# Reactive oxygen species and nuclear factor-kappa B pathway mediate high glucose-induced Pax-2 gene expression in mouse embryonic mesenchymal epithelial cells and kidney explants

Y-W Chen[1], F Liu[1], S Tran[1], Y Zhu[1], M-J Hébert[1], JR Ingelfinger[2] and S-L Zhang[1]

[1]*Université Montréal, Centre hospitalier de l'Université de Montréal (CHUM)-Hôtel-Dieu, Pavillon Masson, Montréal, Québec, Canada and* [2]*Harvard Medical School, Massachusetts General Hospital, Pediatric Nephrology Unit, Boston, Massachusetts, USA*

**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 embryonic mesenchymal epithelial cells (MK4) and *ex vivo* (kidney explant from Hoxb7-green florescent protein (GFP) mice) approaches. Pax-2 gene expression was determined by reverse transcriptase-polymerase chain reaction, 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) cotransfection. 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 nuclear factor kappa B signaling pathway, but not via protein kinase C, p38 mitogen-activated protein kinase (MAPK), and p44/42 MAPK signaling.**

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**Correspondence:** *S-L Zhang, Université Montréal, Centre hospitalier de l'Université de Montréal, Pavillon Masson, 3850 rue Saint-Urbain, Montréal, Québec H2W 1T7, Canada. E-mail: shao.ling.zhang@umontreal.ca*

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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 5-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% 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 via reactive oxygen species (ROS); high glucose inhibits cyclooxygenase-2, resulting in prostaglandin $E_2$ deficiency; and high glucose may induce transcription factors and proto-oncogenes. Kanwar *et al.*[9] 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 extracellular matrix glycoproteins.[14–16] Additionally, kidney-related key proto-oncogenes and transcription factors such as glial cell-derived neurotrophic factor, cRet, and paired-box 2 (Pax-2) may be altered in diabetic embryopathy,[9] although clear experimental evidence is presently lacking. The 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.[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 renal-coloboma syndrome seem to be highly related 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-$\kappa$B) is a major intracellular target in hyperglycemia and oxidative stress.[32,33] NF-$\kappa$B 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 mouse embryonic mesenchymal epithelial cells (MK4) cells and kidney explants, with ROS generation and the NF-$\kappa$B pathway certainly being involved as underlying mechanisms.

## RESULTS

### High glucose stimulates Pax-2 expression in MK4 cells

MK4 cells were incubated in media containing 1% depleted fetal bovine serum (dFBS) and 25 mM different glucose analogs such as D-glucose, D-mannitol, L-glucose, or 2-deoxy-D-glucose. After incubation for 24 h, cells were harvested and analyzed for Pax-2 messenger RNA (mRNA), reverse transcriptase-polymerase chain reaction (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 5 to 25 mM with a maximal effect at 25 mM 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, whereas 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).[36–38] Pax-2 expression was normalized by $\beta$-actin. The 24-h incubation period was used for all subsequent studies.

