Pax2 gene expression, consistent with the fact that Pax2 is a nuclear transcription factor (Figure 7-3, page 106). To confirm our *in vitro* data, we adopted an *ex vivo* model using embryonic kidney explants from Hoxb7-GFP mice. We observed a similar stimulatory effect of high glucose on Pax2 gene expression in our kidney explant system (Figure 7-7, page 110).

10.1.2. High D-glucose Induces ROS Generation in MK4 Cells

Although detailed mechanism(s) by which high D-glucose regulates Pax2 gene expression are far from clear, we hypothesize that it does so via ROS generation. As a result, high D-glucose induces ROS generation in MK4 cells (Figure 7-5A, page 108). These outcomes were suppressed by ROS blockers, such as inhibitors of NADPH oxidase (DPI) and mitochondrial electron transport chain complex I (rotenone), but not by NF-kB inhibitors (PDTC) (Figure 7-5B, page 108). It is unclear whether ROS directly regulate Pax2 gene expression. XO, a form of xanthine oxidoreductase that generates ROS, is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid (406). We employed XO (Figure 7-5C, page 107) to examine the effect of ROS generation on Pax2 gene expression. Indeed, superoxide generated from the XO system directly stimulated Pax2 gene expression in MK4 cells (Figure 7-5D and -5E, page 108). To confirm our *in vitro* data, E16 kidney explants were isolated and cultured for 24 hours in either normal glucose or high glucose with or without H_2O_2. Our results showed that H_2O_2 directly stimulated Pax2 gene expression in E16 kidney explants (Figure 7-7E and -7F, page 110). In diabetic environments or hyperglycemia, increased ROS generation leads to embryonic

dysmorphogenesis. Our findings prompted us to ask an interesting question: does high D-glucose regulate Pax2 gene expression via ROS generation? The data revealed that ROS inhibitors, such as DPI and rotenone, can block the stimulatory effect of high glucose on Pax2 expression in MK4 cells (Figure 7-6, page 109). To confirm our *in vitro* results, we adopted an *ex vivo* model of embryonic kidney explants from Hoxb7-GFP mice. We noted that the ROS inhibitors DPI and rotenone blocked the similar stimulatory effect of high glucose on Pax2 gene expression in our kidney explant system (Figure 7-7, page 110). Our observations also demonstrated that high glucose stimulated Pax2 promoter activity at the transcriptional level, which could be blocked by ROS inhibitors (Figure 7-8A, page 111). Taken together, they disclosed that the stimulatory effect of high glucose on Pax2 gene expression was suppressed by ROS inhibitors in both *in vitro* and *ex vivo* studies, indicating that high glucose regulated Pax2 gene expression via ROS generation.

10.1.3 High D-glucose Induces Pax2 Gene Expression via Activation of the NF-kB Pathway

According to Brownlee's hypothesis, the mechanisms underlying hyperglycemia-induced kidney damage include excessive ROS generation with subsequent PKC and NF-kB activation (291;312). Other investigations have demonstrated that both the p38 MAPK and p44/22 MAPK signaling pathways may be involved in hyperglycemia-induced ROS generation in RPTCs (407;408) and mesangial cells (409;410). To determine if the p38 MAPK, p44/22 MAPK and PKC signaling pathways are involved in the stimulatory effect of high glucose on Pax2 expression, inhibitors of p38 MAPK (SB203580), p44/22 MAPK (PD98059) and PKC (GFX) were tested. Our data showed that p38 MAPK, p44/42 MAPK and PKC inhibitors could not block the stimulatory action of high glucose on Pax2 mRNA

and protein expression in MK4 cells (Figure 7-6, page 108), suggesting that the p38 MAPK, p44/42 MAPK and PKC signaling pathways are not involved in the effect of high glucose on Pax2 gene expression. NF-kB, one of the major intracellular targets of hyperglycemia, can be stimulated by hyperglycemia and activate the expression of downstream genes. In the present studies, we observed that a NF-kB inhibitor (PDTC) could block the stimulatory effect of high glucose on Pax2 expression in MK4 cells (Figure 7-6, page 108). We identified several NF-kB-binding motifs, including 6 of GGrmwkyCCC and 2 of GGGGmyTyy located in a full length 5'-promoter region of Pax2 (AF515729), using Alibaba2.1 sequence searching software (www.gene-regulation.com). We transiently transfected a human PAX2 promoter into MK4 cells. Our data demonstrated that high glucose stimulated Pax2 promoter activity at the transcriptional level, which could be blocked by NF-kB inhibition (Figure 7-8A, page 111). To further understand the mechanisms underlying the NF-kB pathway involved in high glucose-induced Pax2 gene expression, we tested DNIκB α which binds the subunits p50 and p65 complexed in an inactive form, preventing them from translocating to the nucleus. After co-transfection with DNIκBα into MK4 cells, Pax2 promoter activity induced by high glucose was decreased (Figure 7-8B, page 111), suggesting that NF-κB is involved in high glucose-induced stimulation of Pax2 transcription activity. Taken together, our findings confirmed that high D-glucose evokes Pax2 gene expression via activation of the NF-kB pathway, but not via the p38 MAPK, p44/42 MAPK and PKC signaling pathways

10.1.4. Conclusion

The stimulatory influence of high glucose on Pax-2 gene expression is mediated, at least in part, via ROS generation and activation of the NF-κB signaling pathway, but

not via the PKC, p38 MAPK and p44/42 MAPK signaling pathways, as shown in Figure 10-1.

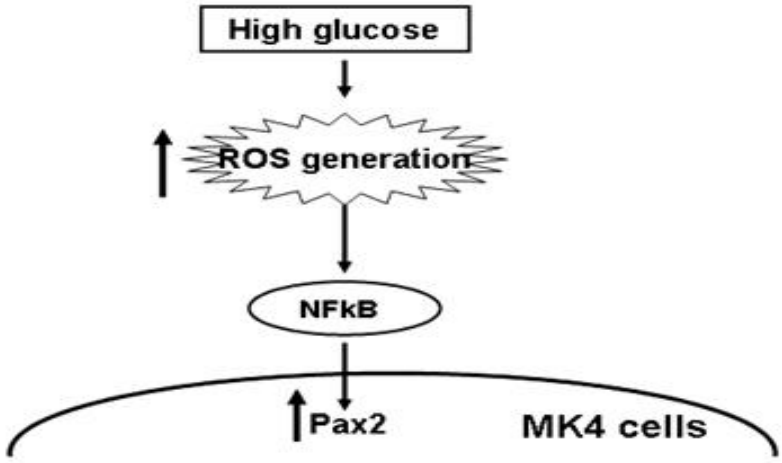

Figure 10-1 *In vitro* working diagram

10.2. To Demonstrate the Influence of a High-glucose Milieu on UB Branching Morphogenesis

The mechanisms of hyperglycemia-induced renal malformation are not clearly understood. Kanwar et al. (411) reported that E13 embryos exposed to 30 mM glucose showed reduced metanephroi size, suggesting that hyperglycemia alters nephrogenesis. In our *in vitro* studies, we demonstrated that the stimulatory effect of high glucose on Pax2 gene expression is mediated, at least in part, via ROS generation and activation of the NF-κB signaling pathway, but not via the PKC, p38 MAPK and p44/42 MAPK signaling pathways. Our *ex vivo* experiments were designed to examine the influence of a high-glucose milieu on UB branching morphogenesis and its underlying mechanism in Hoxb7-GFP-Tg mice as a model with which we would be able to observe UB development under fluorescence microscopy, as described in Section 5.2.1.

10.2.1. High D-glucose Stimulates UB Branching in a Time-dependent Manner

Kidney explants were isolated from time-pregnant mice at E13, and then cultured in either normal or high D-glucose for up to 96 hours with fresh medium changed every 24 hours. As seen in sequential fluorescence microscopic images, high D-glucose stimulated UB branching in a time-dependent manner in comparison to normal D-glucose (Figure 8-1A, -1B, -1C, and -1D, page 136). We also carefully measured metanephroi diameter and found that high glucose reduced it, also in a time-dependent manner (Figure 8-1E, page 136), indicating that high glucose diminished metanephroi size by apoptosis. Other maternal diabetes-related kidney explant studies also demonstrated that hyperglycemia decreased metanephros size, induced UB branching dysmorphogenesis and diminished the population of nascent nephrons (256;294;295).

10.2.2. High D-glucose Specifically Stimulates UB Branching

Growth and branching of the epithelial ureteric tree are critical for metanephros development. Current methods of UB branching analysis are mostly qualitative. Cullen-McEwen et al. (412) noted that the ideal observation time of the recognized architectural “pattern” of the UB tree is between 18 and 48 hours. Based on our results (Figure 8-1, page 136), the stimulatory effect of high glucose occurs after 24 hours of incubation; hence, we undertook 24 hours of stimulation in subsequent studies. E13 kidney explants were incubated for 24 hours in medium that contained 25 mM of different glucose analogues, such as D-mannitol, L-glucose, 2-deoxy-glucose and D-glucose. High D-glucose (25 mM) specifically and dose-dependently stimulated UB branching and increased UB tip numbers (Figure 8-3, page 138), but other glucose analogues, such as D-mannitol, L-glucose and 2-deoxy-glucose, had no impact (Figure 8-2, page 137).

