

# Maternal diabetes modulates kidney formation in murine progeny: the role of hedgehog interacting protein (HHIP)

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## Abstract

**Aims/hypothesis** We hypothesised that maternal diabetes impairs kidney formation in offspring via augmented expression of hedgehog interacting protein (HHIP). Our gene-array results were performed in neonatal kidneys from our murine model of maternal diabetes and indicated that *Hhip* expression was significantly modulated by maternal diabetes.

**Methods** We systematically examined the functional role of HHIP in kidney formation in our murine maternal diabetes model and elucidated the potential mechanisms related to dysnephrogenesis in vitro.

**Results** The kidneys of the offspring of diabetic dams, compared with those of the offspring of control non-diabetic dams, showed retardation of development—small kidneys and less ureteric bud (UB) branching morphogenesis. Augmented HHIP expression was observed in the offspring of diabetic dams, initially localised to differentiated metanephric mesenchyme and UB epithelium and subsequently in maturing glomerular endothelial and tubulointerstitial cells. The heightened HHIP targeting TGF- $\beta$ 1 signalling was associated with dysmorphogenesis. In vitro, HHIP overexpression decreased sonic hedgehog and paired box gene 2 proteins (SHH and PAX2, respectively) and increased transcriptional nuclear factor-kappa B (NF $\kappa$ B, p50/p65), phosphorylation of p53, and TGF- $\beta$ 1 expression. In contrast, overexpression of PAX2 inhibited HHIP and NF $\kappa$ B and activated SHH, N-myc and p27<sup>Kip1</sup> expression. Moreover, high glucose stimulated HHIP

expression, and then targeted TGF- $\beta$ 1 signalling. Thus, PAX2, via a negative autocrine feedback mechanism, attenuated the stimulatory effect of high glucose on HHIP expression.

**Conclusions/interpretation** Maternal diabetes modulates kidney formation in young progeny mediated, at least in part, via augmented HHIP expression.

**Keywords** *Hhip* gene expression · Maternal diabetes · Nephrogenesis

## Abbreviations

Con-offspring	Offspring of non-diabetic dams
DBA	<i>Dolichos biflorus</i> agglutinin
DHH	Desert hedgehog
Dia-offspring	Offspring of diabetic dams
GFP	Green fluorescent protein
HHIP	Hedgehog interacting protein
IHH	Indian hedgehog
Ins-offspring	Offspring of diabetic dams treated by insulin implantation
IUGR	Intrauterine growth restriction
MM	Metanephric mesenchyme
NF $\kappa$ B	Nuclear factor-kappa B
PAX2	Paired box gene 2 protein
qPCR	Quantitative PCR
SHH	Sonic hedgehog protein
STZ	Streptozotocin
Tg	Transgenic
UB	Ureteric bud

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## Introduction

Renal malformations are responsible for 34–59% of chronic kidney disease in children and for 31% of paediatric end-stage

renal disease in the USA [1–3]. Of the many factors associated with congenital abnormalities of the kidney and urinary tract, diabetes mellitus during gestation seems to be one of the key players in both human and experimental settings [4]. For example, when the normal pattern of kidney formation is interrupted in both humans and experimental animal models (e.g. by a high ambient in utero glucose level), kidney abnormalities with a low total nephron number may ensue, either in isolation or as part of multiple malformation syndromes. The incidence of such anomalies appears to be proportional to the degree of maternal hyperglycaemia [5–7]. The underlying mechanisms of such findings are incompletely delineated [6, 8, 9].

We previously reported that severe maternal diabetes (maternal blood glucose concentration >30 mmol/l) in a murine model (C57/BL6 background) impairs kidney formation and is associated with an intrauterine growth restriction (IUGR) phenotype in the offspring. Affected pups have relatively small kidneys/body weight with ~40% fewer nephrons [6, 10, 11]. We performed screening gene profiling of IUGR neonatal kidneys from offspring of dams with diabetes vs kidneys from offspring of control non-diabetic dams (Affymetrix GeneChip Mouse Gene 1.0ST array platform, Affymetrix, Santa Clara, CA, USA), aiming to identify the novel proteins or signalling pathways through which maternal diabetes impairs nephrogenesis.

We analysed our ontology database data using the following: the Affymetrix Expression Console ([www.affymetrix.com](http://www.affymetrix.com)) to normalise the gene database; the Robust Multi-Array Analysis (RMA) algorithm (Partek, St. Louis, Missouri, USA, [www.partek.com](http://www.partek.com)) to compute expression values; a TM4 MultiArrayViewer (Boston, MA, USA, [www.tm4.org](http://www.tm4.org)) to analyse differential expression ( $p$  value threshold of 0.01 and false discovery rate of 0.05). Of the genes showing significant differences between the groups in our murine maternal diabetes model, *Hhip* (fold change, 1,536;  $p=0.007$ ) was upregulated in the neonatal kidneys of offspring from diabetic dams but was expressed normally in insulin-treated dams, as it is highly related to development, signal transduction and proliferation in the analysis of functional pathway clustering.

*Hhip*, which encodes hedgehog interacting protein (HHIP), a transmembrane glycoprotein, was discovered by screening a mouse cDNA expression library for proteins that bound to sonic hedgehog protein (SHH) [12]. As a putative antagonist of the hedgehog signal pathway, HHIP binds to proteins from three mammalian hedgehogs—SHH, Indian hedgehog (IHH) protein and desert hedgehog (DHH) protein—to attenuate their bioactivity [12]. Of the three mammalian hedgehog genes, only expression of *Shh* and *Ihh* has been reported in the developing murine kidney. However, the absence of a renal phenotype in *Ihh*-deficient mice indicates that it might not have a key role in kidney development [13]. SHH

signalling optimally controls the expression of three distinct classes of genes expressed in the nephrogenic zone that are required for normal renal development: early kidney inductive and patterning genes (*Pax2*, *Sall1*); cell-cycle modulators (*Ccnd1*, *Mycn*) and the signalling effectors of the hedgehog pathway (*Ci* homologues, *Gli1*, *Gli2*) [14].

The protein encoded by *Hhip* is expressed in vascular endothelial cells [15] and in cells adjacent to those expressing SHH, positioning it to bind to the SHH [12]. To date, although evidence indicates that cellular expression of HHIP inhibits the activation of SHH signalling and promotes angiogenesis during the formation of a variety of tumours [15, 16], the functional role of *Hhip* expression in developing and mature kidney is incompletely understood.

In the present studies, we first validated the changes in HHIP expression in pregnancies complicated with diabetes and then investigated whether ectopic *Hhip* gene upregulation induced by maternal diabetes could interfere with SHH-targeting gene signalling [14]. We also investigated whether upregulation of HHIP had an impact on kidney formation from birth to 3 weeks in young offspring since in rodents nephrogenesis continues until postnatal day 10 (i.e. murine gestational period between 19 and 21 days) [5]. We then examined potentially involved mechanisms using both in vivo and in vitro studies.