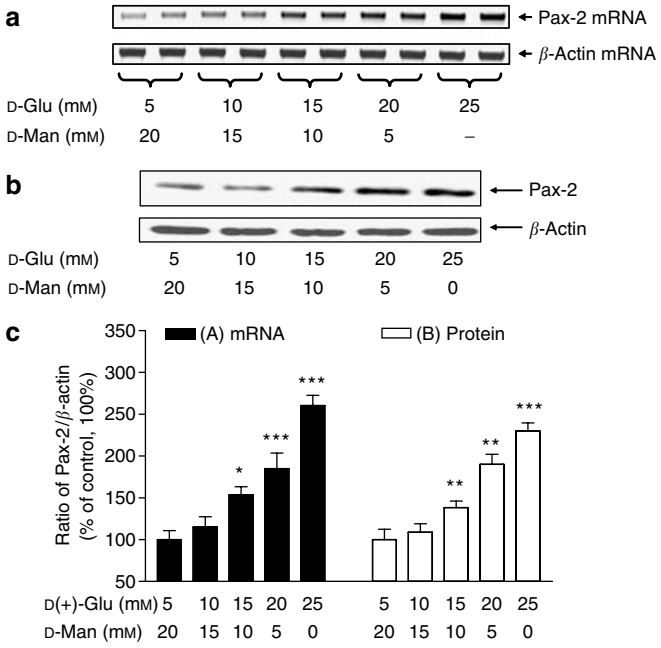


**Figure 1 | High glucose dose-dependent effect.** High D(+) glucose upregulates Pax-2 gene expression in a dose-dependent manner in MK4 cells analyzed by (**a**) RT-PCR and (**b**) Western blot. After synchronized with serum free medium overnight, the quiescent cells were incubated in DMEM with 1% dFBS containing final glucose concentration from 5 mM to 25 mM for 24 h, whereas D-mannitol was supplemented to maintain constant isotonicity or osmolality; (**c**) relative densities of Pax-2 were normalized to $\beta$-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 $\pm$ s.d. of three independent experiments. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.005$.

### 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 b reveal that inhibitors of p38 mitogen-activated protein kinase (MAPK) (SB203580), P44/42 MEK (PD98059), and protein kinase C (PKC) (GF109203) 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 min incubation in high glucose medium, ROS generation began to increase in MK4 cells, and this elevation lasted 60 min (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, d, and e).

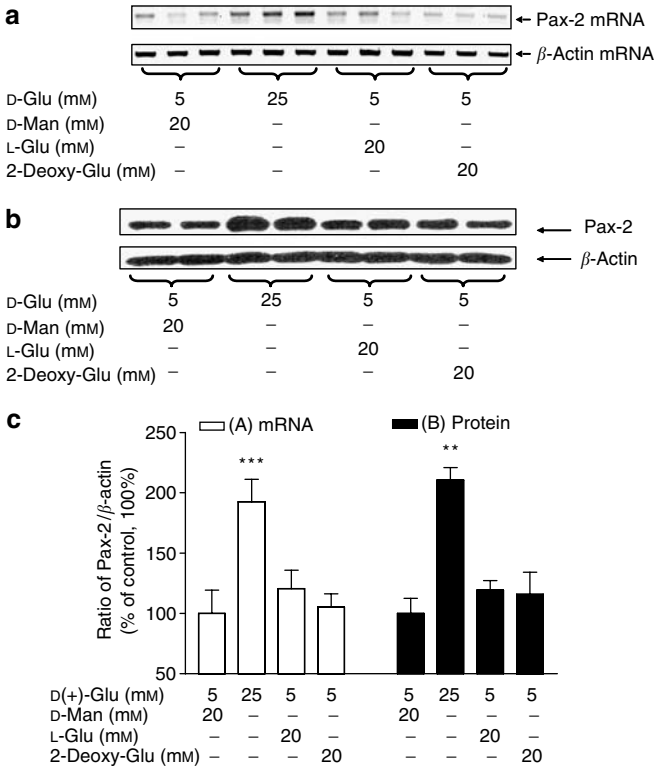


**Figure 2 | High glucose specifical effect.** Pax-2 gene expression is specifically stimulated by high D(+) glucose (25 mM), not by other glucose analogs such as L-glucose, D(+)-Mannitol, or 2-deoxy-D-glucose in MK4 cells analyzed by (**a**) RT-PCR and (**b**) Western blot. The quiescent cells were incubated in DMEM with 1% dFBS containing different glucose analogs in 25 mM as final concentration for 24 h; (**c**) the relative densities of Pax-2 were normalized to β-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± s.d. of three independent experiments. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.005$.