10.2.3. Stimulatory Effect of High D-glucose on UB Branching Morphogenesis is Mediated via Pax2 Gene Expression

We noted increased Pax2 gene expression in MK4 cells and kidney explants, indicating an effect of high D-glucose. Although Pax2 was detected in both UB and MM lineages, immunohistological staining pointed to Pax2 up-regulation by high D-glucose in the area of the UB (Figure 8-4C, page 139). To prove that hyperglycemia *in utero* triggers Pax2 gene expression, we injected STZ to induce gestational diabetes in an *in vivo* model to generate a high-glucose environment during pregnancy. Our data revealed increased Pax2 expression mainly localized in UB in diabetic neonate kidneys compared to the controls (Figure 8-11, page 146). Taken together, these findings suggest that high-glucose stimulation of UB branching morphogenesis is mediated via Pax2 gene expression.

10.2.4. ROS-induced UB Branching Morphogenesis

We reported that high glucose specifically prompts Pax2 gene expression via ROS generation in MK4 cells and E16 kidney explants from Hoxb7-GFP mice. We then attempted to clarify the direct functional impact of ROS on UB branching. We found that H_2O_2 directly increased UB branching in E13 kidney explants (Figure 8-7, page 142). Our results raised an interesting question: does high glucose induce UB branching morphogenesis via ROS generation? In E14 kidney explants, high D-glucose elicited UB branching, and inhibitors of NADPH oxidase (DPI) and mitochondrial electron transport chain complex I (rotenone) blocked the stimulatory effect of high glucose on UB branching morphogenesis (Figure 8-6, page 141).

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen, where it catalyzes the decomposition of H_2O_2 to water and oxygen ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) (413). Morever, high glucose-induced UB branching in E12 kidney

explants could be partially blocked by catalase (Figure 8-8C, page 143). To prove that hyperglycemia *in utero* evokes ROS production, we administered STZ to produce gestational diabetes in an *in vivo* model to generate a high-glucose environment during pregnancy. We isolated E19 kidney explants from the offspring of normal and diabetic mothers, and then measured ROS production. According to our unpublished data on E19 kidney explants from a gestational diabetes model, ROS levels were increased in diabetic compared to control kidneys (Figure 12-1, page 206). Taken together, our results indicate that a hyperglycemic environment *in utero* affects UB branching morphogenesis by an increment in ROS production.

10.2.5. High Glucose Stimulates UB Branching via AKT Signaling

Evidence shows that PI3K/AKT pathway activation plays a crucial role in intracellular signaling leading to hyperglycemia-induced endothelial cell apoptosis (414;415). Indeed, Fonsato et al. (416) demonstrated that down-regulation of Pax2 expression correlated with decreased AKT phosphorylation, suggesting that Pax2 is involved in renal tumor angiogenesis via the PI3K/AKT pathway. Therefore, we pose the interesting question: is high D-glucose-regulated Pax2 gene expression via the PI3K/AKT pathway involved in impairment of the UB lineage? As our results show, high glucose-induced UB branching in E12 kidney explants could be blocked by AKT inhibitor IV (Figure 8-8D, page 143). AKT inhibitor IV suppressed the stimulatory effect of high glucose on Pax2 expression in E17 kidney explants (Figure 8-9, page 144). Indeed, our data indicate that the stimulatory action of high glucose on UB branching and Pax2 gene expression is completely abolished by AKT inhibitor, suggesting that high D-glucose-regulated Pax2 gene expression via the PI3K/AKT pathway is involved in impairment of the UB lineage.

10.2.6. Conclusion

Our findings demonstrated that high glucose alters UB branching morphogenesis via Pax2 gene and protein expression. The stimulatory impact of high glucose seems to be mediated via ROS generation and activation of AKT signaling pathway, as illustrated in Figure 10-2.

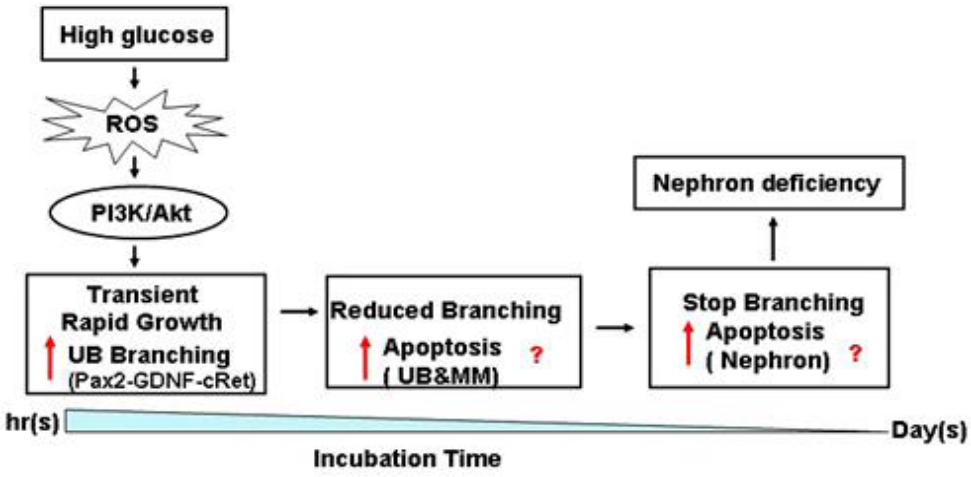

Figure 10-2 ex vivo working diagram

10.3. To Delineate the Fundamental Role of Maternal Diabetes in Modulating Renal Morphogenesis in the Offspring

Diabetes is associated with a high rate of congenital malformations, including urogenital abnormalities (417;418). These congenital malformations result from developmental defects in early organogenesis. Our *in vitro* and *ex vivo* studies have demonstrated that high glucose alters Pax2 gene expression and UB branching morphogenesis. Furthermore, our data showed that high glucose reduced metanephroi size in a time-dependent manner. A study in E13 kidney explants exposed to high glucose for 1 week confirmed our results (294). Moreover, Kanwar et al. (295) reported that apoptosis was increased in E13 metanephroi incubated in high glucose for 4 days. On the basis of our observations and those of others, we

suggested that high glucose induced UB branching dysmorphogenesis, and at the same time, it also triggered cell apoptosis in both UB and nephrons, causing their collapse. We hypothesized that apoptosis is the major mechanism by which renal function is ultimately affected in ODMs over time (our *in vivo* follow-up study). In our *in vivo* short-term experiments, we aimed to dissect the fundamental role of maternal diabetes in modulating renal morphogenesis in the offspring and to investigate their underlying mechanisms.

10.3.1. STZ-induced Gestational Diabetes Model and STZ Toxicity

In our *in vivo* study, we created a high-glucose *in utero* environment in pregnant dams by IP STZ injection (150 mg/kg) at E13, as shown in Figure 9-3. After 2 days of treatment, we implanted insulin minipills in diabetic pregnant dams to normalize blood glucose. We, therefore, had 3 groups: the offspring of non-diabetic pregnant mice serving as controls, the offspring of diabetic pregnant mice, i.e., diabetic offspring, and the offspring of insulin-treated pregnant mice, i.e. treated offspring. Offspring kidneys were harvested at 4 time points after birth: neonatally, at 1 week, 2 weeks and 3 weeks of age. We followed-up by looking at kidney morphology, nephron number, gene expression, and apoptotic events in control and diabetic offspring in this short-term postnatal study. One of our ongoing experiments will examine the fundamental role of maternal diabetes in modulating renal morphogenesis in the offspring of insulin-treated, pregnant mice.

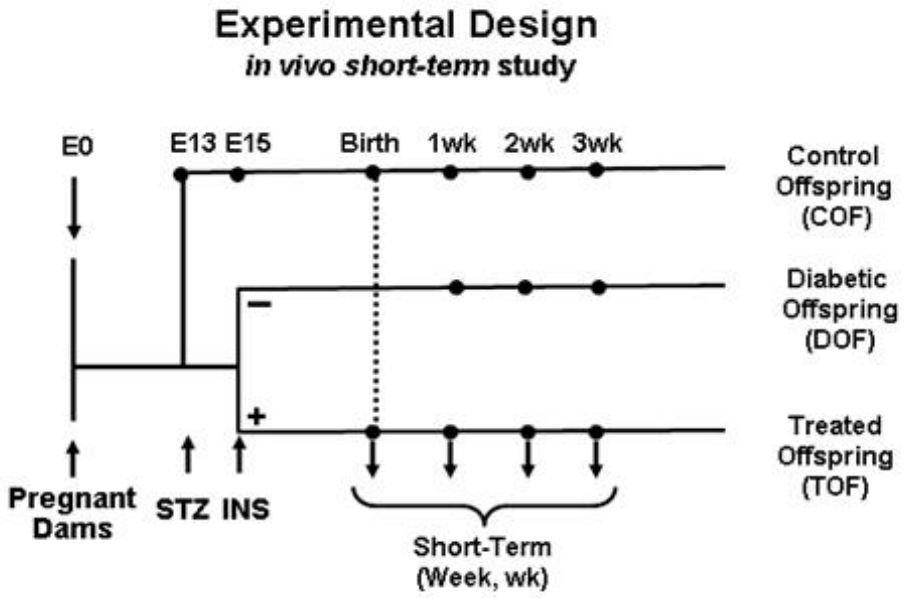

Figure 10-3. Experimental design of short-term *in vivo* study

STZ-induced diabetes has been employed to elucidate the effect of maternal diabetes on fetal growth and development. It has been demonstrated that exposure to intrauterine diabetes may be an important cause of both impaired renal function and hypertension in the offspring (419;420). In these experiments, diabetes was induced before pregnancy, avoiding the possible effect of STZ on the embryos. On the other hand, our diabetic animals were induced during pregnancy. In fact, Tay et al. (421) demonstrated that even with careful optimization of STZ dosing, acute tubular necrosis was superimposed. To determine whether STZ could affect nephrogenesis, we performed additional experiments in Nephrin-CFP mice. We injected STZ IP (150 mg/kg) at E13 in our Nephrin-CFP mice. We followed-up by examining kidney size and nephron number by fluorescence microscopy in E16 kidneys isolated from pregnant mice with 3 different maternal hyperglycemic levels after 3 days of STZ administration. We observed that kidney size and glomeruli number seemed to depend on the level of maternal hyperglycemia (Figure 9-4A, page 175).

