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ABSTRACT

Background. Intrauterine growth restriction (IUGR) leads to low nephron number and higher incidence of renal disease. We hypothesized that IUGR induces early podocyte alterations based on a dysregulation of Wilms' tumour suppressor gene 1 (WT1), a key player of nephrogenesis and mediator of podocyte integrity.
Methods. IUGR was induced in rats by maternal protein restriction during pregnancy. Kidneys were harvested from male offspring at Days 1 and 70 of life. qRT–PCR, immunohistochemistry and electron microscopy were performed in renal tissue. Albuminuria was assessed by enzyme-linked immunosorbent assay.
Results. At Day 70 of life, higher albuminuria and overt alterations of podocyte ultrastructure were detected in IUGR animals in spite of normal blood pressure. Moreover, we found increased glomerular immunoreactivity and expression of desmin, while synaptopodin and nephrin were decreased. Glomerular immunoreactivity and expression of WT1 were increased in IUGR animals at this time point with an altered expressional ratio of WT1 +KTS and −KTS isoforms. These changes of WT1 expression were already present at the time of birth.
Conclusions. IUGR results in early podocyte damage possibly due to a dysregulation of WT1. We suggest that an imbalance of WT1 isoforms to the disadvantage of −KTS affects nephrogenesis in IUGR rats and that persistent dysregulation of WT1 results in a reduced ability to maintain podocyte integrity, rendering IUGR rats more susceptible for renal disease.

INTRODUCTION

Numerous epidemiological studies have shown that intrauterine growth restriction (IUGR) is associated with a

significantly reduced nephron number and a higher incidence of chronic renal disease later in life [1, 2]. This observation can be partly explained by Brenner's hypothesis which suggests that the reduction in nephron number leads to compensatory glomerular hyperfiltration and arterial hypertension which in turn induce glomerular damage [3]. In order to describe a pathogenetic link between early effects of IUGR and the development of disease in adulthood, Barker [4] introduced the term of 'fetal programming', assuming that an adverse intrauterine environment leads to a lifelong (epi-) genetic conditioning with altered cellular differentiation and tissue architecture rendering former IUGR individuals more susceptible for organ dysfunction, independent from other risk factors.

The abundance of pathogenetic factors which are capable of inducing IUGR is reflected by a variety of animal models, leading to IUGR [5]. In our study, we used the model of maternal protein restriction, because it is easy to handle, highly reproducible and frequently used to investigate cardiovascular consequences of IUGR [6]. This model leads to a significant reduction in nephron number at the time of birth [7] and to a more severe course of mesangioproliferative glomerulonephritis later in life [8]. In accordance with reports from other groups, we did not observe any secondary hypertension in IUGR animals in this model [9, 10].

Previous studies in our model had revealed discreet signs of glomerulosclerosis after IUGR [8]. Glomerulosclerosis is frequently caused by a dysfunction of podocytes resulting in adhesions of the podocyte to the Bowman's capsule and subsequent matrix expansion [11]. Moreover, a relevant crosstalk between podocytes and mesangial cells was described conveying profibrotic signals from the podocyte to the mesangium [12]. Thus, the podocyte with its complex molecular structure plays a central role in the regulation and maintenance of glomerular function [13]. In view of these data, we decided to more thoroughly examine glomerular changes with a focus on podocyte alterations. We hypothesized that alterations of podocyte architecture and integrity after IUGR may render kidneys more susceptible for glomerular disease later in life. In conjunction with the evidence for a dysregulation of nephrogenesis, we became interested in the potential role of the Wilms' tumour suppressor gene 1 (WT1), an important factor of growth and differentiation which regulates kidney organogenesis and development [14, 15]. WT1 plays an essential role in the maintenance of podocyte function and integrity [16]. The functionality of WT1 is regulated by alternative splicing, leading to isoforms with different functional specifications. Alternative splicing of exon 9 and the resulting WT1 isoforms WT1 +KTS and −KTS affect embryonic renal development [17]. For example, the severe urogenital and renal dysplasia in patients with Frasier syndrome is associated with an altered +KTS/−KTS ratio [18].

In this study, we tested the hypothesis that in our rat model of maternal protein restriction IUGR leads to early structural and functional podocyte alterations based on a primary dysregulation of WT1 in the absence of arterial hypertension.