## Methods

**Animal models** We used the *Hoxb7*-green fluorescent protein (GFP) transgenic (Tg) (*Hoxb7*-GFP-Tg, C57/BL6 background) mouse for the current study; this mouse is fertile with a normal phenotype at birth and during adult life [6, 7]. *Hoxb7*-GFP-Tg mice (GFP expression specifically in ureteric bud [UB] driven by *Hoxb7* promoter), provided by F. Costantini (Columbia University Medical Center, New York, NY, USA) [17, 18], were engineered to allow UB branching morphogenesis to be visualised in real time in vivo as reported previously [6, 7].

As reported previously, we used a murine maternal diabetes model [6, 7, 11] induced by a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich Canada, Oakville, ON, Canada) at a dose of 150 mg/kg of body weight at E13 gestation age. Neonatal kidneys of offspring from three groups of dams (non-diabetic, diabetic and diabetic dams treated by insulin implantation at E15 [Linshin, Scarborough, ON, Canada]) were used.

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

**Cell lines** The MK4 cell line [11, 19] was obtained from S. S. Potter (Children's Hospital Medical Center, Cincinnati, OH, USA) and is representative of late embryonic metanephric mesenchyme (MM), as it undergoes mesenchymal-to-epithelial conversion. MK4 cells express genes typical of late MM, including *Pax2*, *Pax8*, *Wnt4*, *Cdh6*, collagen IV and *Lfb3* (also known as *Hnf1*) [20]. Mouse UB tip cells were provided by J. Barasch (Columbia University, New York, NY, USA) and were generated from microdissected UBs of E11.5 mouse embryos transgenic for simian virus 40 large T antigen (Immorto-mouse) and which express epithelial and ureteric genes (*Ret*, *Met*) without hepatocyte growth factor [21]. The plasmids pcDNA3.1/Hhip and pcDNA3.1/Pax2 were gifts from A. McMahon (Harvard University, Cambridge, MA, USA) [12] and P. Goodyer (McGill University, Montreal, QC, Canada) [22], respectively.

We used both MK4 and UB tip cells to represent the MM and UB epithelium, respectively, and examined *Hhip* gene expression and HHIP regulation with or without a high glucose (25 mmol/l D-glucose) insult in MK4 cells. Unless otherwise indicated, all control cells were incubated in low-glucose (5 mmol/l D-glucose) medium containing 20 mmol/l D-mannitol to ensure equi-osmolar control.

**Counting of UB tips and measurement of neonatal kidney size** Neonatal kidneys of offspring from three groups of dams (non-diabetic, diabetic and diabetic dams treated by implanted insulin) were dissected aseptically and the number of UB tips in each group was assessed as reported previously [7]. Neonatal kidney size (length [mm], width [mm] and area [mm<sup>2</sup>]) was measured from the two-dimensional GFP images of freshly isolated neonatal kidneys using Image-J 1.48 (NIH, USA, <http://imagej.nih.gov/ij/>).

**Real-time quantitative PCR** Real-time quantitative PCR (qPCR) (the Fast SYBR green mastermix kit and the 7500 Fast real-time PCR system; Applied Biosystems, Life Technologies, Foster City, CA, USA) was performed as reported previously [6, 7, 11]. The final real-time qPCR product was also reviewed by Typhoon Trio Variable Mode Imager (GE Health Care, Bio-Sciences, Uppsala, Sweden). The following primers were used: *Hhip* forward 5'-CCCATCGGCTCTTCATTCTA-3', reverse 5'-CCTTTCGTCTCCTCCCTTTA-3' (NM\_020259.4).

**Western blot, immunohistochemistry and immunofluorescence** Western blot analysis, immunohistochemical staining and immunofluorescence were performed essentially according to an established procedure as reported previously [6, 7, 11, 19]. The following antibodies were used: monoclonal anti-HHIP and  $\beta$ -Actin antibodies (Sigma-Aldrich Canada); *Dolichos biflorus* agglutinin (DBA)-FITC (Vector Laboratories, Burlington, ON, Canada) staining for UB identification [7]; anti-SHH, -nuclear

factor-kappa B (NF $\kappa$ B) (p50/p65), -N-myc, -CD31 and -p53 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-paired box gene 2 protein (PAX2) antibody (Covance, Richmond, CA, USA); anti-phospho-p53 (Ser 15) (Abcam, Cambridge, MA, USA); anti-p27<sup>Kip1</sup> (BD Transduction Laboratories, Mississauga, ON, Canada); anti-TGF- $\beta$ 1 antibody (R&D Systems, Burlington, ON, Canada); phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) antibody (New England Biolabs, Whitby, ON, Canada) and Smad2/3 antibody (Cedarlane-Millipore, Burlington, ON, Canada).

**Statistical analysis** Statistical significance between the experimental groups was analysed by one-way ANOVA, followed by the Bonferroni test, using Graphpad Software, Prism 5.0 ([www.graphpad.com/prism/Prism.htm](http://www.graphpad.com/prism/Prism.htm)). Three or four separate experiments were performed for each protocol. Data are expressed as means $\pm$ SD. A probability level of  $p \leq 0.05$  was considered to be statistically significant.