Furthermore, we also isolated E16 kidneys from pregnant mice in normal maternal glucose with or without STZ administration at E13. Our data showed that STZ had no toxic effect on the kidney embryos (Figure 9-4B, page 175). Since STZ did not induce hyperglycemia in 100% of our cases, we isolated E16 and E18 kidneys from pregnant mice with normal maternal glucose levels and STZ administration at E13. Our data disclosed that kidney size and the number of glomeruli formed seemed to be independent of STZ administration or the length of exposure to STZ (Figure 9-4C, page 175). Taken together, we believe it is unlikely that a small amount of STZ exerts toxicity in fetuses *in utero* in our model.

10.3.2. LBW is Associated with Maternal Diabetes

As described above, clinically, 2 opposite abnormal situations of fetal growth are associated with maternal diabetes, as illustrated in Figure 10-4. In our studies, we adapted an experimental model of severe maternal diabetes, which provides a hyperglycemic environment *in utero.* We are focusing on a model of fetal restriction because LBW is often accompanied by fewer glomeruli and perinatal programming in adulthood. A study of STZ-induced diabetes rat models also showed that the mean body weights of offspring from STZ mothers were decreased by about 10% compared to the controls (422). Microsomic babies were found among the offspring of diabetic mothers (Figure 9-1, page 172), which could be explained by IUGR. Severely diabetic dams displayed dehydration, and severe hyperglycemia in the mothers actually decreased uterine blood flow, which greatly affected the transport of nutrients to the fetus. Our severely diabetic dams presented dehydration and sickness, resulting in maternal undernutrition. Fetal IUGR can occur in response to maternal undernutrition (423). From birth to weaning (3 weeks), the offspring of diabetic mothers manifested significantly reduced body weight in comparison to

control offspring (Figure 9-2, page 173). Our data indicated that severe maternal hyperglycemia and decreased uteroplacental blood flow caused fetal malnutrition and delayed fetal growth.

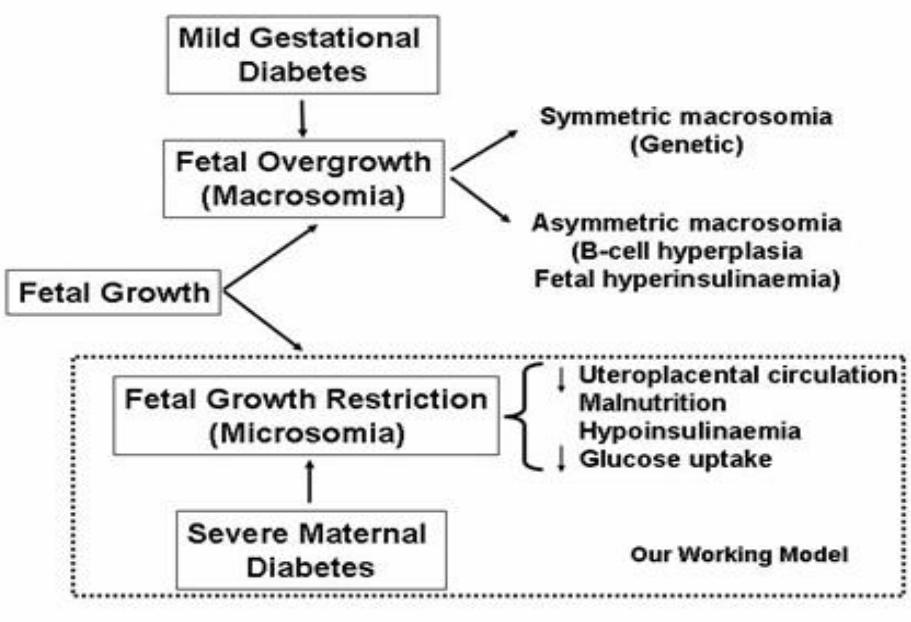

Figure 10-4. Maternal diabetes working model

The relationship between LBW and nephron number has been well-documented. Manalich et al. (151) counted glomeruli in coronal sections of the renal cortex in 35 neonates and found fewer nephrons in the kidneys of newborns with LBW. Next, we were interested in evaluating nephron endowment in our *in vivo* model.

10.3.3. Hyperglycemia Delays Renal Formation

Sustained exposure of the fetus to high glucose concentrations may result in diabetic embryopathy characterized by a multitude of congenital birth defects, including renal system anomalies (86). Kanwar et al. (411) have demonstrated that exposure to hyperglycemia *in utero* impairs nephrogenesis in mice, leading to reduced kidney size. Another study in STZ-induced diabetes rat models showed that

the kidneys from STZ fetuses were slightly smaller than those of their normal counterparts (422). To confirm that hyperglycemia impairs nephrogenesis, hematoxylin/eosin (HE) staining was used to view renal morphology, and Dolichos Biflorus Agglutinin-FITC staining was undertaken for UB identification. Less UB branching and a smaller cortex zone with small size glomeruli were observed in the kidneys of ODMs (Figure 8-10C, page 145). We also noted that the offspring of diabetic dams had smaller kidneys with reduced average glomerular volume (Figure 9-3A, page 174) and 40% fewer nephrons on average (Figure 9-3C, page 174) compared to control offspring. We found that glomerular volume (V_G) was persistently lower in ODMs from birth until the age of 2 weeks (Figure 9-3B, page 174) compared to control animals. The glomerular volume (V_G) in 3 week-old offspring from diabetic mice was not significantly different as compared to that from nondiabetic mice (Figure 9-3B, page 174). Neverthless, the trend to catch up glomerular volume at the age of 3 weeks indicated adaptation to a greater GFR per glomerulus (hyperfiltration), resulting from reduced nephron endowment. Glomerular enlargement seems to be associated with compensatory hypertrophy in the face of nephron deficit. Lower birthweights or intrauterine growth retardation undoubtedly contributes to lower nephron number. Reduction in kidney mass with increasing age suggests that many missing nephrons have been lost over life. Although, we did not demonstrate any data on the nephron number at age of 3 week-old, we believe it is impossible that the nephron number are recovered at age of 3 week-old. Several studies have demonstrated that exposure to high glucose *in utero* increases the synthesis of ECM components in developing kidneys (422;424). Although we did not report any results on glomerular hypertrophy in ODMs, we believe that glomerular assessment in the offspring may show hypertrophy. Taken

together, our data indicate that exposure to hyperglycemia *in utero* delays or retards renal formation.

10.3.4. Nascent Nephron Hypoplasia is Due to Apoptosis

More than a decade ago, Brenner et al. (124) proposed the hypothesis that a low nephron number at birth is associated with essential hypertension and a tendency to progressive loss of renal function after renal injury. Apart from genetic factors, maternal hyperglycemia or maternal diabetes during pregnancy seems to play an important role in the pathogenesis of fewer nephrons (425). In experimental animals, STZ-induced diabetes during pregnancy leads to a highly significant reduction of nephron numbers in ODMs (117;256). This nephron deficit may be a risk factor for the development of chronic renal disease and hypertension in adulthood. Our results showed that the neonatal kidneys of ODMs had 40% fewer nephrons on average compared to the controls (Figure 9-3C, page 174). Furthermore, in the study Amri et al. (256), nephrons were counted by glomeruli isolation, whereas in our experiments, we adapted Bertram's protocol, which necessitates cutting whole kidneys into serial sections each of 7 microns. We stained each slide with HE and took pictures of every 10^{th} section. After carefully counting the glomeruli on each section, we could mathematically extrapolate the total number of nephrons in the kidneys. We postulated that nascent nephron hypoplasia is due to rapid apoptosis, resulting from maternal hyperglycemia observed in glomerular cells. Studies have demonstrated that a high-glucose milieu triggers increased apoptosis in diabetic embryopathy (426;427). In our investigations, apoptosis was detected by more than one method, such as TUNEL assay and active caspase-3 staining. The most important finding was increased apoptosis in embryonic kidneys in diabetic pregnant dams, indicating augmented apoptosis in embryos exposed to a diabetic milieu. In normal

nephrogenesis, apoptosis is normal and necessary during kidney development in association with cell proliferation and differentiation. We observed more apoptotic cells in already-differentiated MM, specifically in the collapsed nephron region in neonatal and 1-week-old kidneys (Figure 9-5C, page 176). Furthermore, we found increased activation of caspase-3 in the kidneys of ODMs compared to control animals (Figure 9-6, page 177). We noted significantly increased active caspase-3 in renal tubules, possibly proximal tubules. During normal nephrogenesis, formation of the rodent kidney is complete by 2 weeks after birth. In the newborn kidney, the tubules are still differentiating, so that augmented active caspase-3 may localize in either collecting tubules or UB. Different cell types play different functional roles in the glomerulus. Excessive apoptosis of any cell type can lead to abnormal renal function in the diabetic condition. Studies have demonstrated that early loss of podocytes underlies filtration barrier deterioration in DN (428), and suggested that podocyte apoptosis represents a novel, early mechanism leading to DN (314). We also used double fluorescent staining for WT-1 and active caspase-3 antibodies to show that glomerular podocytes undergo apoptosis under hyperglycemia in developing glomeruli (Figure 9-5B, page 176). Our data indicate that exposure to hyperglycemia *in utero* triggers podocyte apoptosis during nephrogenesis, resulting in a low nephron number at the time of birth.