MATERIALS AND METHODS

Animal procedures

All procedures performed on animals were carried out in accordance with the guidelines of the American Physiological Society, conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the local government authorities (Regierung von Mittelfranken, AZ 621-2531.31-14/05, -12/06 and -31/09). Virgin female Wistar rats were obtained from Charles River (Sulzfeld, Germany) and were housed in a room maintained at 22 ± 2°C and exposed to a 12 h dark/light cycle. The animals were allowed unlimited access to standard chow (#1320, Altromin, Lage, Germany) and tap water. IUGR was induced by maternal protein restriction as described previously [8]. Ten dams were time-mated by the appearance of sperm plugs and then fed a casein-based diet containing 17.2% protein (# C1000, Altromin) or an isocaloric casein-based diet containing 8.4% protein (# C1003, Altromin) throughout pregnancy. Sodium content (0.25%) of both diets was equal. Rats delivered spontaneously and the litters were immediately reduced to six male pups per dam to guarantee equal lactation conditions. During lactation, rat mothers were fed standard chow. The offspring was nursed by their mothers until weaned at Day 21 to standard chow. The animals used for experiments were derived from five litters in each group. At 10 weeks of age, 10 pups of the low protein-treated group (IUGR) and 10 pups of the normal protein-treated group (non-IUGR) were sacrificed. In order to compare the findings obtained from our rat model of IUGR with an animal model of severe podocyte injury, we used the rat model of deoxycorticosterone-acetate (DOCA)-salt-induced hypertension, as described previously [19]: in short, rats underwent left unilateral nephrectomy (UNX). After 2 weeks, 21-day-release DOCA pellets containing 50 mg DOCA (Innovative Research of America, Sarasota, FL) were implanted subcutaneously after incision of the right flank. After 21 days, a second replacement pellet was implanted. Uninephrectomized control rats underwent sham operation. Procedures were performed under isoflurane anaesthesia. The animals had free access to 1% isotonic saline (NaCl 0.9%). After 6 weeks of DOCA treatment, the experiment was terminated. At this time point, DOCA-treated rats had developed high blood pressure and glomerulopathy as revealed by albuminuria and histological markers of glomerular damage [19].

Blood pressure measurement

As described previously [9], intra-arterial blood pressure measurements were performed in 70-day-old IUGR and non-IUGR rats. Animals were anaesthetized by intraperitoneal application of ketamine (20 mg/kg; Ketavet, Pfizer GmbH, Karlsruhe, Germany) and medetomidine (0.5 mg/kg; Domitor, Pfizer GmbH). Catheters were implanted in the right femoral artery and tunnelled subcutaneously. After antagonization with 0.2 mL atipamezol (5 mg/mL, Antisedan, Pfizer GmbH), animals woke up within 3–5 min. Animals

were allowed to recover for 2 h. After this, mean arterial blood pressure was recorded by a polygraph (Hellige, Freiburg, Germany) in conscious animals for 30 min.

Urine and plasma analysis

In 70-day-old IUGR and non-IUGR rats, blood was collected from indwelling catheters. Plasma creatinine and urea were analysed using an automatic analyser Integra 800 (Roche Diagnostics, Mannheim, Germany). Twenty-four hours before sacrifice, animals were housed in metabolic cages to collect urine for quantification of urine volume and albumin excretion. Albuminuria was assessed by enzyme-linked immunosorbent assay (Bethyl Laboratories, Biomol, Hamburg, Germany).

Tissue preparation

Animals were deeply anaesthetized by intraperitoneal application of ketamine (Ketavet) 100 mg/kg body weight and midazolam (Dormitor) 5 mg/kg body weight. Retrograde perfusion with NaCl 0.9% was performed via the abdominal aorta. The left kidney was removed and weighted. Portions of the cortex were immediately snap-frozen in liquid nitrogen for mRNA analysis or in Cryoblock (Medite, Burgdorf, Germany) to prepare cryosections. Another portion of renal tissue was fixed in methyl Carnoys solution (60% methanol, 30% chloroform, 10% glacial acetic acid) or 3% paraformaldehyde.

Electron microscopy

The remaining kidney was perfusion fixed with 3% glutaraldehyde. After removing, the kidney was weighted and dissected in a plane perpendicular to the interpolar axis, yielding slices of 1 mm width. The slices were embedded in Epon-Araldite, ultrathin sections (0.08 μm) were cut and stained with uranyl acetate and lead citrate for qualitative electron microscopic investigations using a Zeiss electron microscope (EM 107, Zeiss Co., Oberkochen, Germany) [20].

Immunohistochemistry

After fixation in methyl Carnoys solution or 3% paraformaldehyde, the tissues were dehydrated and embedded in paraffin. Three micrometre thin sections were cut with a Leitz SM 2000 R microtome (Leica Instruments, Nussloch, Germany).

Paraffin sections were layered with the primary antibody and incubated overnight. After addition of the secondary antibody (dilution 1:500, biotin-conjugated, goat anti-rabbit IgG or rabbit anti-mouse IgG, all from Dianova, Hamburg, Germany), the staining procedures were carried out by a peroxidase detection method as described before [21]. Each slide was counterstained with haematoxylin. Double stainings were performed using immunofluorescence: primary antibodies were applied simultaneously overnight at 4°C. Sections were then incubated with secondary antibodies, CY2-labelled antimouse IgG and CY3-labelled antirabbit IgG (both from Dianova) at the same time for 2 h. As a negative control, we used equimolar concentrations of preimmune rabbit or mouse immunoglobulin G replacing the primary antibody. To detect podocyte damage, sections were stained with a monoclonal antibody to desmin (Dako, Hamburg, Germany) in a dilution of 1:50, a monoclonal antibody to synaptopodin (Progen, Heidelberg, Germany) in a dilution of 1:100 and a polyclonal antibody to nephrin (Acris Antibodies, Hiddenhausen, Germany) was used on cryosections in a dilution of 1:100 [19]. Expansion of desmin, synaptopodin or nephrin in the glomeruli was evaluated in a Leitz Aristoplan microscope (Leica Instruments) using Metavue software (Visitron Systems, Puchheim, Germany). The stained area was expressed as the percentage of the glomerular cross-section.