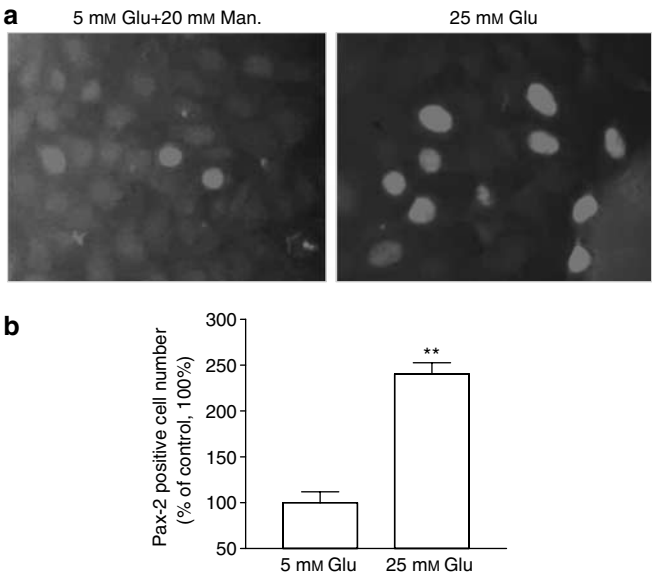


**Figure 3 | Pax-2 immunofluorescence staining.** High glucose upregulates Pax-2 expression in MK4 cells as shown by (**a**) immunofluorescence staining (original magnification × 400). Quiescent cells were incubated in either 5 mM or 25 mM D-glucose DMEM containing 1% dFBS for 24 h; (**b**) The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± s.d. of three independent experiments. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.005$.

### Inhibitors of nicotinamide adenine dinucleotide phosphate hydrogenase oxidase, mitochondrial electron transport chain complex I, and NF-κB 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 diphenylene iodonium chloride (DPI) and rotenone, but not pyrrolidinedithiocarbamate (PDTC) (Figure 5b). Figure 6 shows that inhibitors of nicotinamide adenine dinucleotide phosphate hydrogenase oxidase (DPI), mitochondrial electron transport chain complex I (rotenone), and NF-κB 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-κB 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-green florescent protein (GFP) mice were isolated and cultured *ex vivo* as shown in Figure 7a. After culturing the explants for 24 h in either normal glucose (5 mM) or high glucose (25 mM) Dulbecco's modified Eagle's medium (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 c. 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 7e (quantitative RT-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-κB signaling pathway (Figure 8a). Moreover, after cotransfection 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-κB signaling pathway.

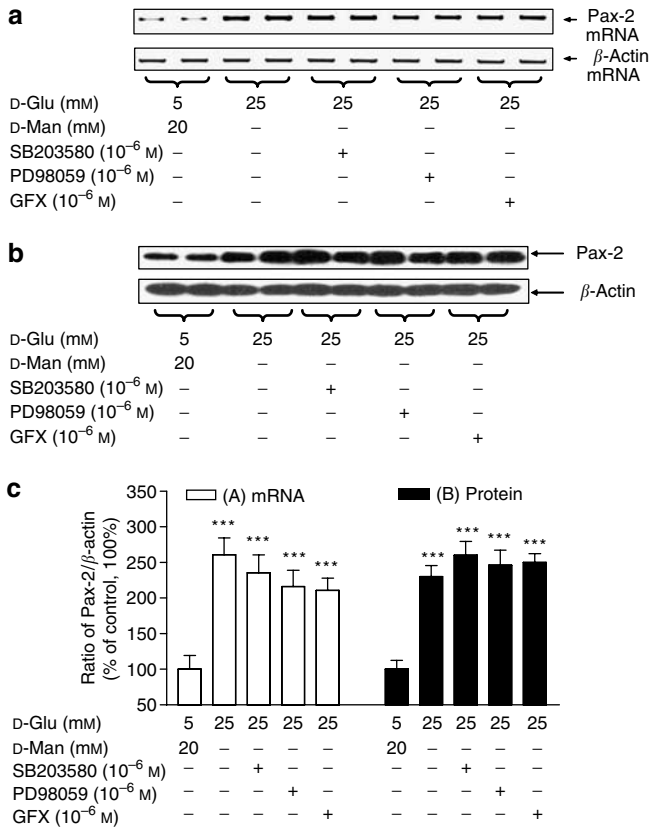


**Figure 4 | p38 MAPK, p44/42 MAPK, and PKC inhibitor effect.** Effect of SB203580, PD98059, and GFX on Pax-2 gene expression stimulated by high glucose in MK4 cells as analyzed by (**a**) RT-PCR and (**b**) Western blot; Quiescent cells were incubated in either 5 mM or 25 mM D-glucose DMEM containing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