10.3.5. Effect of Hyperglycemia and Intrarenal RAS Activation on Renal Damage

The intrarenal RAS plays a fundamental role in nephrogenesis. All components of the RAS are expressed in the metanephric kidney in rodents during kidney development. As noted in Section 4.2.6.2, genetic interruption of RAS components in mice elicits a series of renal malformations. Evidence suggests activation of the intrarenal RAS in DN. Both clinical and animal studies have demonstrated that

activation of the intrarenal RAS contributes to tubular apoptosis in DN (316;429). We wanted to show that the RAS in hyperglycemia *in utero* triggers increased renal damage by apoptosis. We observed that Agt and renin, 2 major contributors to Ang II production, are up-regulated in the kidneys of ODMs (Figures 9-7, 9-8 and 9-9, page 178-180). Cells in the JG apparatus are responsible for secreting renal renin in the normal kidney, but cells positive for renin staining appear to be localized in the glomeruli or tubular region in the kidneys of ODMs. Specifically, this shift in renin expression with increased Agt expression might be capable of stimulating local Ang II formation, contributing to heightened RAS activation. Our data revealed that cleaved active caspase-3 immunostaining is increased in the proximal tubules of ODMs with strong staining density of Agt mostly in the proximal tubule portion of the diabetic offspring kidney. There was a strong correlation between apoptosis and RAS activation in the same cells, suggesting that RAS activation is associated with apoptotic events causing renal damage.

10.3.6. Effect of Hyperglycemia and NF-kB Activation on Renal Damage

The NF-kB pathway has been reported to be a major intracellular target in hyperglycemia and oxidative stress (325;430). Since we demonstrated that high glucose induced Pax2 gene expression through the NF-kB pathway, we wanted to investigate if NF-kB involvement in hyperglycemia *in utero* triggers increased apoptosis and renal damage. The mechanisms associated with NF-kB-mediated apoptosis are not clear. However, there is evidence that NF-kB activation regulates apoptosis (431). Up-regluation of subunits p50 and p65 has been reported in tubular epithelial cells of proteinuric kidneys (327). By immunohistochemistry, we demonstrated up-regulation of subunits p50 and p65 in the kidneys of offspring born to diabetic mothers, and these subunits were translocated from the cytosol to the

nucleus in the RPTCs of ODMs (Figure 9-10A, page 181). We believe that the NF-kB pathway regulates apoptosis in proximal tubules. By gel motility shift assay, we showed that NF-kB activation was greater in the neonatal kidneys of ODMs compared to their controls (Figure 9-10B, page 181). Our data indicated that a high glucose environment *in utero* triggers the apoptosis of nascent nephrons, possibly via activation of the NF-kB pathway.

10.3.7 Conclusion

Our findings demonstrated that maternal diabetes impairs renal development and induces nascent nephron cell apoptosus via intrarenal RAS and NF-kB signaling, as shown in Figure 10-5.

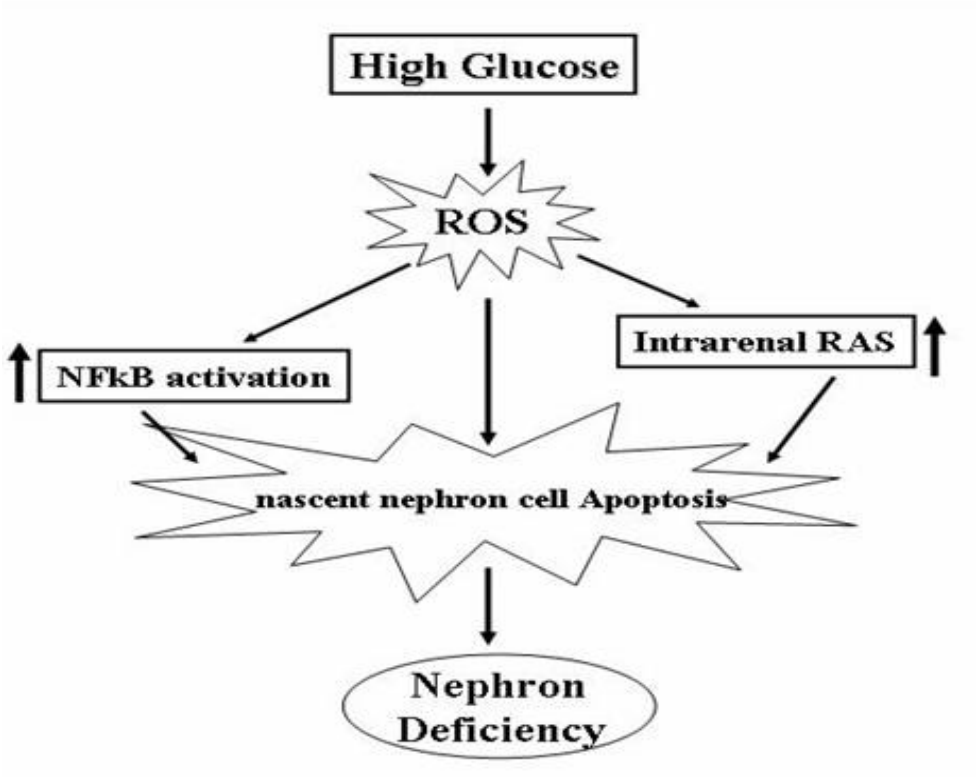

Figure 10-5. *In vivo* working diagram

In summary, the adverse environment created *in utero* by maternal hyperglycemia obstructs kidney development in the offspring and increases vulnerability of the kidneys exposed to different pathological processes in adult life. Our results have shown that high D-glucose elevates Pax2 gene expression and apoptosis in MK4 cells; high D-glucose alters UB branching morphogenesis; glomerular podocytes undergo apoptosis and components of the intrarenal RAS are overexpressed in the kidneys of ODMs.

In utero exposure to diabetes could cause lower nephron number and induced hypertension and renal disease in adult offspring. The offspring of diabetic rats displayed elevated systolic blood pressure and renal failure. Our ongoing long-term *in vivo* research will study if nephron underendowment induced by maternal diabetes is a major mechanism of the perinatal programming of hypertension as illustrated in Figure 10-6 as our working model.

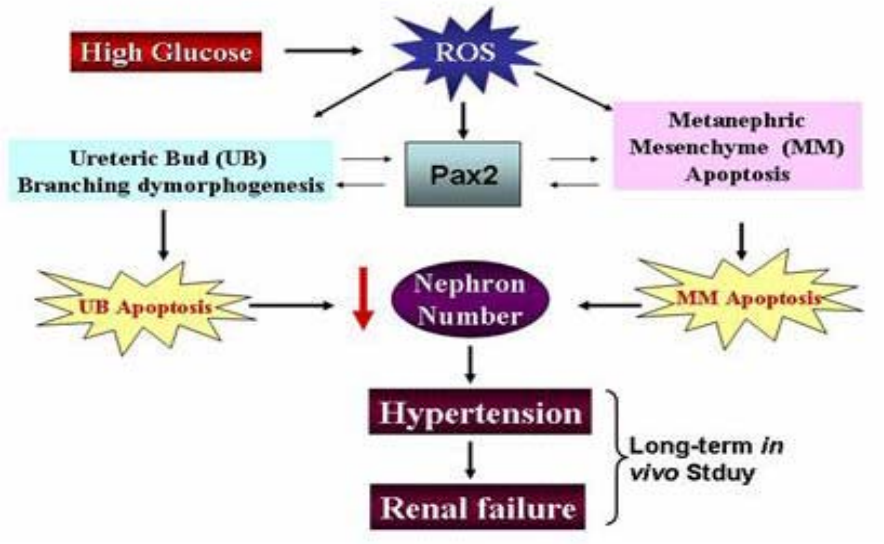

Figure 10-6 Our working model.

CHAPTER 11: RESEARCH PERSPECTIVES

11.1 MATERNAL DIABETES AND PERINATAL PROGRAMMING

11.1.1. Maternal Diabetes and Perinatal Programming

Gestational diabetes or hyperglycemia occurring during pregnancy is linked with an increased risk of complications not only in mothers but also in their offspring. Accumulating evidence, from both animal studies and epidemiological data, indicates that fetal beta cell hyperplasia and hyperinsulinemia induce irreversible changes leading to heightened risk of T2DM, obesity, cardiovascular disease and hypertension in adulthood (432).