To assess the size of the nephrogenic zone in neonatal kidneys, median sagittal sections were stained with a mouse monoclonal antibody detecting proliferative cell nuclear antigen (PCNA, Dako) used in a dilution of 1:50. The strongly PCNA-positive nephrogenic zone, defined as the cortical area of neonatal kidneys containing glomerular precursors until the stadium of comma-shaped bodies, was evaluated by measuring its expansion, generating 10 values evenly distributed throughout the section. To analyse apoptosis in neonatal kidneys, sections were stained with a rabbit polyclonal antibody to active caspase-3 (DCS Diagnostics, Hamburg, Germany) in a dilution of 1:50. Cells positive for active caspase-3 were counted in six randomly selected medium power views of the nephrogenic zone.

For detection and quantification of WT1-positive cells in mature differentiated glomeruli, sections were co-stained with a polyclonal antibody to WT1 (NeoMarkers, Fremont), a monoclonal antibody to synaptopodin (Progen) as a marker for differentiated glomeruli and 4′,6-diamidino-2-phenylindole (DAPI) (Santa Cruz Biotechnology, Heidelberg, Germany). The number of WT1-positive cells and total glomerular cell numbers were assessed by counting WT1 and DAPI-positive nuclei in at least 20 randomly selected glomeruli per kidney section.

Glomerular geometry

The size of glomerular area was evaluated in periodic acid-Schiff-stained renal sections with a Leitz Aristoplan microscope (Leica Instruments) using Metavue software (Visitron Systems) as described in detail [22].

Real-time RT–PCR

Renal tissue was homogenized in 500 μL of RLT buffer reagent (Qiagen, Hilden, Germany) with an ultraturrax for 30 s and total RNA was extracted from homogenates with RNeasy Mini columns (Qiagen) according to the standard protocol. First-strand cDNA was synthesized with TaqMan reverse transcription reagents (Applied Biosystems, Darmstadt, Germany) using random hexamers as primers. Final RNA concentration in the reaction mixture was adjusted to 0.1 ng/μL. Reactions without Multiscribe reverse transcriptase were used as negative controls for genomic DNA contamination. RT-products were diluted 1:1 with dH_2O before PCR procedure. PCR was performed with an ABI PRISM 7000 Sequence Detector System and SYBR Green or TaqMan reagents (Applied Biosystems) according to the manufacturers' instructions. All samples were run in triplicate. Specific mRNA levels were calculated and

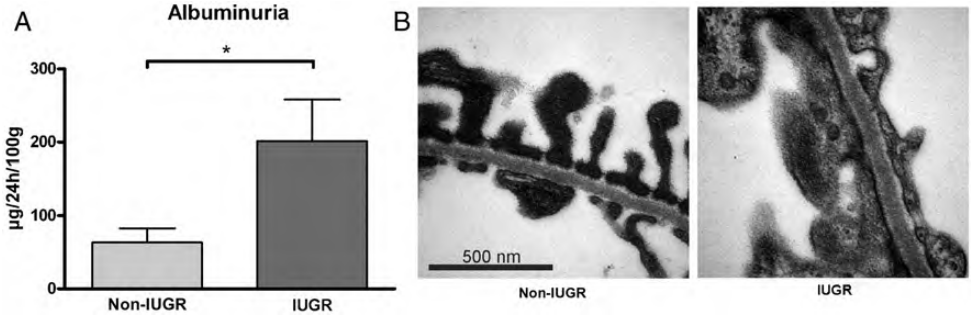

FIGURE 1: Podocyte damage in IUGR rats at Day 70 of life. (**A**) Albumin excretion. (**B**) Representative electron micrographs of the glomerular capillary wall and podocyte foot processes (uranyl acetate and lead citrate staining). *P < 0.05.

normalized to 18S rRNA as a housekeeping gene with the $\Delta\Delta C_T$ method as specified by the manufacturer (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_040980.pdf). Primer Express software (Perkin Elmer, Foster City, CA) was used to design primer pairs. For detection of WT1, the forward primer was 5′-AGGACTGCGAGAGAAGGTTTTCT-3′; the reverse primer was 5′-TGGAATGGTTTCACACCTGTGT-3′. For detection of WT1 +KTS, the forward primer was 5′-ACCACCTGAAGACCCACACC-3′; the reverse primer was 5′-GCGCAAACTTTTTCTGACAACTG-3′; the probe was 5′-TCATACAGGTAAAACAAGTGAAAAGCCCTTCAGC-3′. For detection of WT1 −KTS, the forward primer was 5′-ACCACCTGAAGACCCACACC-3′; the reverse primer was 5′-GCGCAAACTTTTTCTGACAACTG-3′; the probe was 5′-CATACAGGTGAAAAGCCCTTCAGCTGTCG-3′. For detection of Pax2, the forward primer was 5′-CAAGCCCGGAGTGATTGG-3′; the reverse primer was 5′-CAGCAATCTTGTCCACCACTTTG-3′. For detection of Six2, the forward primer was 5′-CGGGACAAAGTGGACAATGG-3′; the reverse primer was 5′-CTCCAAAGGATCTCAAAAGCAACT-3′. For detection of p53, the forward primer was 5′-ATGATATTCTGCCCACCAC-3′; the reverse primer was 5′-TAACAACTCTGCAACATCCT-3′. For the detection of the housekeeping gene 18s, the forward primer was 5′-TTGATTAAGTCCCTGCCCTTTGT-3′; the reverse primer was 5′-CGATCCGAGGGCCTCACTA-3′.