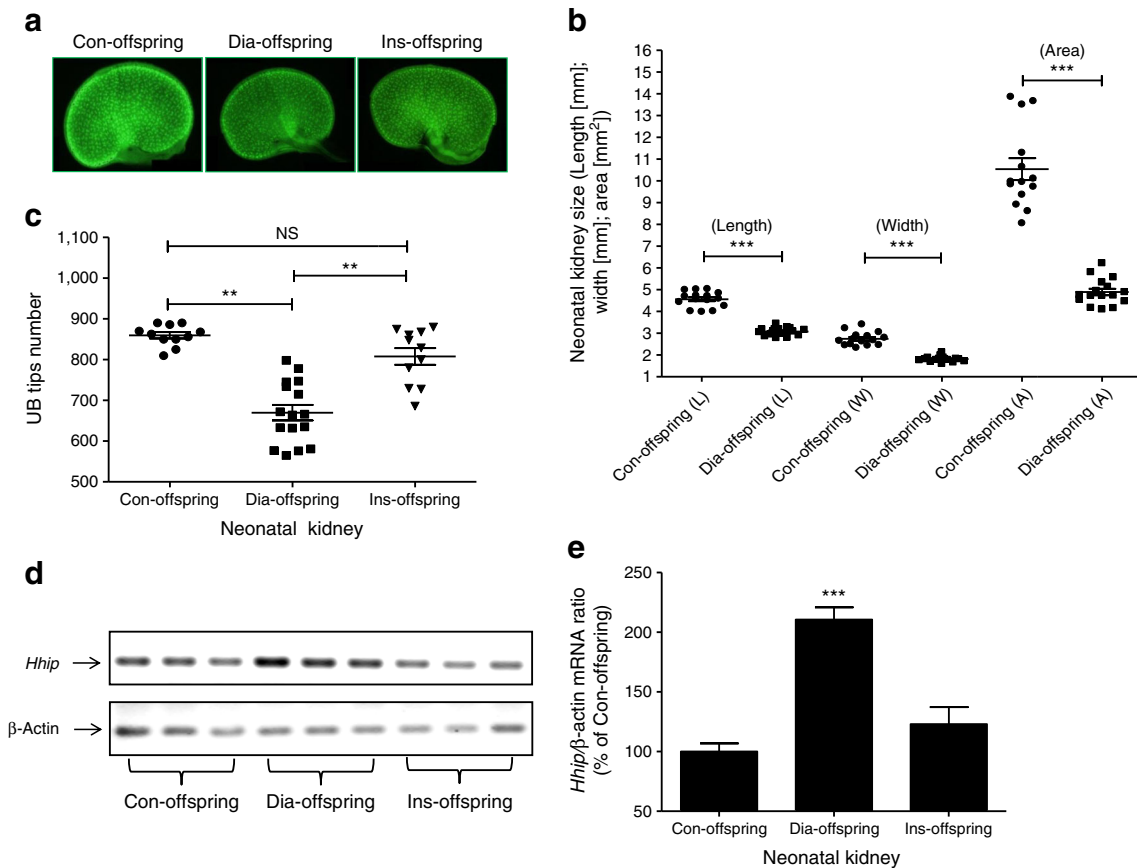
## Results

**UB branching morphogenesis** We compared UB branching morphogenesis in neonatal kidneys of three subgroups of Hoxb7-GFP-Tg murine lines' offspring (Con-offspring, the offspring of non-diabetic dams; Dia-offspring, the offspring of diabetic dams; Ins-offspring, the offspring of diabetic dams treated by insulin implantation) (Fig. 1a). At birth, maternal blood glucose concentration (mmol/l) was as follows: non-diabetic dams, 9.03 $\pm$ 1.75; diabetic dams, 29.36 $\pm$ 2.54; diabetic dams treated by insulin implantation, 15.8 $\pm$ 1.36.

Compared with Con-offspring, Dia-offspring with IUGR phenotype had smaller neonatal kidney/body weight ratio as previously reported [6], smaller kidney size (Fig. 1b) and fewer UB tips (Con-offspring, 859.8 $\pm$ 7.74; Dia-offspring, 669.9 $\pm$ 18.96) (Fig. 1c). The impairment of UB branching was ameliorated by insulin administration to the mothers (Ins-offspring, 807.7 $\pm$ 20.52) (Fig. 1c).

**Hhip gene expression and localisation in vivo** We subsequently validated and confirmed *Hhip* mRNA expression by real-time qPCR (Fig. 1d, e) and HHIP protein expression by immunohistochemistry (Fig. 2a) and immunofluorescence staining (Fig. 2b). Consistent with the data obtained from our prior microarray analysis, HHIP expression was significantly increased in the neonatal kidneys of IUGR Dia-offspring.

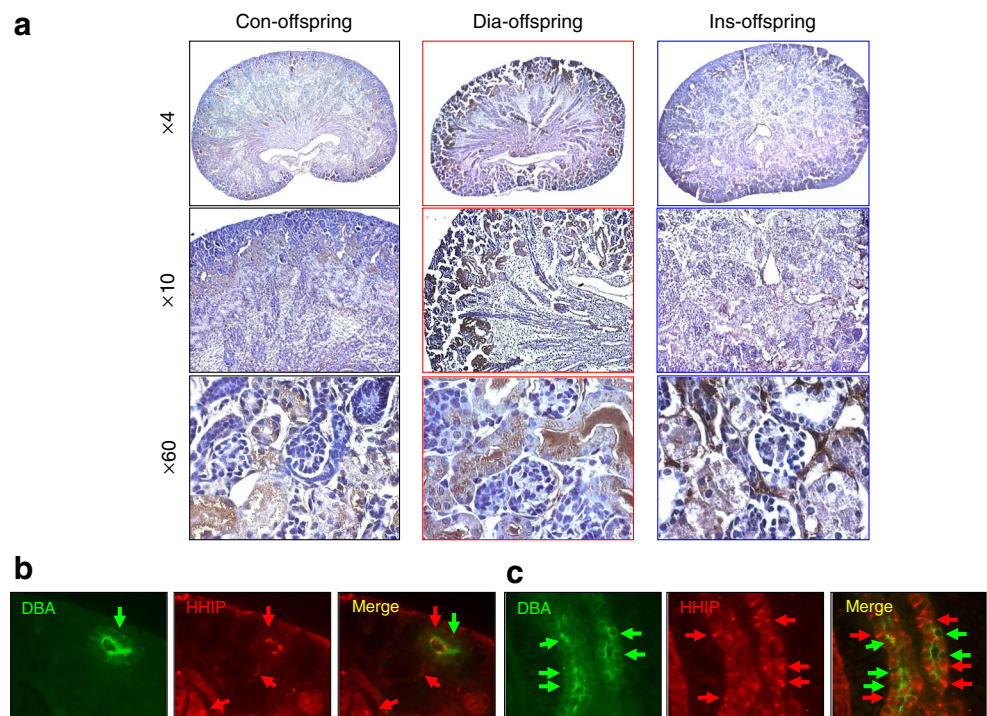
We next followed the time course of intrarenal HHIP expression and its localisation pattern during late kidney formation in the offspring (from day 1 to 3 weeks). In the kidneys of Con-offspring, HHIP expression was detected in both UB and MM lineages on day 1 (Fig. 2) and in the collecting duct system at 1 week (Fig. 3). After kidney formation was