## DISCUSSION

The present studies demonstrate that high glucose stimulates Pax-2 gene expression in mouse embryonic metanephric mesenchymal 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-κB 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 metanephric mesenchyme 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.[39–42] Nielsen *et al.*[43] reported that the prevalence of renal malformations such as renal agenesis and congenital abnormalities of kidney and urinary tract have a strong association with pre-gestational maternal diabetes, more than cardiovascular congenital abnormalities or multiple congenital abnormalities.

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 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.[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 (25 mM) specifically and dose dependently stimulated Pax-2 gene expression in MK4 cells, whereas 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-κB inhibitors. These data are consistent with our previous observation that high glucose induces ROS generation in immortalized renal proximal tubular cells.[44,45] In order to confirm that ROS directly regulate Pax-2 gene expression, we examined the effect of the 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-κB 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[44,45] and mesangial cells.[46,47] 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 block the high glucose action on Pax-2 gene expressions at both protein and mRNA levels, suggesting that p38 MAPK, p44/42 MAPK, and PKC signaling pathways are

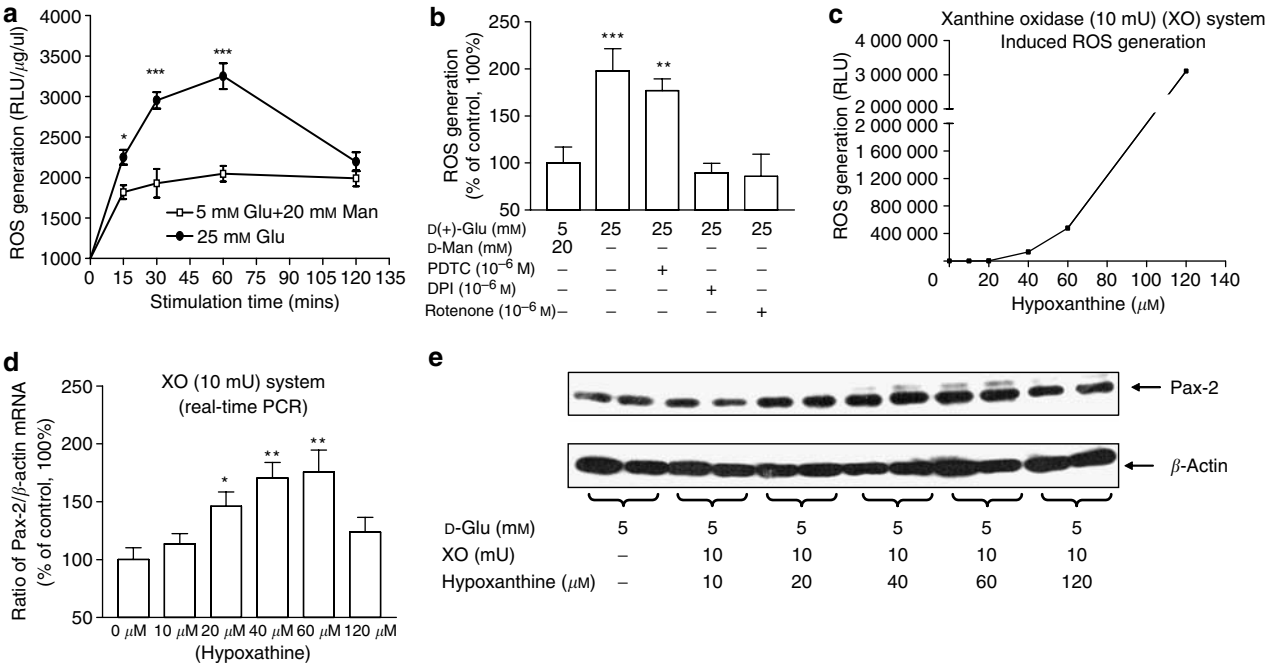


**Figure 5 | High glucose induced ROS on Pax-2 gene expression.** High glucose time dependently stimulates ROS generation in (**a**) MK4 cells , that is blocked by ROS inhibitors, but not (**b**) NF-κB inhibitors analyzed by lucigenin assay. Moreover, superoxide generated from XO system (**c**) directly stimulates Pax-2 gene expression in MK4 cells in a dose-dependent manner, shown here as analyzed by (**d**) real-time PCR and (**e**) Western blot; (**a**) Quiescent cells were incubated in