11.1.2. Maternal Diabetes Related to the Development of Obesity

Excessive growth has been reported in the offspring of women with diabetes during pregnancy, including both GDM and PGDM (433). The offspring of Pima Indian women with pre-existent T2DM and GDM presented fetal macrosomia, and were larger and heavier at every age compared to the offspring of non-diabetic women (434). Weiss et al. (435) showed that the offspring of women with T1DM had significantly higher BMI by age 5-15 years compared to those of control women. The Growing Up Today Study in 9- to 14-year-old white, non-Hispanic children disclosed that among 465 subjects whose mothers had GDM, 17.1% were at risk of being overweight and 9.7% were overweight in early adolescence (436). The mechanisms by which intrauterine exposure to maternal diabetes increases the risk of offspring obesity are not entirely understood. At age 5-9 years, the offspring of Pima women with diabetes or impaired glucose tolerance during pregnancy have higher fasting insulin concentrations than those of women with better glucose tolerance during pregnancy, indicating that relative hyperinsulinemia may be a precursor of childhood obesity (437). Also, leptin, a hormone secreted by adipocytes

and the placenta, seems to be a potential mechanism for later development of obesity in offspring exposed to diabetes *in utero* (438). These findings suggest that exposure to a diabetic environment *in utero* increases the risk of obesity in childhood and early adulthood.

11.1.3. Maternal Diabetes Induces Impaired Glucose Tolerance, Insulin Resistance and T2DM in the Offspring

Several clinical studies have disclosed that exposure to maternal diabetes *in utero* raises the risk of metabolic syndrome, including glucose intolerance, insulin resistance, and T2DM in the offspring in later life (439). Impaired glucose tolerance is one of the long-term complications in ODMs (107). In a study in Germany, the prevalence of impaired glucose tolerance was increased in the offspring of mothers with pre-existent T1DM and GDM (440). Dabelea et al. (441) reported that among Pima Indians, there was significantly more T2DM in the 5-34-year-old offspring of diabetic women than in those of nondiabetic women. In populations with a high prevalence of diabetes in pregnancy, such as North American Pima Indians, a U-shaped curve has been found, with high rates of diabetes also occurring in those who had HBW (≥4,500 g) (72). Wei et al. (442) confirmed a U-shaped correlation between birth weight and the risk of T2DM, which also afflicated those who weighed ≥4,500 g at birth in Taiwan. Segar et al. (443) showed that exposure to a hyperglycemic milieu during the last third of gestation results in insulin resistance in the offspring of STZ-induced diabetic rats. The ODMs of STZ-induced diabetic rats had impaired glucose tolerance compared to their controls (420). These findings indicate that exposure to an intrauterine diabetic environment heightens the risk of metabolic syndrome, including glucose intolerance, insulin resistance, and T2DM in the offspring in later life.

11.1.4. Maternal Diabetes Related to the Development of Hypertension and Renal Disease

Associations between exposure to maternal diabetes and offspring blood pressure have been examined (444). *In utero* exposure to diabetes can cause lower body weight and induce cardiovascular abnormalities in adult offspring (390). A study in Chicago showed that the offspring of diabetic mothers had significantly higher systolic and mean arterial blood pressure than those of nondiabetic controls (433). Another investigation, in 42 Pima Indians aged 7-11 years, established that intrauterine exposure to diabetes is a significant determinant of high systolic blood pressure during childhood (445). Project Viva, a recent cohort study in 1,238 mother-child pairs, revealed that children with intrauterine exposure to GDM had higher systolic arterial blood pressure at age 3 years than the controls (446). In Pima Indians, the offspring of mother who had diabetes during pregnancy were at higher increased risk of elevated albuminuria (447). Amri et al. (256) demonstrated that fetuses exposed *in utero* to hyperglycemia had a 13% decline in birth body weight and a 10-35% decrease in nephron numbers. The ODMs of STZ-induced diabetic rats had elevated systolic blood pressure, increased glomerular area, and reduction in the GFR and renal plasma flow (RPF) compared to the offspring of nondiabetic mothers (448). Magaton et al.(420) showed that ODMs had increased systolic blood pressure, glomerular hypertrophy, a decreased GFR from age 2 months, and a diminution of Ang 1-7 concentration, indicating early functional kidney impairment. Rocha et al. noted that, in comparison to control groups, ODMs had hypertension from 8 weeks onwards, glomerular hypertrophy from 3 months, a significantly decreased GFR and RPF from 3 months, and a reduction in glomeruli number in 12-month-olds (419). These findings suggest that exposure to a hyperglycemic

environment *in utero* increases the risk of renal disease and hypertension in later life.

11.1.5. Possible Mechanism(s) of Maternal Diabetes in Perinatal Programming of Hypertension

Exposure to maternal diabetes *in utero* may induce irreversible changes leading to an heightened risk of T2DM, obesity, cardiovascular disease and hypertension in adult life. One of the best known renal mechanisms involved in perinatal programming of hypertension may be the reduction of nephron numbers in patients and animals models as a function of birth weight or IUGR (141;449). Diabetes is associated with a high frequency of congenital malformations, including urogenital abnormalities. These congenital malformations result from developmental defects occurring in early organogenesis. Studies have demonstrated that maternal diabetes is a novel risk factor for inborn nephron deficits. Congenital nephron deficiency is a risk factor for chronic renal disease and hypertension progression in adulthood. Multiple mechanisms are involved in maternal diabetes impacting nephron deficiency, such as hyperglycemia, ROS, apoptosis, and NF-kB. As described in Section 4.2, hyperglycemia *in utero* impairs nephrogenesis, culminating in a reduced number of nephrons. Furthermore, the crucial role of high glucose-induced apoptosis in experimental DN has been demonstrated. Kanwar et al. (295) have reported that *in vitro* exposure of metanephroi to high glucose concentration increases apoptosis. The intrarenal RAS is another important candidate. As described in Section 4.2.6, deficiency, mutation or the abnormal expression of intrarenal RAS genes during organogenesis in experimental models often results in abnormal kidneys, with a reduction in nephron numbers. It has been demonstrated that intrarenal RAS activation plays a key role in the development of hypertension

in diabetes (450). A study of maternal low-protein diets showed that renal AT1R expression was increased after IUGR in rats (451). Animal experiments have documented heightened renal renin expression in experimental DN, suggesting that hyperglycemia activates the intrarenal RAS (388). In rats, hyperglycemia *in utero* produces long-lasting hypertension in the male offspring with enhanced tissue ACE activity (391).

11.1.6. Effects of Perinatal Programming by Hyperglycemia in Adult Offspring of Diabetic Mothers (Age 20 Weeks)

Population studies reporting the multiple long-term consequences of maternal hyperglycemia explain the concept of perinatal programming. Rasch et al. (452) established a link between the RAS and perinatal programming and adult cardiovascular diseases, such as hypertension. However, the mechanisms of programming remain unclear. The long-term part of our project will investigate the effects of perinatal programming by hyperglycemia *in utero* on diabetic adult offspring (age 20 weeks), as shown in Figure 11-1 as our experimental design in this long-term *in vivo* study.

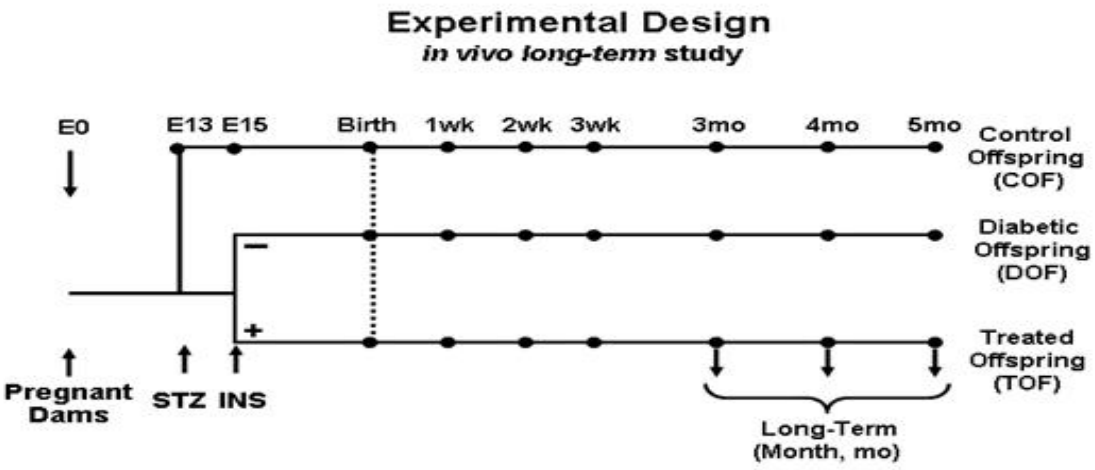

Figure 11-1 Experimental design of our long-term *in vivo* study

Brochu et al. (453) reported that the offspring of rats born with IUGR presented differences in the development of hypertension and plasma renin activity between male and female adults, suggesting the importance of gender in outcomes during adulthood after IUGR. It would, therefore, be appropriate to separate the results of our future long-term study by sex. Our short-term experiment showed that the body weight of diabetic offspring was lower than that of control offspring. In our preliminary investigations, we found that the body weight of diabetic adult offspring was lower than that of control adult offspring, in both males and females, as seen in Figures 11-2 and 11-3.