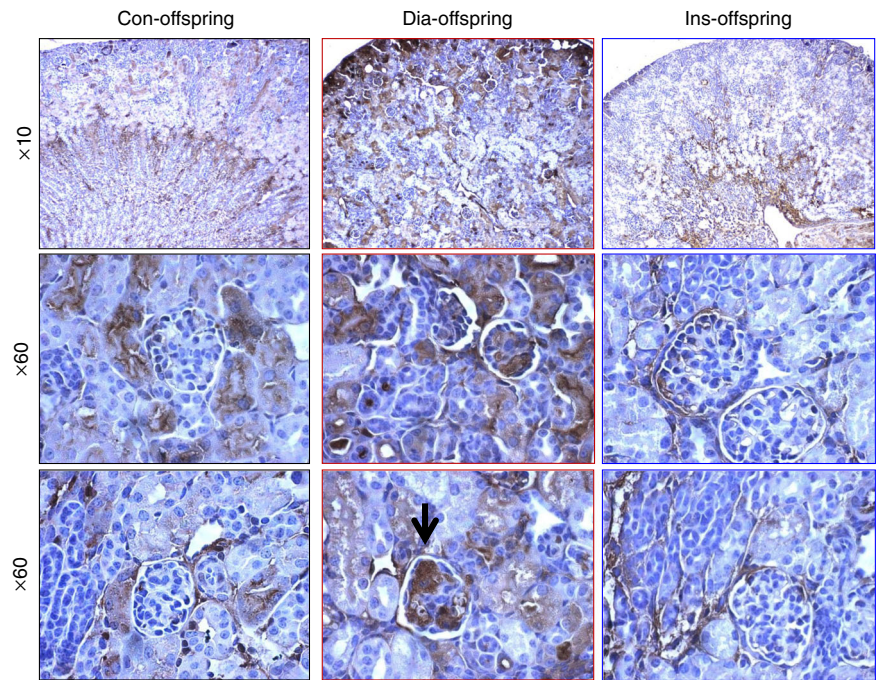
**Fig. 1** (a) GFP images of freshly isolated neonatal kidney (magnification  $\times 4$ ). (b) Neonatal kidney size; Length, L; Width, W; Area, A. (c) The number of UB tips in neonatal kidney. Circles, Con-offspring; squares, Dia-offspring; inverted triangles, Ins-offspring. (d, e) Real-time qPCR analysis of *Hhip* mRNA expression in neonatal kidney. (d) Typhoon gel

image. (e) The relative density of *Hhip* mRNA was compared with its own  $\beta$ -actin mRNA. Con-offspring values were considered as 100%. Each point represents the mean  $\pm$  SD of three independent experiments.  $**p \leq 0.01$ ;  $***p \leq 0.001$

**Fig. 2** (a) Immunohistochemical staining showing HHIP expression in neonatal kidneys. Black frame, Con-offspring; red frame, Dia-offspring; blue frame, Ins-offspring (magnification  $\times 4$ ,  $\times 10$  and  $\times 60$ , as shown). (b, c) Co-localisation, by immunofluorescence, of HHIP (red arrows) and DBA (green arrows) as well as HHIP/DBA merge in UB tips in renal cortex (b) and UB trunks in renal medulla (c) of the neonatal kidneys of Dia-offspring (magnification  $\times 60$ )



**Fig. 3** Immunohistochemical staining showing HHIP expression in 1-week-old kidneys. Black frame, Con-offspring; red frame, Dia-offspring; blue frame, Ins-offspring (magnification  $\times 10$  and  $\times 60$ ). The glomerular nodular HHIP expression is indicated by the arrow head

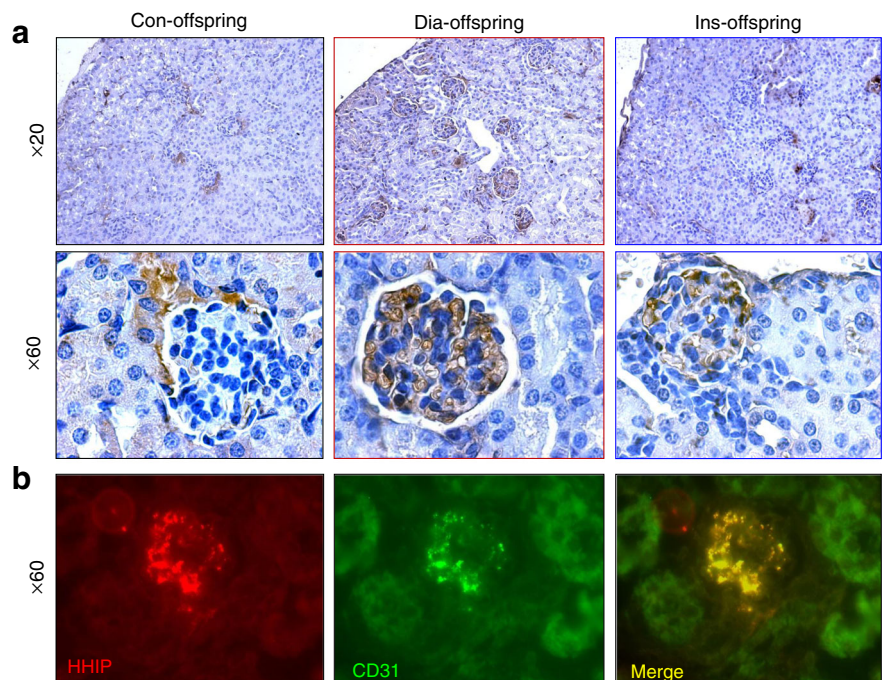


completed (3 weeks), very limited HHIP expression was observed and was localised to mature glomerular endothelial and adjacent tubulointerstitial cells (Fig. 4).

In contrast, the enhanced HHIP expression remained in the nephrogenic zone and was detected in both UB and MM epithelium in the kidneys of Dia-offspring (Fig. 2a). An increased amount of secreted HHIP and intracellular HHIP was detected adjacent to UB tips (Fig. 2b) and UB trunks (Fig. 2c) (DBA-FITC staining for UB identification [7]) in the

neonatal kidneys. By 1 week of age, this increased HHIP expression was progressively evident in glomeruli, ultimately forming a nodular expression pattern (Fig. 3). At 3 weeks, the Dia-offspring had only small amounts of HHIP in the tubulointerstitium (Fig. 4a) but HHIP was strongly expressed in glomerular endothelial cells in the neonatal kidney cry-sections, as assessed by immunofluorescence (confirmed by co-localisation with CD31, a marker of endothelial cells) (Fig. 4b). Furthermore, the pattern of HHIP expression in

**Fig. 4** HHIP expression in 3-week-old kidneys. (a) Immunohistochemical staining showing HHIP. Black frame, Con-offspring; red frame, Dia-offspring; blue frame, Ins-offspring (magnification  $\times 20$  and  $\times 60$ ). (b) Co-localisation, by immunofluorescence, of HHIP (red) and CD31 (green), as well as HHIP/CD31 merge (yellow), in 3-week-old kidneys of Dia-offspring (magnification  $\times 60$ )