either 5 mM glucose DMEM or 25 mM containing 1% dFBS for periods of 15 min to 2 h, 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 5 mM 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 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 h. Each point represents the mean ± s.d. of three independent experiments. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.005$.

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-κB signaling pathway.

NF-κB 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 IκB and it is activated through a common pathway, which involves the phosphorylation-induced proteasome-mediated degradation of the inhibitory subunit, IκB. Upon stimulation, a serine kinase cascade is activated leading to the phosphorylation of IκB and dissociating from the NF-κB heterodimer. This event primes IκB as a substrate for ubiquitination and subsequent degradation in the cytoplasm. The NF-κB 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), pro-inflammatory cytokines (e.g., tumor necrosis factor-α and interleukin-1β), receptor for advanced glycation end products, 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.[48,49] In order to further understand the underlying mechanisms of the NF-κB 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-κB 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 six of GGrmwkyCCC and two 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

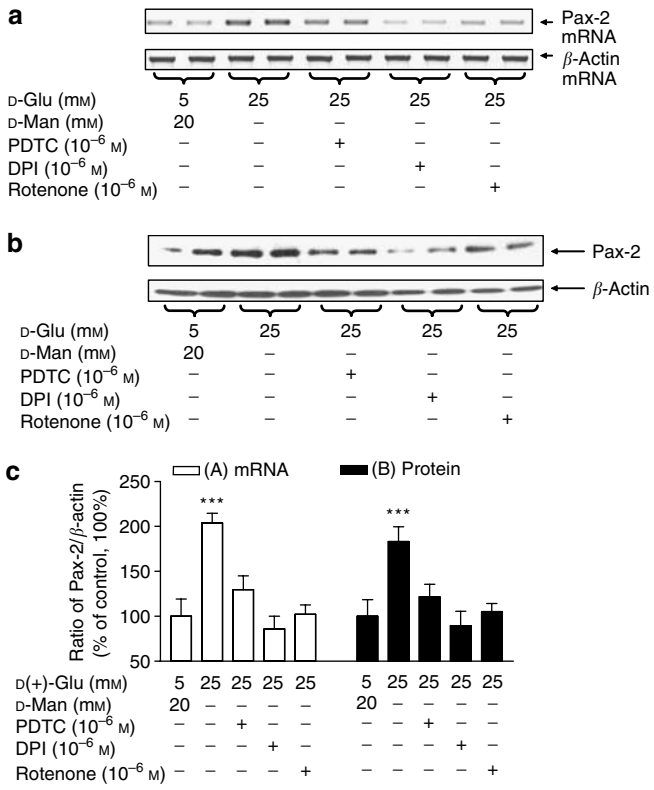


**Figure 6 | Inhibitory effect of ROS and NF-κB inhibitors.** The inhibitory effect of PDTC, DPI, and rotenone on Pax-2 gene expression upregulated by high glucose in MK4 cells analyzed by (**a**) RT-PCR and (**b**) Western blot; quiescent cells were incubated in either 5 mM or 25 mM D-glucose DMEM containing 1% dFBS for 24 h with or without inhibitors; (**c**) the relative densities of Pax-2 were compared with β-actin. The normalized Pax-2 level in cells incubated in 5 mM glucose was considered the control (100%). Each point represents the mean ± s.d. of three independent experiments. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.005$.