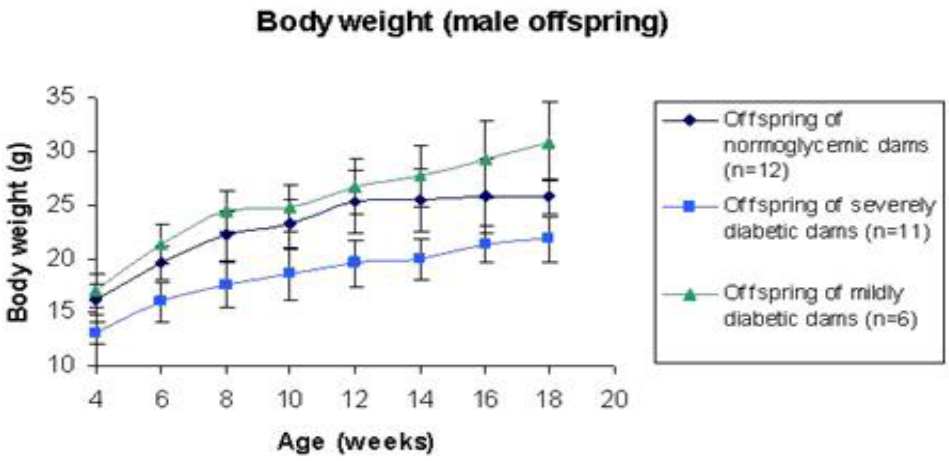

Figure 11-2. Body weight of male adult offspring

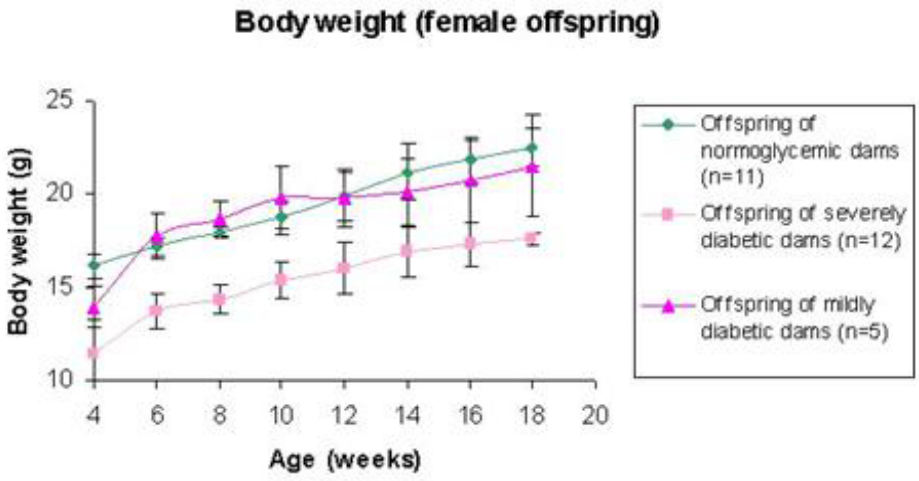

Figure 11-3. Body weight of female adult offspring

According to Barker's hypothesis, LBW is inversely correlated with blood pressure. As discussed in Section 11.1.4, exposure to a diabetic environment *in utero* can cause lower body weight and induce cardiovascular abnormalities in adult offspring. Our preliminary studies pose interesting questions as to whether adult diabetic offspring develop hypertension. Our short-term experiments showed that hyperglycemia *in utero* impaired the intrarenal RAS and induced the apoptosis of glomerular cells, resulting in low nephron numbers in ODMs. According to Brenner's hypothesis, low nephron numbers lead to glomerular hyperfiltration, culminating in glomerular hypertrophy. We postulate that compensatory glomerular hypertrophy is a gradual predisposition to hypertension in ODMs Thus, we can check the progression of renal failure (decreased GFR and elevated albuminuria) in offspring exposed to hyperglycemia *in utero*, as illustrated in Figure 11-4 as our working model.

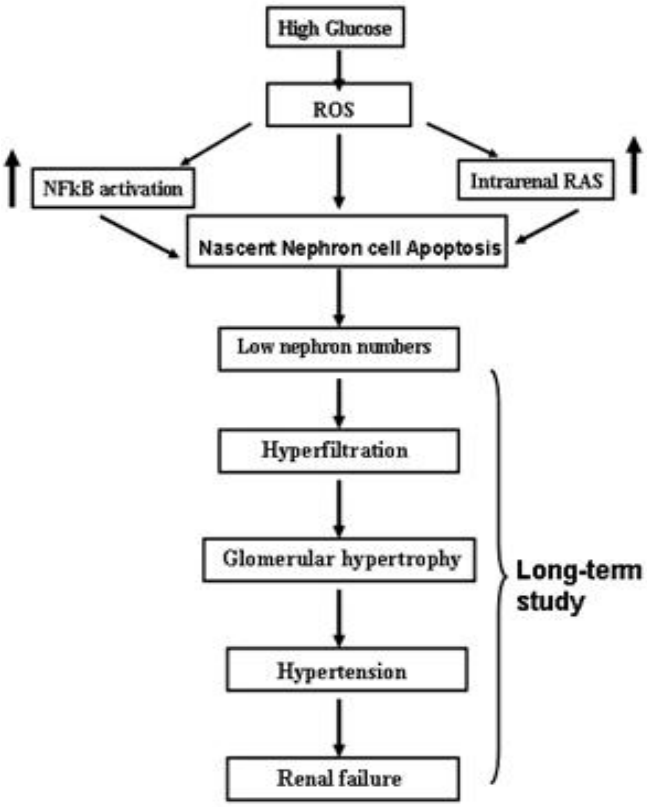

Figure 11-4. Our working model for long-term *in vivo* study

11.2. Underlying Mechanism by Which NF-kB Promotes Apoptosis via the p53 Pathway in UB and MM Lineages

Increased apoptosis in diabetic embryopathy has been reported both *ex vivo* and *in vivo*. Hyperglycemia-induced apoptosis is one of the major mechanisms affecting renal morphology. The high-glucose environment in diabetes mellitus has been linked to ROS generation, followed by apoptosis, and to a number of cytokines and cytokine-like factors. We previously observed *in vitro* that high glucose specifically induced Pax2 gene expression in MK4 cells and kidney explants via ROS generation and the NF-kB pathway. We also discerned that high glucose increased cell apoptosis in MK4 cells due to ROS generation (454). Linking this finding to apoptosis, a first possibility might be that NF-kB activation evokes proapoptotic genes, including p53, which could lead to apoptosis of RPTCs. In our preliminary studies, we noted that high D-glucose stimulates NF-kB (p50/p65) gene expression in MK4 cells, as seen in Figure 11-5.

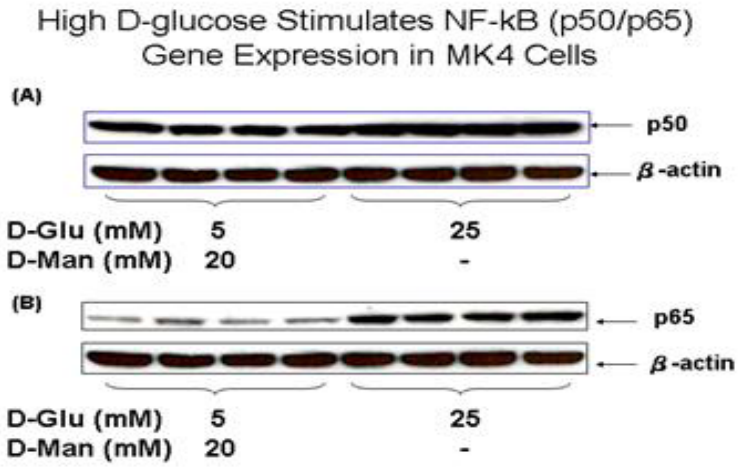

Figure 11-5. High D-glucose stimulates NF-kB (p50/p65) gene expression in MK4 cells

We also found that high D-glucose stimulates p53 phosphorylation (Ser15) and p53 translocation in MK4 cells, as depicted in Figure 11-6.

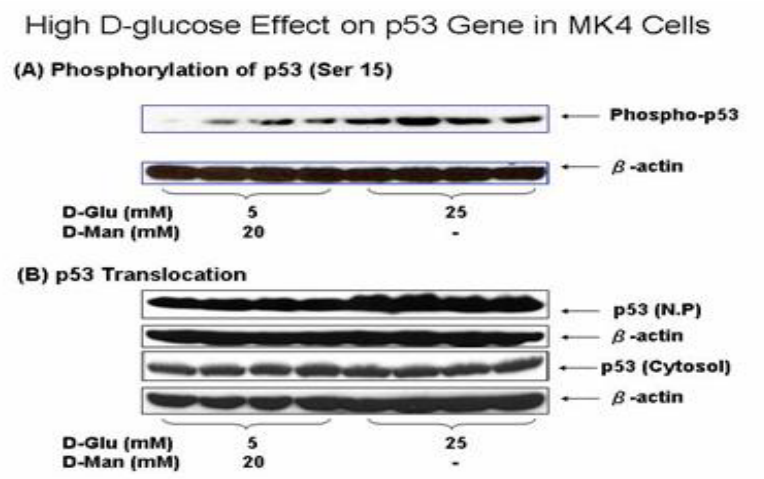

Figure 11-6. High D-glucose effect on p53 gene in MK4 cells

The mechanisms of hyperglycemia-induced apoptosis mediated by the NF-kB-induced p53 pathway are not clear. p53 Tg mice show smaller kidneys and nephron deficiency resulting from increasing apoptosis in the undifferentiated mesenchyme (356). Studies have demonstrated that NF-kB-binding sites are present in the promoter regions of p53 (339), and NF-kB can activate p53 expression (340). Our preliminary experiments raise interesting questions: 1) does high D-glucose stimulate apoptosis via the NF-kB pathway in MK4 cells? 2) does high D-glucose have an impact on the p53 gene (phosphorylation or translocation) via the NF-kB pathway in MK4 cells? We are interested in studying the underlying mechanism by which NF-kB promotes apoptosis via the p53 pathway in UB and MM lineages in MK4 cells, as shown in Figure 12-3 as our working model. We also want to investigate the short-term *in vivo* model to demonstrate whether p53 expression and NF-kB (p50/p65) gene expression are up-regulated in the kidneys of diabetic offspring.