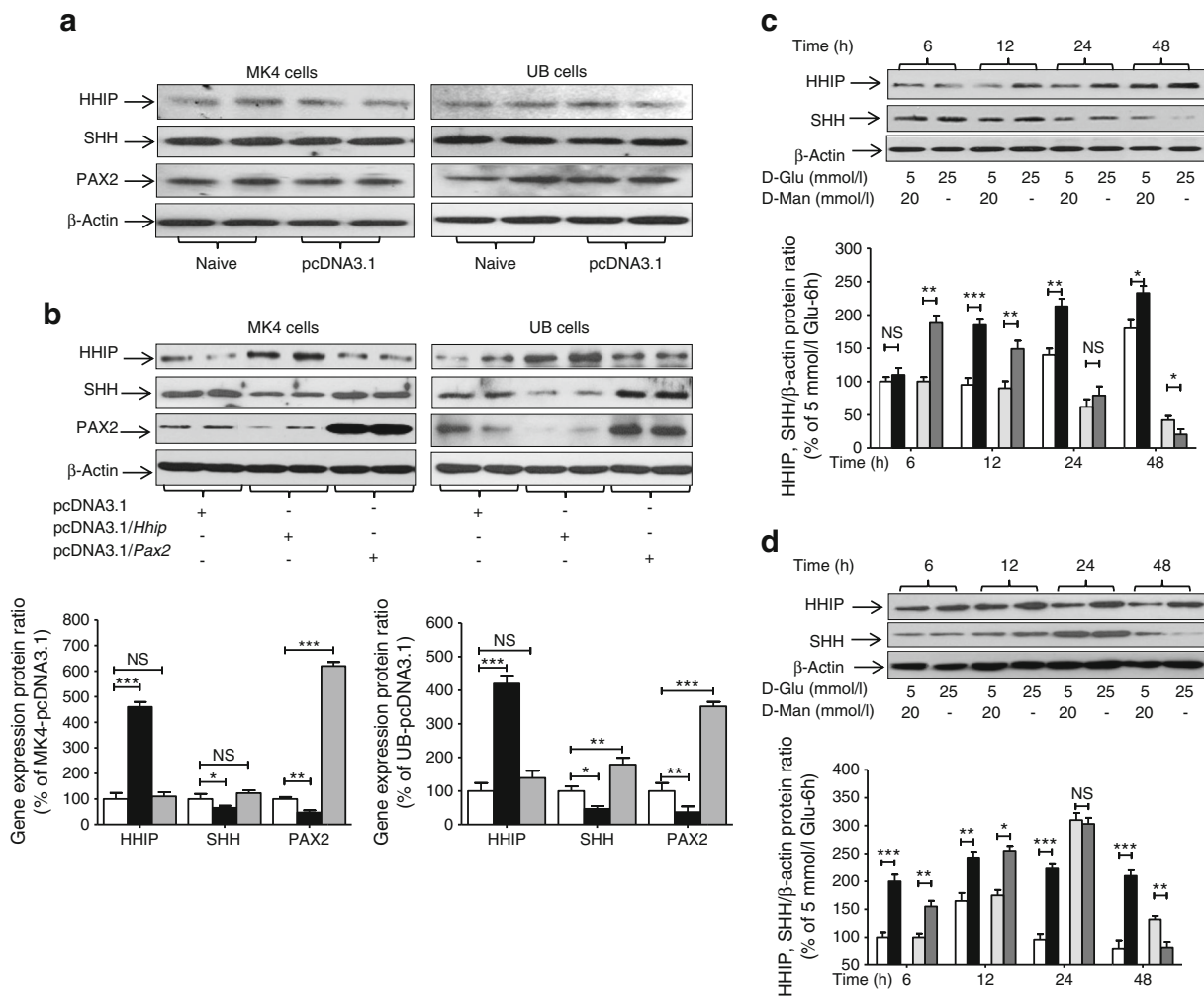


the kidneys of Ins-offspring was essentially the same as that in the Con-offspring.

**HHIP expression and regulation in vitro** Western blotting showed that HHIP, SHH and PAX2 were basally expressed in both MK4 and UB cells as well as in cells transfected with the empty vector plasmid, pcDNA3.1 (1 µg/ml) (Fig. 5a), a control for the rest of transfection experiments. Overexpression of *Hhip* by transient transfection of pcDNA3.1/*Hhip* (1 µg/ml) into both MK4 and UB tip cells (Fig. 5b) inhibited both SHH and PAX2 expression. Conversely, overexpression of PAX2 by transient transfection with pcDNA3.1/*Pax2* (1 µg/ml) increased SHH and decreased HHIP expression (Fig. 5b).

We tested the effect of a high-glucose milieu (25 mmol/l D-glucose) on HHIP and SHH expression in MK4 (Fig. 5c) and UB tip cells (Fig. 5d). High glucose persistently stimulated HHIP expression in a time-dependent manner, whereas SHH expression was only initially increased by high glucose within 24 h, then decreased rapidly after 48 h in both cell lines (Fig. 5c, d).

We then performed studies using the MK4 cell line, as it is derived from MM lineages, to examine high-glucose milieu regulation on HHIP expression, since we had noted that the augmented HHIP expression in the offspring of dams with diabetes was seen in the developing proximal portion of the nephron, which is mainly derived from differentiated MM lineages. High glucose (25 mmol/l D-glucose) stimulated



**Fig. 5** HHIP, PAX2 and SHH protein expression analysed by western blot. **(a)** Basal expression in naive MK4 and UB cells as well as the cells transfected with pcDNA3.1 plasmid (1 µg/ml). **(b)** MK4 and UB tip cells were transfected with the plasmids pcDNA3.1 (white bars), pcDNA3.1/*Hhip* (black bars) and pcDNA 3.1/*Pax2* (grey bars) at 1 µg/ml, respectively; the gene expression protein ratio in cells transfected with pcDNA3.1 plasmid was considered to be 100%. **(c, d)** The time course of high glucose effect on HHIP and SHH expression in naive MK4 cells

**(c)** and UB tip cells **(d)** was analysed by western blot using 20 mmol/l D-mannitol (D-Man) as an osmotic control (white bars, 5 mmol/l D-glucose [D-Glu]/HHIP; black bars, 25 mmol/l D-glucose/HHIP; light-grey bars, 5 mmol/l D-glucose/SHH; dark-grey bars, 25 mmol/l D-glucose/SHH). The gene expression protein ratio in 5 mmol/l glucose medium at the 6 h incubation period were considered to be 100%. Each point represents the mean ± SD of three independent experiments. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

HHIP expression in a time- and dose-dependent manner in naive MK4 cells but other glucose analogues such as L-glucose, D-mannitol and 2-deoxy-D-glucose did not (Fig. 6a–c). We also observed that insulin ( $10^{-6}$  mol/l) inhibited the stimulatory effect of high glucose on *Hhip* gene expression (as assessed by western blotting of HHIP) in MK4 cells (Fig. 6d).