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.

## MATERIALS AND METHODS

### Reagents

Normal glucose (5 mM D-glucose DMEM (Cat. No. 12320) was purchased from Invitrogen Inc. (Burlington, Ontario, Canada). D(+)-glucose, L-glucose, D-mannitol, 2-deoxy-D-glucose, DPI, rotenone, GF109203X, PDTC, xanthineoxidase, 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, New York, NY, USA),[50,51] 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).[20,52] MK4 cells were from Dr S Steven Potter (Division of Developmental Biology, Children's Hospital Medical Center, Cincinnati, OH, USA).[53] Dominant negative IκBα (DN IκBα) plasmid (pcDNA3.1/DN IκBa) from Dr John SD 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.

dFBS, depleted of endogenous steroid and thyroid hormones was prepared by incubation with 1% activated charcoal and 1% AG 1 × 8 ion-exchange resin (Bio-Rad Laboratories Inc., Richmond, CA, USA) for 16–24 h at room temperature, as described previously.[36,37] Although endogenous steroid and thyroid hormones have been removed from FBS, the purpose of adding 1% dFBS into either 5 mM glucose or 25 mm DMEM after rendering them quiescent with serum free medium is to support the cells during the additional 24 h experimental period until harvested, as demonstrated previously.[36,37]

### Culture of MK4 cells

The MK4 cell line is representative of late embryonic metanephric mesenchyme as it undergoes mesenchymal to epithelial conversion.[53] 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).[53] 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 37°C.

### *Ex vivo* embryonic kidney culture

Embryonic kidneys were isolated from timed pregnant Hoxb7-GFP mice at embryonic stage E16[50,51] under sterile conditions and cultured either in normal glucose or high glucose DMEM supplied with 1% dFBS. These mice specifically express the 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 h 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 70–80% 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 h, cells were processed for immunofluorescence investigation as reported previously.[36,37] Immunofluorescence images were recorded with a Olympus 1 × 71 Microscope (CARSEN, ON, Canada). The images are presented at × 400 original magnification.

### Western blotting

Western blots were performed as in previous studies.[36,37] Briefly, small aliquots (20–50 μl) of homogenized cell sample were subjected to 10% sodiumdodecylsulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride 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.