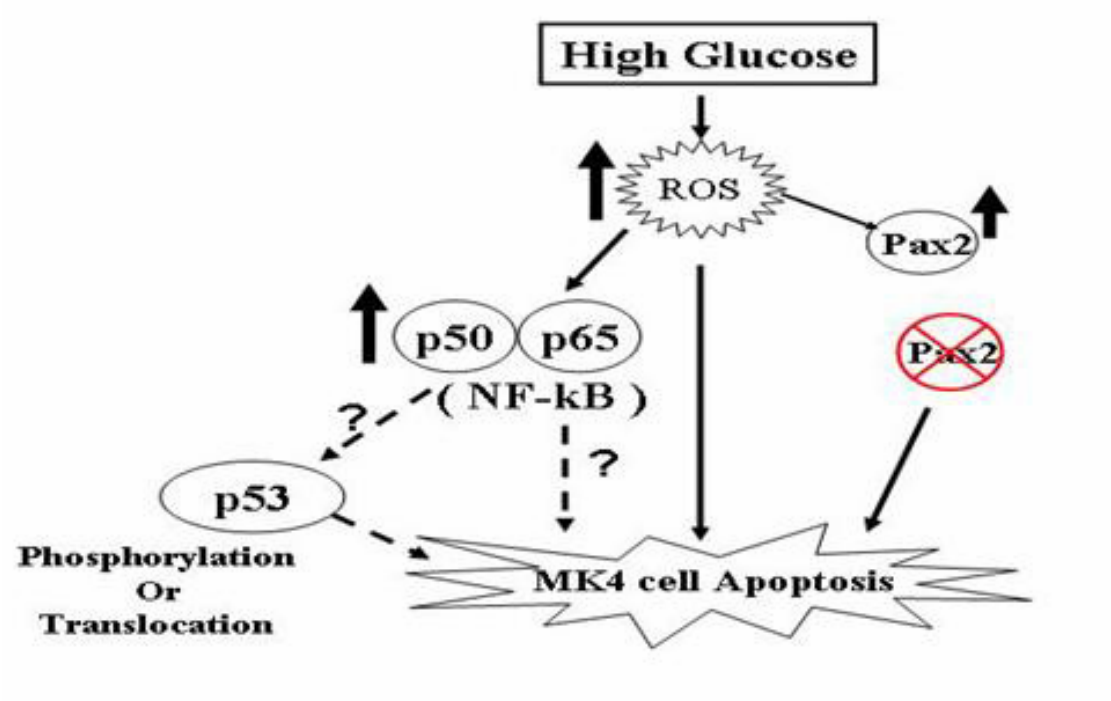

Figure 11-7. Our working model

11.3. To Determine Whether an Antioxidant System (Catalase) can Protect against the Effects of Hyperglycemia *in utero* on Embryonic Organogenesis Affected by Increased ROS generation

Studies suggest that antioxidants can reverse high glucose-induced renal damage (455). In an investigation of diabetic embryopathy, Ryu et al. (456) established that ROS are increased in rat embryos with congenital malformations. Oxidative damage is high in diabetic culture medium, and nitric oxide protects against diabetes-induced teratogenicity in a dose-dependent manner. Our laboratory showed that ROS play a role in nephrogenesis both *in vitro* and *ex vivo*. Using E19 kidney explants from a gestational diabetes model, our unpublished data indicate that ROS generation occurs in diabetic kidneys compared to control kidneys (Figure 12-1, page 206). Our data demonstrate that catalase partially blocks high glucose-induced UB branching in E12 kidney explants. Our unpublished findings suggest that

catalase inhibits the stimulatory effect of high glucose on Pax2 gene expression (Figure 12-2, page 207). Brezniceanu et al. (316) reported that catalase overexpression attenuated ROS and apoptosis stimulation in the kidneys of diabetic mice *in vivo*. In their other study, they (457) showed that db/db mice developed obesity, hyperglycemia, hypertension, and albuminuria but db/db rCAT-Tg mice had normal blood pressure compared to db/db mice. These results raise interesting questions: 1) could an antioxidant system (catalase) protect against the effects of an hyperglycemic environment *in utero* on embryonic organogenesis impacted by an increase in ROS generation? 2) could an antioxidant (catalase) prevent the perinatal programming of hypertension in an hyperglycemic environment *in utero*? In the future, we are going to study catalase Tg mice to investigate the pathogenesis of nephropathy resulting from maternal diabetes. Catalase Tg mice were developed by Dr. John S.D. Chan's laboratory. These animals specifically overexpress catalase in proximal tubules driven by kidney androgen-regulated protein promoter 2 (KAP2), as illustrated in Figure 11-8.

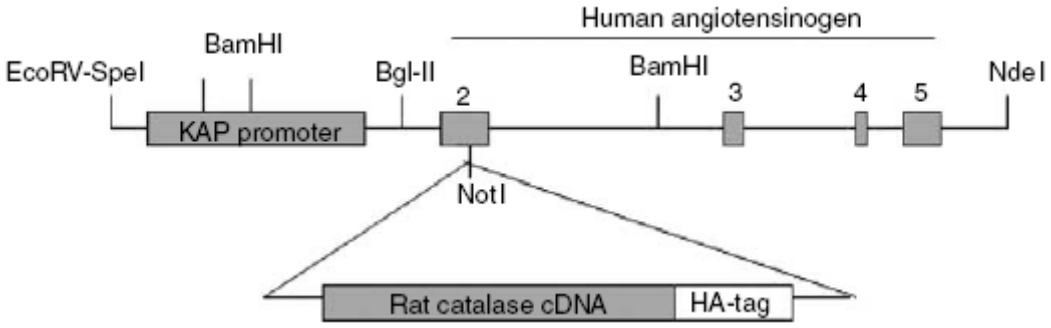

Figure 11-8. KAP2-rCAT construct (adapted from Brezniceanu et al.: Kidney Int 2007) (316)

This Tg mouse model with antioxidant enzyme overexpression will serve to explore our hypothesis on the role of ROS in gestational diabetes, as shown in Figure 11-9 as our experiment design.

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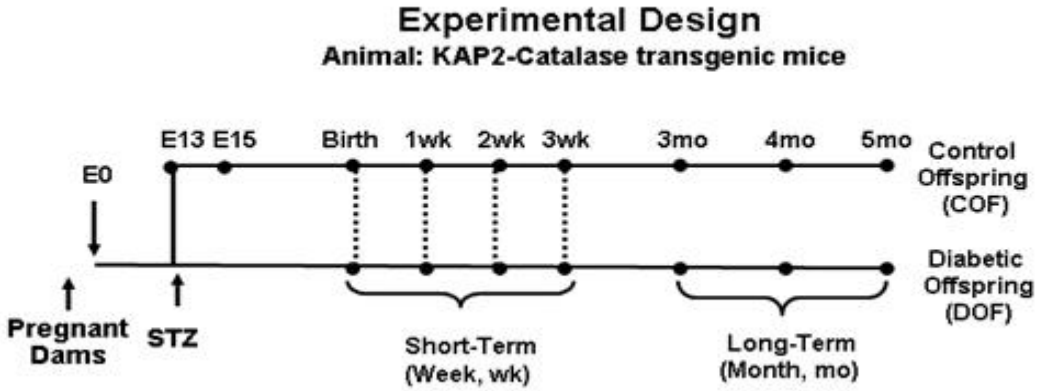

Figure 11-9. Our experiment design.

Thus, we can check whether an increase in the antioxidant defense system will protect against heightened ROS production in kidneys exposed to an hyperglycemic environment *in utero* and attenuate apoptosis in gomerular or proximal tubules, as depicted in Figure 12-10 (our working model). In addition, we will be able to establish if this antioxidant defense system will normalize the effect of high glucose on UB branching morphogenesis after ROS generation by crossing Hoxb7/GFP mice with Catalase Tg mice.