We used MK4 cells transfected with the plasmid, pcDNA3.1 (MK4-vector) (1  $\mu$ g/ml) as controls, since the basal expression in naive and transfected MK4 cells were essentially identical (Fig. 5). A high-glucose milieu stimulated transcriptional NF $\kappa$ B (p50/p65) and phosphorylation of p53 in MK4-vector cells. Overexpression of *Hhip* in MK4 cells alone significantly increased NF $\kappa$ B (p50/p65) and p53 expression, including phosphorylation of p53 (Fig. 7a), and this activation was further enhanced by exposure to high glucose. In contrast, overexpression of *Pax2* in MK4 cells significantly decreased HHIP and NF $\kappa$ B (p50/p65) expression; further, this inhibition was unaffected by high glucose (Fig. 7b). In addition, overexpression of *Pax2* in MK4 cells resulted in significantly increased SHH, N-Myc and p27<sup>Kip1</sup> expression and high-glucose exposure had no additional effect.

**TGF- $\beta$ 1 expression in vivo and in vitro** In vitro, we observed overexpression of *Hhip* in MK4 cells resulted in increased TGF- $\beta$ 1 and phosphorylated Smad2/3 expression and that

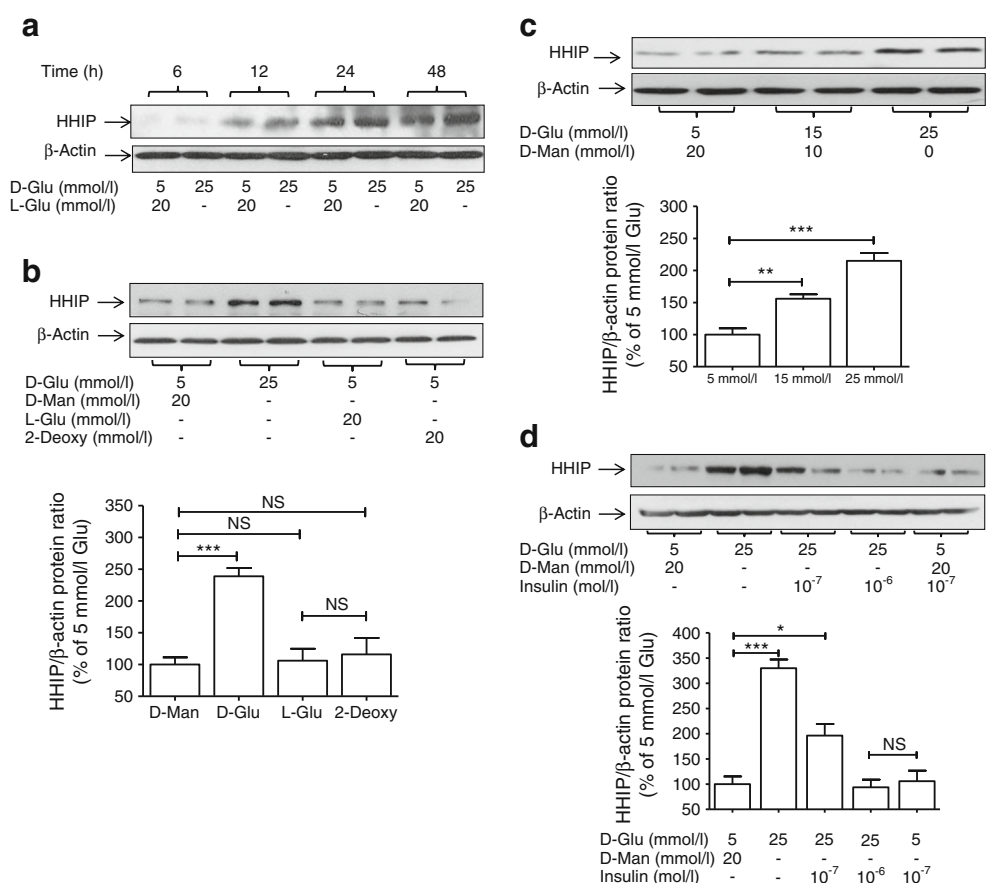
this activation was further enhanced by high-glucose media (Fig. 8a). Recombinant TGF- $\beta$ 1 upregulated HHIP and phosphorylated Smad2/3, but downregulated p27<sup>Kip1</sup> expression in a dose-dependent manner in naive MK4 cells (Fig. 8b). In vivo studies validated these findings—TGF- $\beta$ 1 expression was significantly increased in the kidneys of Dia-offspring from day 1 to 3 weeks of age (Fig. 8c). The heightened TGF- $\beta$ 1 expression displayed a similar immunohistochemical pattern to HHIP in mature glomerular endothelial and adjacent tubulointerstitial cells (Fig. 8d).

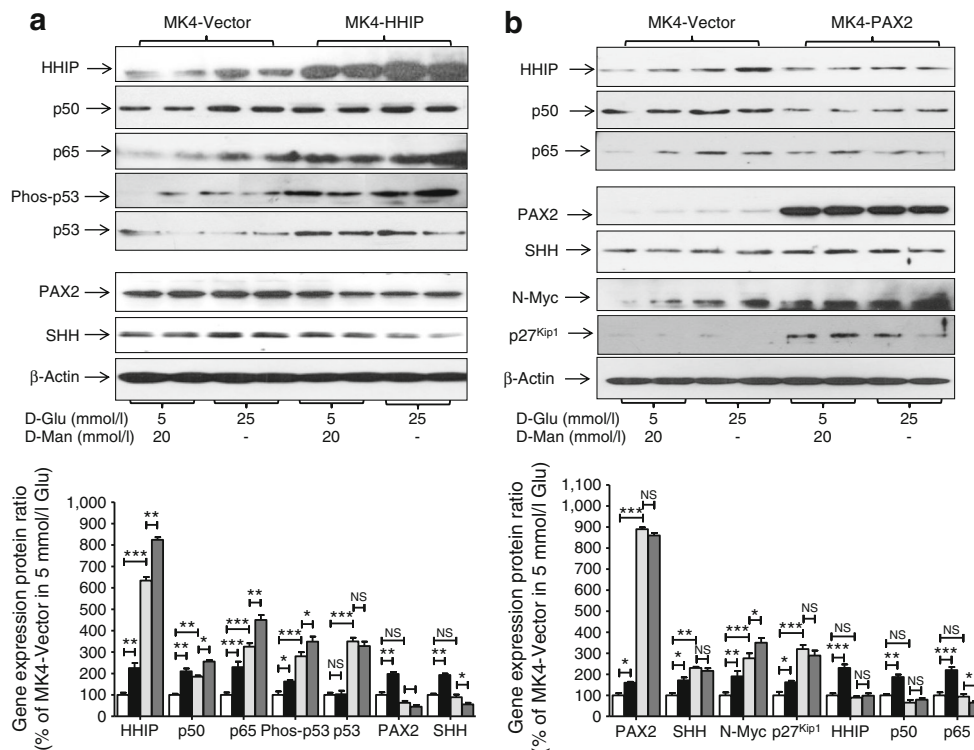
## Discussion

The present study suggests that heightened ectopic HHIP expression targets NF $\kappa$ B, p53 and TGF- $\beta$ 1 expression, which is associated with dysmorphogenesis in young Dia-offspring. We would suggest that overexpression of *Pax2* may create a negative autocrine feedback on HHIP regulation and may attenuate the stimulatory effect of high glucose on *Hhip* expression in vitro.