Detection of WT1 +KTS/−KTS ratios

The ratios of WT1 splice variants +KTS and −KTS were detected using RT–PCR (forward primer: 5′-GTGAAACCATTCCAGTGTAAAAC-3′, reverse primer: 5′-GCCACCGACAGCTGAAGGGC-3′). PCR products were separated using a 12% polyacrylamide gel similar to that described in Hammes *et al.* [17]. Quantification of signals was performed using Luminiscent Image Analyser LAS 1000 (Fuji-Film, Berlin, Germany) and evaluated with Aida 4.15 Image Analyser software (Raytest, Berlin, Germany). Ratios of +KTS and −KTS were calculated. Ratios of WT1 splice variants were assessed in kidneys of IUGR and non-IUGR animals at Days 1 and 70 of life as well as in kidneys of uninephrectomized rats with DOCA-salt induced hypertension and uninephrectomized normotensive controls.

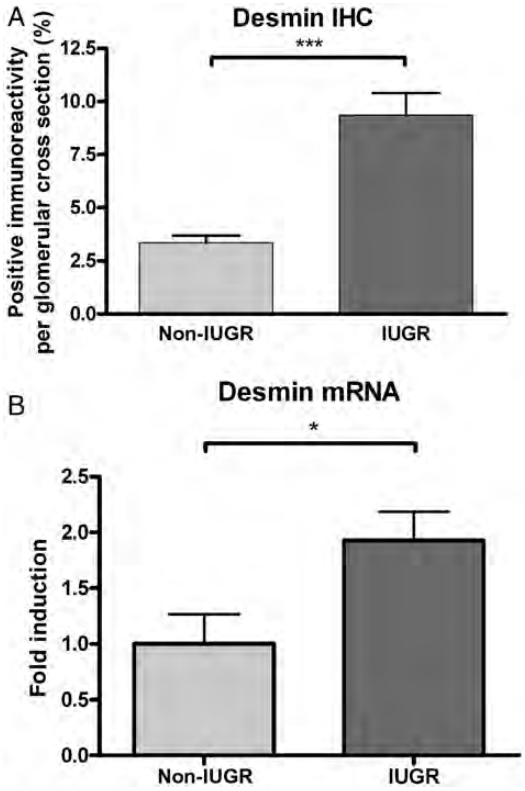

FIGURE 2: Desmin expression in glomeruli of IUGR rats at Day 70 of life. (**A**) Desmin protein abundance in glomerular cross-sections as assessed by immunohistochemistry (IHC). (**B**) Desmin mRNA expression in renal cortical tissue as assessed by real-time PCR analysis. *P < 0.05; ***P < 0.001.

Analysis of data

All data are expressed as means ± standard error of the mean. Differences between IUGR animals and controls were assessed using Student's *t*-test. Two-way analysis of variance (two-way ANOVA) was performed to examine the effect of the two independent factors 'diet (IUGR)' and 'age' on +KTS to −KTS ratios and WT1-positive glomerular cells. Results were considered significant when the P-value was <0.05. Student's *t*-test and two-way ANOVA were carried out using SPSS Statistics, Release 20 (IBM Software Group, Chicago, IL).