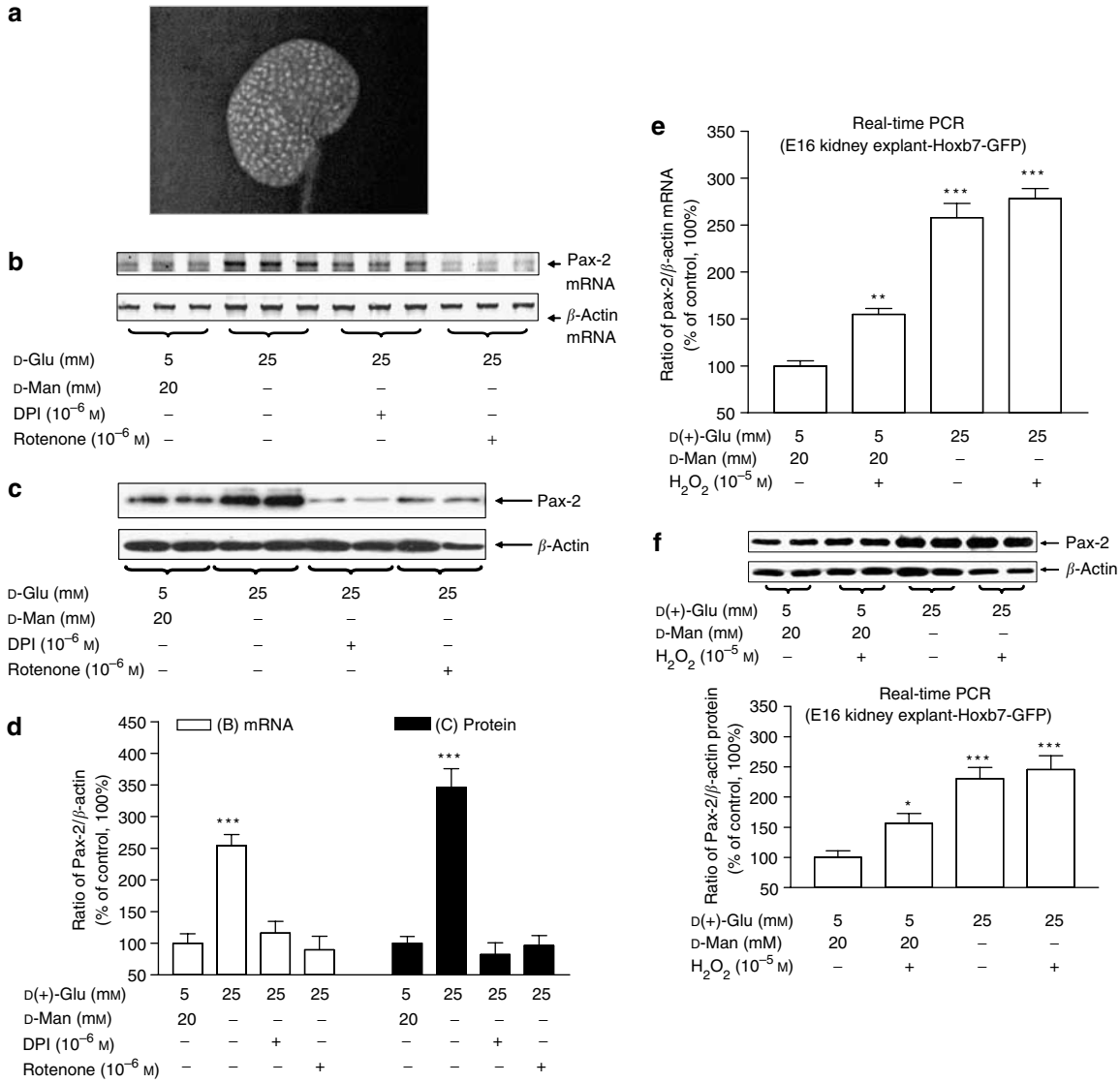


**Figure 7 | High glucose effect on Pax-2 gene expression *ex vivo*.** *Ex vivo* study. Kidney explant isolated from Hoxb7-GFP mice on embryonic day 16 (**a**); the inhibitory effect of DPI and rotenone on Pax-2 gene expression was upregulated by high glucose in renal explant analyzed by (**b**) RT-PCR and (**c**) Western blot, respectively; (**d**) the relative densities of Pax-2 were compared with β-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 (**e**) quantitative RT-PCR, and (**f**) Western blot, respectively. Each point represents the mean ± s.d. of three independent experiments. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.005$.

### ROS generation

ROS production was quantified by the lucigenin method.[44,45] 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 min to 2 h. 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 *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (20 mM), pH 7.4. After washing, the cells were resuspended in Krebs buffer with 1 mg/ml bovine serum albumin, and cell concentration was adjusted to $1 \times 10^7$ in 900 μl 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.).[36,37] 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.[36,37] For internal control, we deployed primers specific for mouse β-actin[54] (forward