Using the *Hoxb7*-GFP-Tg mouse model to permit visualisation of UB branching morphogenesis in neonatal kidneys, which continue to form nephrons after birth, we previously

**Fig. 6** Effects of high D-glucose (25 mmol/l, D-Glu) on HHIP expression in naive MK4 cells. **(a)** Time course analysed by western blot using 20 mmol/l L-glucose (L-Glu) as an osmotic control. **(b)** Effect of the glucose analogues D-glucose (D-Glu), L-glucose (L-Glu), D-mannitol (D-Man) and 2-deoxy-D-glucose (2-Deoxy), on HHIP expression analysed by western blot. **(c)** Dose-dependent effect of glucose analysed by western blot. **(d)** Insulin's inhibitory effect analysed by western blot. The gene expression protein ratio in 5 mmol/l glucose medium with 20 mmol/l D-mannitol at the 24 h incubation period was considered to be 100%. Each point represents the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$





**Fig. 7** Effect of high glucose on different genes of MK4 transfected cells analysed by western blot. **(a)** MK4 cells transfected with the plasmids pcDNA3.1 (1 µg/ml) (MK4-Vector) in D-glucose (D-Glu) 5 mmol/l (white bars) and 25 mmol/l glucose (black bars) or pcDNA3.1/*Hhip* (1 µg/ml) (MK4-*Hhip*) in 5 mmol/l glucose (light-grey bars) and 25 mmol/l glucose (dark-grey bars). **(b)** MK4-Vector in 5 mmol/l glucose (white bars) and 25 mmol/l glucose (black bars); MK4 cells transfected

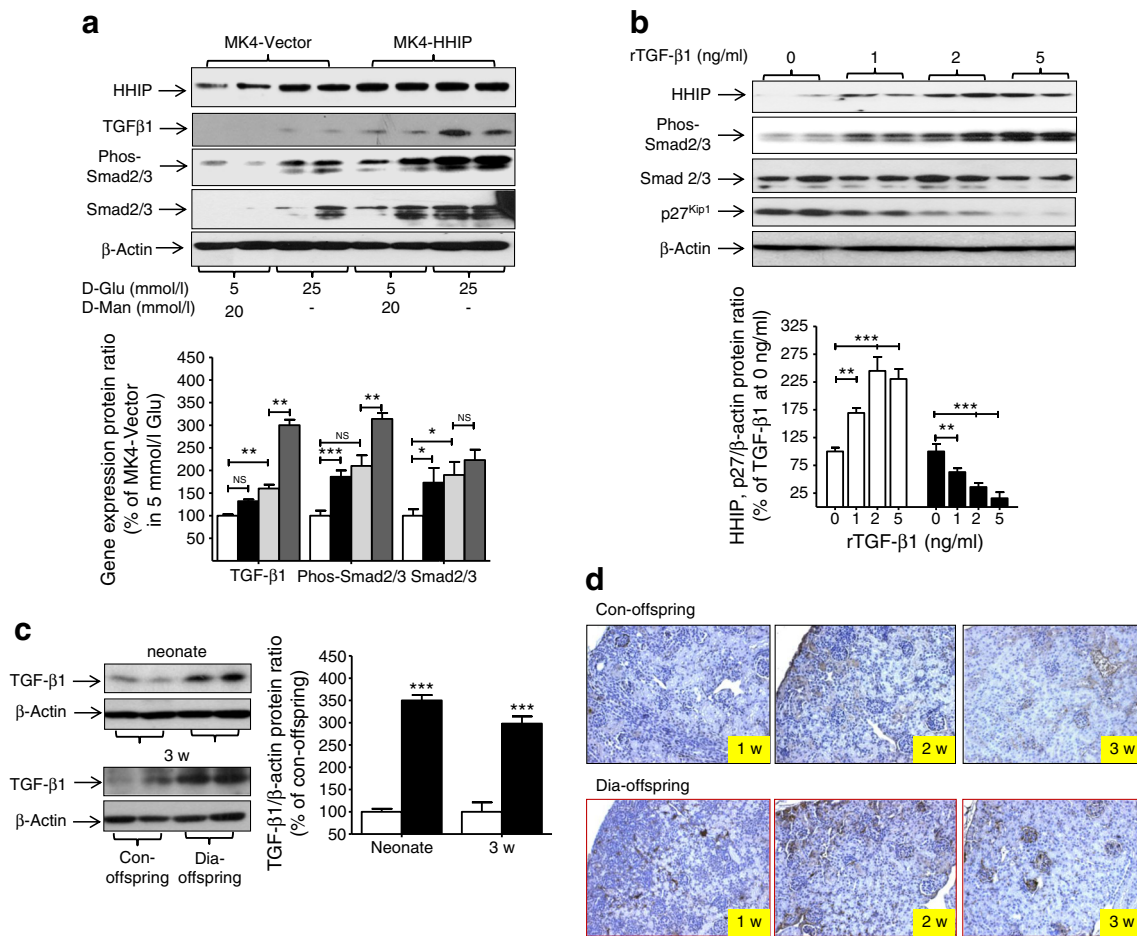
with the plasmid pcDNA3.1/*Pax2* (1 µg/ml) (MK4-*Pax2*) in 5 mmol/l glucose (light-grey bars) and 25 mmol/l glucose (dark-grey bars). The gene expression protein ratio in MK4-Vector cells in 5 mmol/l glucose medium with 20 mmol/l D-mannitol (D-Man) were considered to be 100%. Each point represents the mean ± SD of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

reported that maternal diabetes may impair UB branching morphogenesis. This ultimately impairs kidney formation [6, 11], resulting in renal dysplasia or hypoplasia, likely via increased cell apoptosis or decreased cell proliferation in the differentiated MM and UB epithelium [6, 11]. The underlying mechanisms appear to be associated with impaired mesenchymal-to-epithelial conversion linked to PAX2 and N-myc interaction [6, 7, 19, 22] and/or excessive nascent nephron apoptosis via activation of NFκB (p50/p65) and p53 pathways [6, 11].