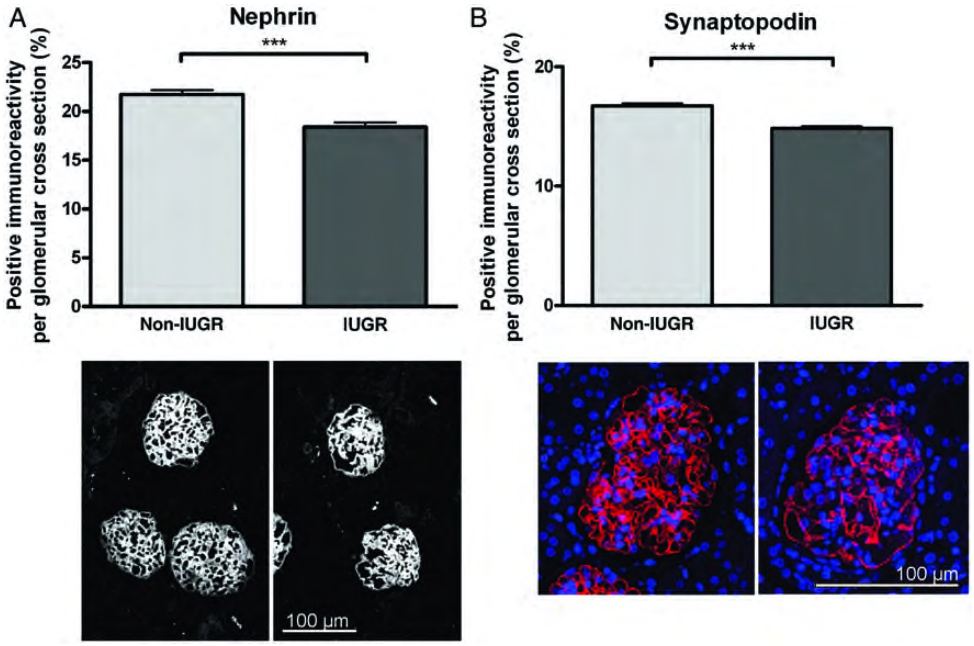

FIGURE 3: Detection of podocyte markers in glomeruli of IUGR rats at Day 70 of life. (**A**) Evaluation of nephrin immunoreactivity with representative photomicrographs (white colour). (**B**) Evaluation of synaptopodin immunoreactivity with representative photomicrographs (red colour). Costaining with DAPI to detect nuclei (blue). ***P < 0.001.

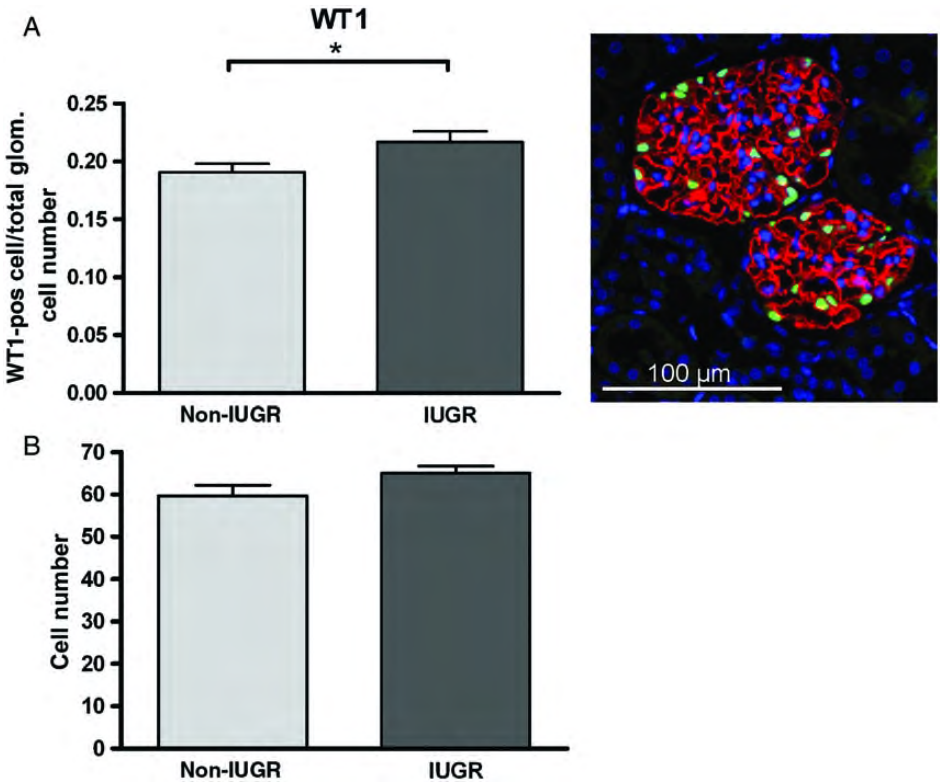

FIGURE 4: WT1-positive glomerular cells and total glomerular cell number in glomeruli of IUGR rats at Day 70 of life. (**A**) WT1-positive cell counts. (**B**) Representative photomicrograph of immunofluorescent detection of WT1-positive cell nuclei (green), costained with synaptopodin (red) and DAPI to detect nuclei (blue). (**C**) Total glomerular cell counts. *P < 0.05; n.s. not significant.

RESULTS

A significant reduction in birth weights in the IUGR offspring in comparison with non-IUGR animals was detected (4.93 ± 0.11 g in IUGR versus 6.40 ± 0.12 g in non-IUGR, P < 0.001). Within the first weeks of life, former IUGR animals caught up with their body weights, so that at Day 70 of life, body weights no longer showed any significant differences between IUGR and non-IUGR animals (367.0 ± 6.36 g in IUGR versus 374.8 ± 3.85 g in non-IUGR, P > 0.05). As described previously, at this age, total glomerular numbers in the kidneys of IUGR rats were reduced ~27%, while the total and mean glomerular volumes were not different in IUGR and non-IUGR rats [8]. This was confirmed in the present study revealing significantly reduced relative kidney weights in the IUGR group (0.47 ± 0.02% in IUGR versus 0.61 ± 0.01% in non-IUGR, P < 0.001), while on the other hand, there were no significant differences in glomerular size between IUGR and non-IUGR animals at Day 70 of life as assessed by measuring glomerular perimeters (388.4 ± 4.28 µm in IUGR versus 387.1 ± 8.57 µm in non-IUGR, P > 0.05). Mean arterial blood pressure was equal in IUGR and non-IUGR rats at Day 70 of life (101.3 ± 2.69 mmHg in IUGR versus 105.70 ± 1.64 mmHg in non-IUGR, P > 0.05).