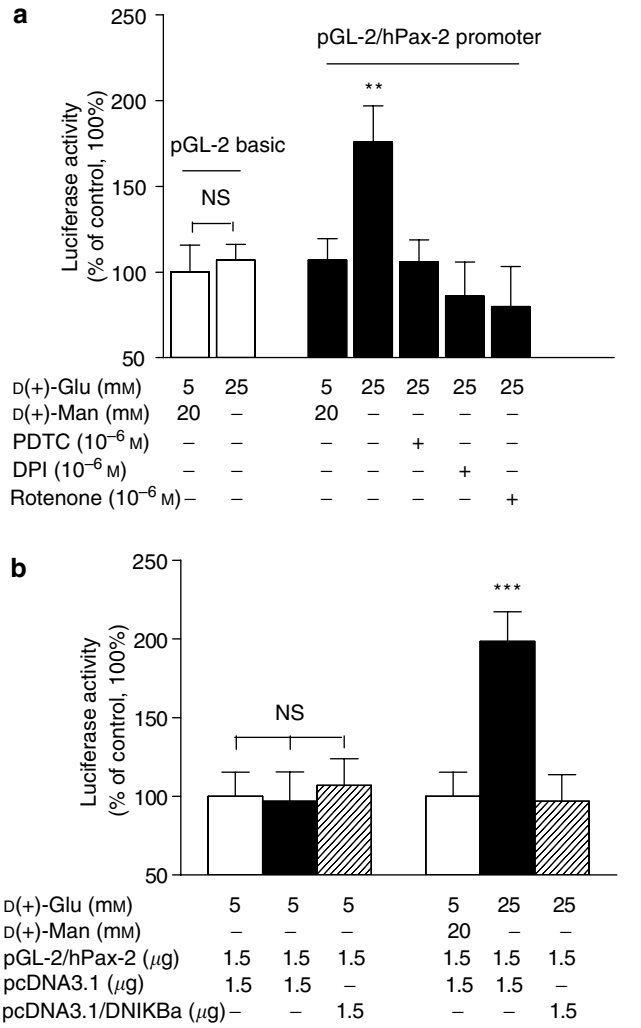


**Figure 8 | High glucose effect on the full length of 5′-franking region of Pax-2 promoter activity in MK4 cells.** (**a**) MK4 cells grown in 12-well plates were transiently transfected with 1.5 $\mu$g of pGL-2/hPax-2 plasmid by Lipofectamine 2000, with pGL-2 basic vector serving as control; DN IKBa effect on high glucose induced Pax-2 promoter activity. (**b**) MK4 cells were transiently cotransfected with 1.5 $\mu$g of each pGL-2/hPax-2 and pcDNA3.1/DNIKBa plasmid by Lipofectamine 2000, whereas pcDNA3.1 plasmid serving as the control. Promoter activity was measured by luciferase assay kit with *renilla luciferase* as an internal control after 24 h 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 $\pm$s.d. of three independent experiments. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.005$.

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 $\beta$-actin cDNA (X03672)).

### Real-time-PCR (quantitative RT-PCR)

Quantitative RT-PCR were performed as in previous studies.[36] In brief, first-strand cDNA was produced from 2 $\mu$g 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°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 threshold cycle value was measured to determine starting copy number of target genes using the standard curve. Lower value of threshold cycle 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 $\beta$-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 $\beta$-actin cDNA [NM_007393]).[36]

### 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)[20,52] 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.), whereas pGL-2 basic vector serving as control. I$\kappa$B$\alpha$ is an inhibitor of NF-$\kappa$B. To study the effect of DN I$\kappa$B$\alpha$ on high glucose induced Pax-2 promoter activity, we transiently cotransfected both plasmids: pGL-2/hPax-2 and pcDNA3.1/DN IκBa, into MK4 cells, whereas both pGL-2 and pcDNA3.1 basic vector served as controls. MK4 cells grown in 12-well plates were transfected with 1.5 $\mu$g of each plasmid. After a 24 h 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 one-way analysis of variance followed by the Bonferroni test as appropriate. Three to four separate experiments were performed for each protocol. Data are expressed as means $\pm$s.d. A probability level of $P \leqslant 0.05$ was considered statistically significant.

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