Consistent with our previous studies [6, 11] with the same phenotype in neonatal kidneys, we validated the data obtained from our screening microarray analysis and observed that *Hhip* expression was significantly increased in the neonatal kidneys of Dia-offspring. Those data led us to speculate whether the augmented HHIP expression induced by high-glucose milieu either directly or indirectly controls SHH-targeting genes involved in cell proliferation, cell fate and cell communication, influencing processes such as epithelial–mesenchymal transition [23, 24]. We further speculated as to whether or not the increased *Hhip* expression could activate NFκB (p50/p65) and p53 signals and then impact on kidney formation.

During normal kidney development, the balance of HHIP–SHH signalling appears to be critical in the control of a series of sequential events of nephrogenic processes [25, 26]. Upon high glucose insult, for example, in our murine model, the offspring of diabetic dams had a markedly different expression pattern of HHIP when compared with offspring of normal non-diabetic dams. As compared with the limited HHIP expression in Con-offspring (i.e. neonate—UB and MM lineages; age 1 week—collecting duct system), a significant amount of HHIP was detected in the nephrogenic zone of the still developing kidneys of Dia-offspring at the neonatal stage (i.e. secreted HHIP adjacent to UB tips) and at 1 week old (i.e. a nodular expression pattern in glomeruli). These data suggested that HHIP might delay or disorient the usual gradient SHH expression pattern establishment from distal (high) to proximal (low) [25, 26], since HHIP is expressed in cells adjacent to those expressing SHH to antagonise its activity [12]. Our in vitro studies in MK4 and UB tip cells with or without high-glucose media elucidated a potential counterbalance between *Hhip* and *Shh* gene expression in both differentiated UB and MM epithelium, and hint that this interaction is critical in the nephrogenic process. Thus, the examination of the functional role of HHIP during the entire period of





**Fig. 8** HHIP and TGF- $\beta$ 1 expression in vitro (**a**, **b**) and in vivo (**c**, **d**). (**a**) TGF- $\beta$ 1 expression in MK4 cells transfected with the plasmids pcDNA3.1 (1  $\mu$ g/ml) (MK4-Vector) in 5 mmol/l glucose (white bars) and 25 mmol/l glucose (black bars) or pcDNA3.1/*Hhip* (1  $\mu$ g/ml) (MK4-*Hhip*) in 5 mmol/l glucose (light-grey bars) and 25 mmol/l glucose (dark-grey bars). The gene expression protein ratio in MK4-Vector cells in 5 mmol/l glucose medium with 20 mmol/l D-mannitol (D-Man) was considered as 100%. (**b**) The dose-effect of recombinant TGF- $\beta$ 1 (rTGF- $\beta$ 1) on HHIP, phospho-Smad2/3, Smad 2/3 and p27<sup>Kip1</sup>

expression in naive MK4 cells. The gene expression protein ratio in rTGF- $\beta$ 1 (0 ng/ml) was considered as 100%. (**c**) Western blot performed in neonatal and 3-week-old kidneys of Con-offspring and Dia-offspring. Con-offspring values were considered as 100%. (**d**) Immunohistochemical analysis of TGF- $\beta$ 1 expression in 1-, 2- and 3-week-old kidneys of Con-offspring (black frame) and Dia-offspring (red frame) (magnification  $\times$ 20). Each point represents the mean  $\pm$  SD of three independent experiments. \* $p$   $\leq$  0.05; \*\* $p$   $\leq$  0.01; \*\*\* $p$   $\leq$  0.001

nephrogenesis with respect to SHH signalling in different renal cell lineages, as well as in the in utero high-glucose environment, merits further investigation.

Alternatively, the heightened HHIP expression might alter the SHH-controlled expression of kidney formation genes, such as *Pax2*, *Sall1*, *Mycn*, *Ccnd1*, *Gli1* and *Gli2* [14], in the nephrogenic zone, subsequently resulting in the development of renal abnormalities. For instance, *Pax2* as a 'kidney-specific' master gene is expressed in both UB and MM lineages, normally optimising UB branching and mesenchymal-to-epithelial transition during kidney development [27–29]. Mutations or deficiency of *Pax2* cause an increase in cellular apoptosis [30–32], associated with renal hypoplasia [30, 33, 34]. In the current study, we observed that overexpression of *Hhip* significantly downregulated genes such as *Shh* and *Pax2*; in contrast, overexpression of *Pax2* increased *Shh* and

decreased *Hhip* gene expression in both UB and MK4 cells, underscoring the functional interaction between HHIP, SHH and PAX2.

Thus, we focused on a differentiated MM epithelium (i.e. MK4 cells), since the augmented HHIP expression remained in the developing proximal portion of the nephron in the Dia-offspring, which is mainly derived from differentiated MM lineages. We designed further experiments to elucidate whether the excess amount of HHIP with or without high glucose could modulate the mesenchymal-to-epithelial conversion and nascent nephron apoptosis by studying the MM cell proliferation genes (i.e. *Shh*, *Pax2*, *Mycn* and p27<sup>Kip1</sup> genes) and apoptosis genes (i.e. genes encoding NF $\kappa$ B [p50/p65] and p53). Indeed, upon exposure to high D-glucose, but not other glucose analogues, there was specific stimulation of *Hhip* gene expression in a time- and dose-dependent manner.

Insulin appears to inhibit the stimulatory effect of high glucose on *Hhip* gene expression in vitro. Moreover, our current data suggest that PAX2 may be involved in a negative autocrine feedback loop to prevent the high-glucose stimulatory effect on *Hhip* gene expression by promoting MM cell proliferation genes (i.e. *Shh*, *Pax2*, *Mycn* and p27kip1 genes) and inhibiting MM cell apoptosis genes (i.e. genes encoding NFκB [p50/p65] and p53).

In the current study, we observed that the distribution of HHIP expression is similar to that of TGF-β1 and that TGF-β1 expression was significantly increased in young Dia-offspring with dysmorphogenesis occurring, suggesting that there is functional interaction between HHIP and TGF-β1. It is well-known that TGF-β1 decreases renal branching morphogenesis via its negative feedback mechanisms [35, 36]. Compelling evidence suggests that TGF-β1 plays a key role in glomerular endothelial cell damage [37, 38], production of extracellular matrix and development of renal interstitial fibrosis in diabetic nephropathy [39–41]. In studies in vitro, we further demonstrated that the increase of HHIP expression either by overexpression of *Hhip* or by the induction of a high-glucose milieu might target TGF-β1 signalling, which, in turn, may lead to events such as phosphorylation of Smad2/3 and decreases in p27<sup>Kip1</sup> expression.

In conclusion, the present studies suggest that impaired nephrogenesis induced by maternal diabetes is associated with augmented *Hhip* gene expression. HHIP targets TGF-β1 signalling, resulting in kidney formation impairment in young Dia-offspring. PAX2 appears to be involved in a negative autocrine feedback loop on *Hhip* gene expression, attenuating the stimulatory effect of high glucose on *Hhip* gene expression.

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