Searching for valid signs of pathological podocyte alterations at Day 70 of life, we detected a significantly increased albumin excretion in IUGR rats compared with non-IUGR (Figure 1A). Moreover, electron microscopy revealed overt alterations of podocyte ultrastructure in former IUGR animals with enlargement of podocytes and foot process effacement (Figure 1B). We also detected significantly higher plasma levels of urea in IUGR animals (66.89 ± 2.53 mg/dL in IUGR versus 52.43 ± 2.84 mg/dL in non-IUGR, P < 0.01), while levels of plasma creatinine were not significantly different between IUGR and non-IUGR rats at this time point [0.334 ± 0.02 mg/dL in IUGR versus 0.288 ± 0.02 mg/dL in non-IUGR animals, not significant (n.s.)]. Immunoreactivity of desmin, which is a marker of podocyte damage, was augmented in glomeruli of 70-day-old IUGR rats (Figure 2A). In accordance with this observation, we could also detect higher cortical mRNA expression levels of desmin in the cortex of these kidneys by RT–PCR (Figure 2B). Furthermore, immunohistochemistry revealed a significant reduction in nephrin (Figure 3A) and synaptopodin (Figure 3B) in IUGR animals at this time point.

In contrast, a significantly higher number of WT1-positive glomerular cells in IUGR animals at Day 70 of life was detected (Figure 4A) with no changes in total glomerular cell numbers in both IUGR and non-IUGR groups (Figure 4C). Consistent with this finding, cortical mRNA expression levels of WT1 were significantly augmented in IUGR compared with non-IUGR rats (Figure 5A). Furthermore, evaluation of WT1

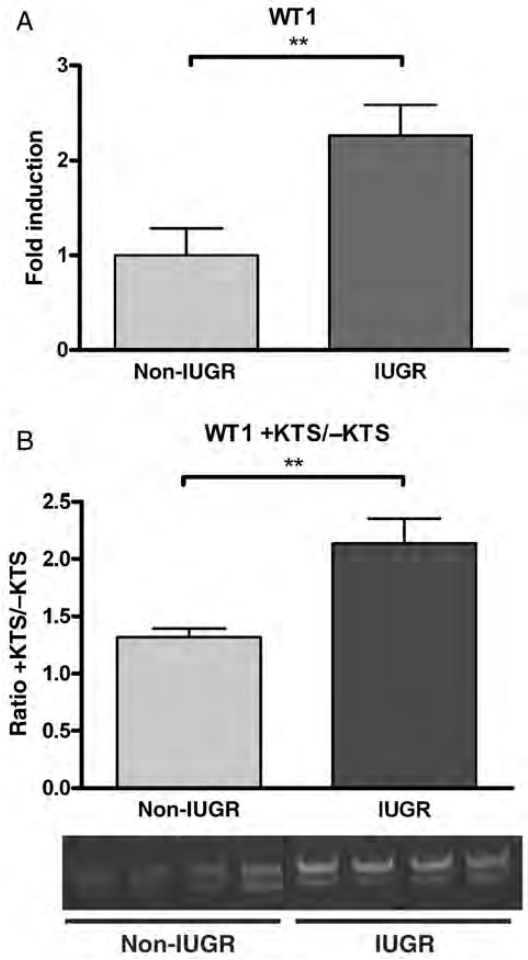

FIGURE 5: WT1 mRNA expression in cortical tissue of IUGR rats at Day 70 of life. (**A**) Total WT1 mRNA expression. (**B**) Expression ratios of WT1 splice variants +KTS and −KTS with exemplary acrylamide gel separation of both isoforms. The upper band represents the +KTS variant at the expected size of 117 bp, the lower band represents the −KTS variant at the expected size of 108 bp. **P < 0.01.

Table 1. mRNA-expression of WT1 +KTS and WT1 −KTS isoforms

	Neonatal		D70	
	Non-IUGR	IUGR	Non-IUGR	IUGR
WT1 +KTS	1.00 ± 0.16	1.66 ± 0.13*	1.00 ± 0.34	3.17 ± 0.90#
WT1 −KTS	1.00 ± 0.12	1.32 ± 0.06*	1.00 ± 0.34	2.35 ± 0.39#

*P < 0.05 versus non-IUGR neonatal.
#P < 0.05 versus non-IUGR D70.