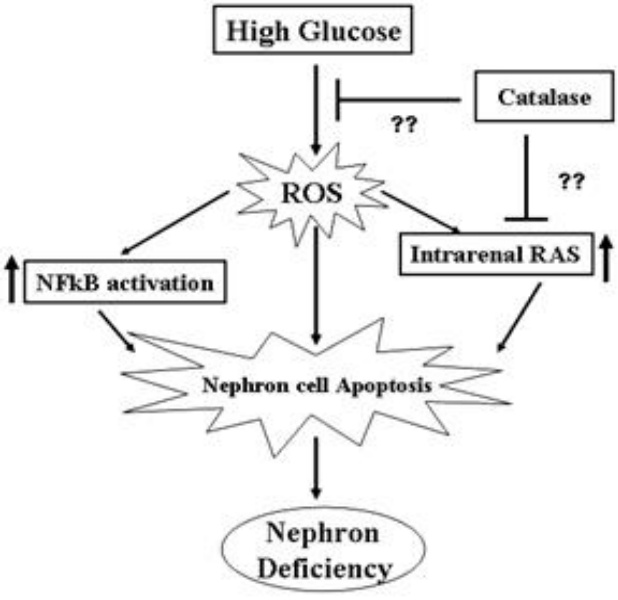

Figure 11-10. Our working model

CHAPTER 12: UNPUBLISHED DATA

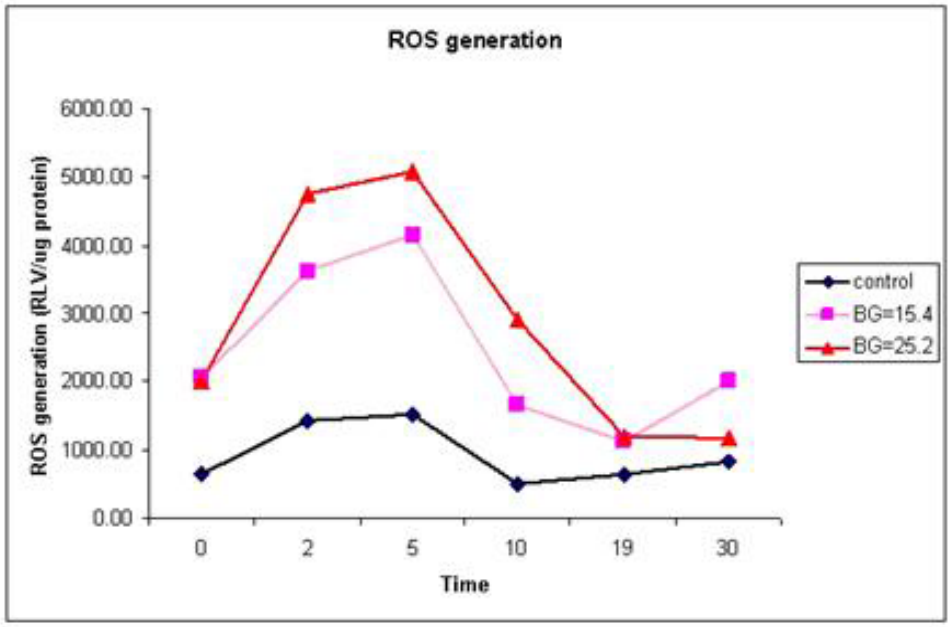

Figure 12-1. Hyperglycemia *in utero* induced ROS generation in E19 kidney explants. The whole E19 kidney explants were isolated from the offspring of either control mice or streptozotocin (STZ)-induced diabetic mice (mildly diabetic dam (BG=15.4 mM) and severely diabetic dam (BG=25.2mM).) ROS generation was assessed by lucigenin mothod, and the final value of ROS generation was normalized by the protein concentration of sample.

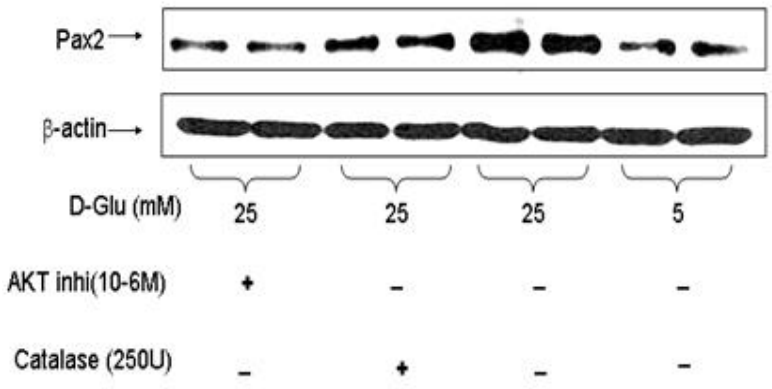

Figure 12-2. Catalase inhibits the stimulatory effect of high glucose on Pax2 gene expression in MK4 cells. After synchronized with serum free medium overnight, the quiescent cells were incubated in either 5mM or 25mM D-glucose DMEM with or without Catalase or AKT inihibitor containing 1% dFBS for 24 hours.

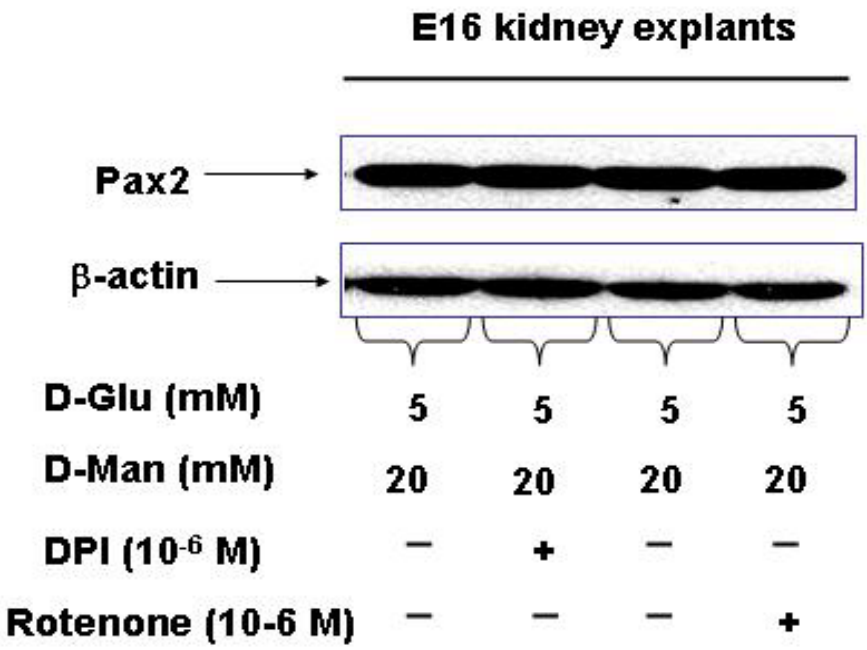

Figure 12-3 Inhibitory effect of diphenylene iodinium (DPI) and rotenone on Pax-2 gene expression in E16 kidney explants. E16 kidney explants were cultured in 5 mM glucose DMEM with or without DPI (10-6 M) and rotenone (10-6 M) for 24 h. The Pax-2 gene expression was analyzed by Western blot.

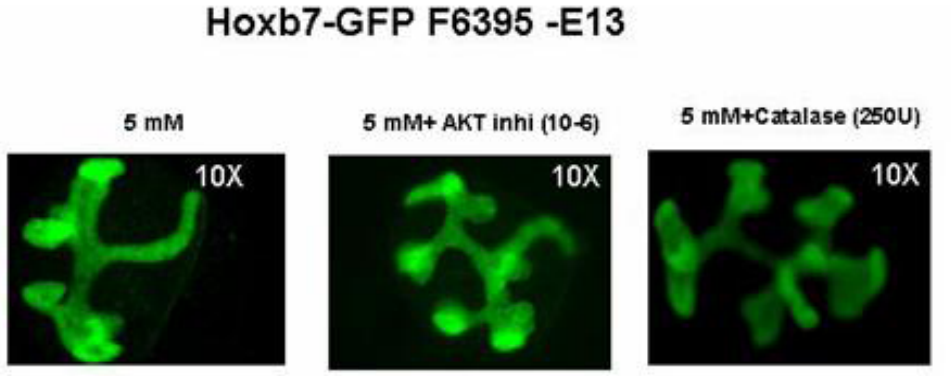

Figure 12-4 The effect of catalase and AKT inhibitor on UB branching morphogenesis in E13 kidney explants. E13 kidney explants were incubated in 5 mM glucose DMEM in the absence or presence of catalase (250 U) and AKT inhibitor (10^{-6}M) for 24 h. The images were recorded by fluorescence microscope.

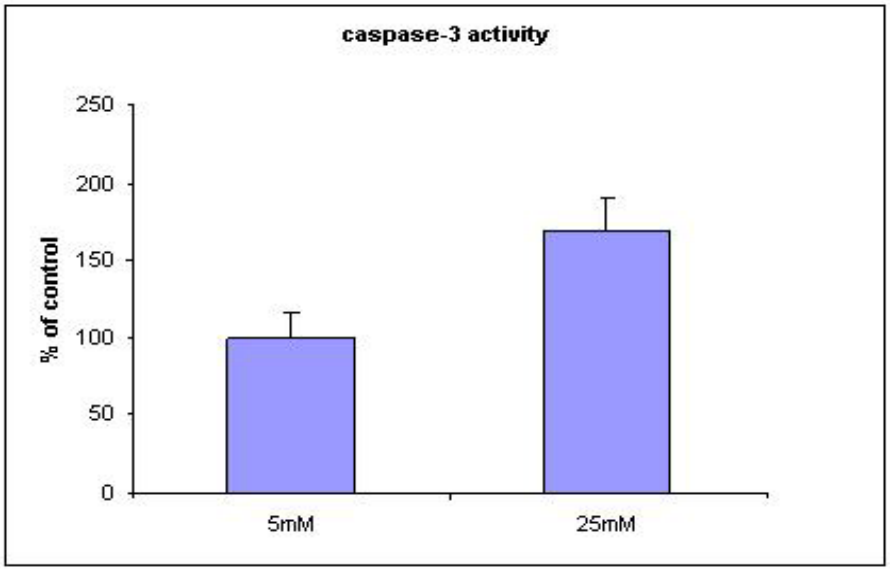

Figure 12-5 Analysis of Caspase-3 activity .Caspase-3 activity was measured with a commercially available kit according to the manufacturer's instructions (Medical and Biological Laboratories,Woburn, MA, USA) . The MK3 cells were incubated in DMEM with 1% dFBS containing either 5mM glucose or 25mM glucose for 24 h

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