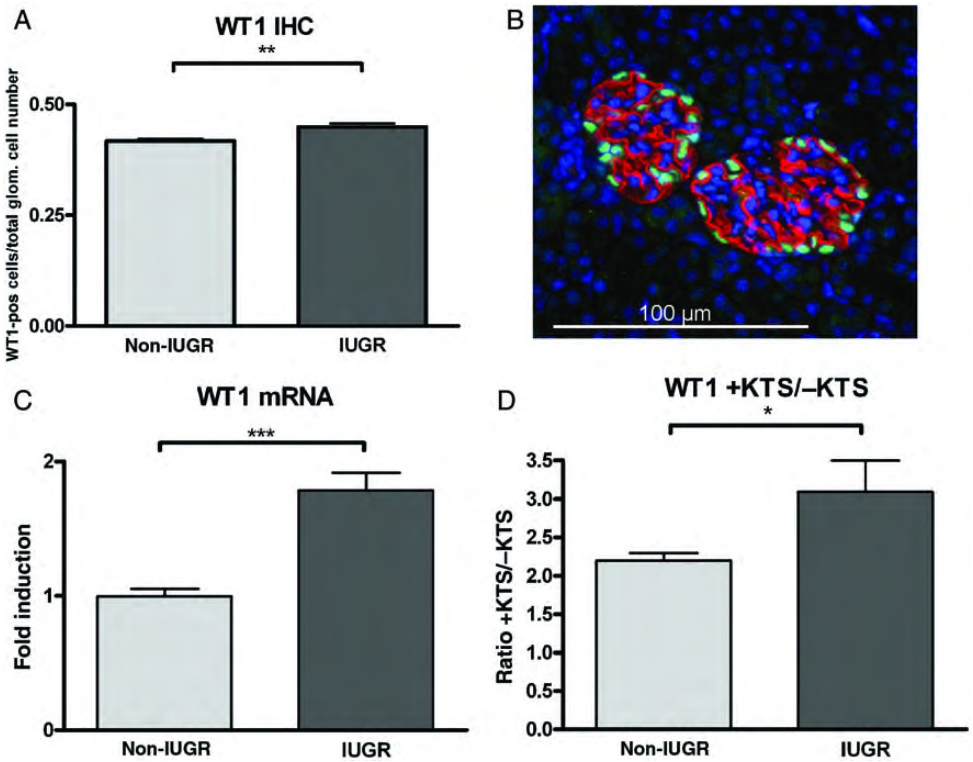

FIGURE 6: WT1 expression in renal tissue of neonatal IUGR rats. (**A**) WT1-positive cell counts in capillary glomeruli. (**B**) Representative photomicrograph of immunofluorescent detection of WT1-positive cell nuclei (green), costained with synaptopodin (red) and DAPI to detect nuclei (blue). (**C**) Total WT1 mRNA expression, (**D**) expression ratios of WT1 splice variants +KTS and −KTS. *P < 0.05; **P < 0.01; ***P < 0.001.

Table 2. P-values from two-way ANOVA

	WT1 +KTS/−KTS ratio	WT1-positive cells/totalglomerular cell number
Diet	0.001	0.001
Age	<0.001	<0.001
Diet×age	0.858	0.727

exon 9 splice variants +KTS and −KTS showed a significant alteration of the expression of these two isoforms (Table 1) and the expressional ratio +KTS/−KTS in IUGR animals at Day 70 (Figure 5B).

Consistent with the findings in rats at Day 70, we detected a higher percentage of WT1-positive glomerular cells in kidneys of neonatal IUGR rats (Figure 6A) and a significant renal overexpression of WT1 mRNA (Figure 6C). The assessment of the WT1 isoforms showed a consistently increased expression of WT1 +KTS and −KTS (Table 1) as well as an increased expressional ratio +KTS/−KTS in the kidneys of neonate IUGR animals (Figure 6D).

By two-way ANOVA, we could confirm that 'age' and 'diet (IUGR)' had significant effects on the WT1 +KTS/−KTS ratio and on the number of WT1-positive glomerular cells in the absence of a relevant interaction of these two factors (Table 2).

Analysing the expressional ratio of WT1 +KTS/−KTS isoforms in kidneys of a model of severe podocyte injury (DOCA-salt-induced hypertensive rats and normotensive controls) revealed no significant differences between the two groups (1.74 ± 0.06 in UNX-DOCA animals versus 1.86 ± 0.09 in control animals, n.s.).

The mRNA expression of both renal developmental transcription factors Pax2 (Figure 7A) and Six2 (Figure 7B) associated with the WT1 pathway was significantly increased in neonates after IUGR. The proliferative capacity of neonatal kidneys was assessed after staining with PCNA, showing a significantly reduced width of the nephrogenic zone in IUGR compared with non-IUGR animals (Figure 8). Apoptosis was more frequent in the nephrogenic zone of IUGR compared with non-IUGR animals, as revealed by an increase in cells positive for active caspase-3 (Figure 9A) and an increase in the expression of p53 in neonatal kidneys of IUGR animals (Figure 9C).

DISCUSSION

The main finding of this study is that in our rat model of maternal protein restriction, IUGR leads to overt structural alterations in the glomeruli of male rats at Day 70 of life in the absence of arterial hypertension. Moreover, we could detect persistently altered mRNA expression patterns of total WT1 and its isoforms +KTS and −KTS in the male offspring from the time of birth. In context with our observations of an

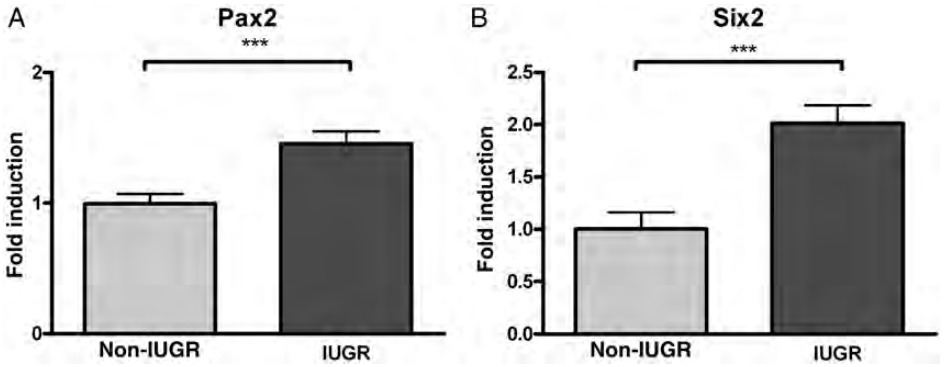

FIGURE 7: Expression of transcription factors involved in the WT1 pathway in renal development in kidney tissue of neonatal IUGR rats. (**A**) mRNA expression levels of Pax2. (**B**) mRNA expression levels of Six2. ***P < 0.001.

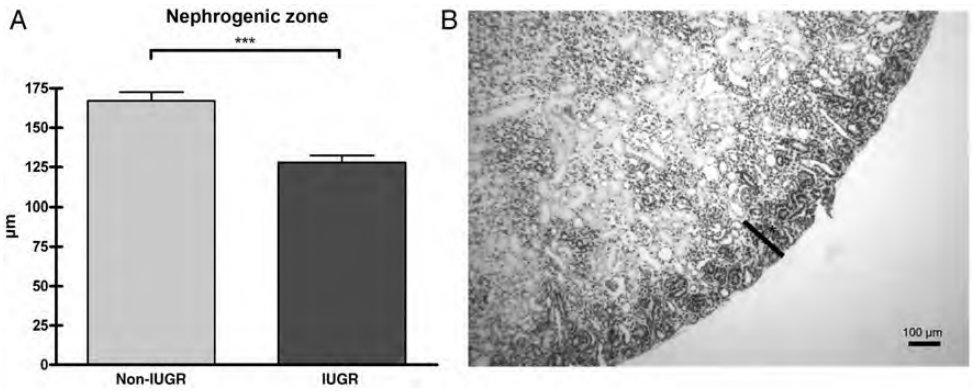

FIGURE 8: Evaluation of the nephrogenic zone in kidneys of neonatal IUGR rats. (**A**) Measurements of the size of the nephrogenic zone. (**B**) Exemplary photomicrograph of a PCNA-stained neonatal kidney. The thickness of the nephrogenic zone is indicated by a black bar. ***P < 0.001.

aggravated course of glomerular disease in former IUGR animals [8], one might postulate that IUGR leads to a dysregulation of WT1, important for glomerular development and differentiation and consequently to early detectable alterations of podocyte structure and integrity, which make these animals more susceptible for the development of glomerular injury later in life.

Previous studies detected glomerular damage in IUGR rats at an age of 90 or 180 days, which paralleled an increase in blood pressure [23]. For this reason, it was not possible to discriminate between direct effects and effects secondary to hypertension in this model. In contrast, in our study, no increase in blood pressure was observed, although a slight but significant increase in glomerular matrix expansion after IUGR was detected [8]. Thus, it is conceivable that the changes detected in the glomeruli of our IUGR animals are not a consequence of increased arterial blood pressure.

Podocyte damage in 70-day-old male IUGR animals was detected using different techniques. On the basis of a significantly higher albuminuria in IUGR animals, we performed electron microscopy, which revealed structural podocyte alterations and foot process effacement. Furthermore, we detected an increase in immunoreactivity of desmin, which is a valid marker of podocyte injury [24], in 70-day-old IUGR animals. Nephrin and synaptopodin, both markers of mature differentiated podocytes [25], appeared reduced, further arguing for podocyte injury in these animals. WT1 is also regarded as a marker of podocyte maturity and differentiation [25]. As a consequence of pathological podocyte alterations, WT1 expression is commonly reduced [16, 26, 27]. Thus, it was surprising that the evaluation of WT1-positive glomerular cells and of the mRNA expression levels of WT1 revealed an up-regulation of WT1. The fact that the total glomerular cell number and the size of glomeruli did not differ between IUGR animals and controls argues for an overexpression of WT1 by podocytes in structurally altered glomeruli of IUGR animals more likely than an increase in podocyte number after IUGR.

WT1 plays a crucial role in early kidney development, which is characterized by the interaction of the metanephric mesenchyme and the epithelial tissue of the ureteric bud [28]. Especially for the growth and differentiation of the latter, WT1 seems to be absolutely necessary [29]. In comma and S-shaped bodies, the precursors of mature glomeruli, high expression levels of WT1 are detectable and decrease with progressing glomerular differentiation. In the mature kidney, WT1 is specifically expressed in podocytes [14]. Besides its important role during embryonic development, WT1 is also a crucial factor for podocyte function and integrity. For this reason, the observation of an overexpression of WT1 in IUGR compromised kidneys already at the time of birth, where kidney development is still ongoing, led to the assumption of a functional dysregulation of WT1. One of the mechanisms to modulate WT1 functionality is alternative splicing of WT1, resulting in the insertion of the three amino acids KTS in its zinc finger

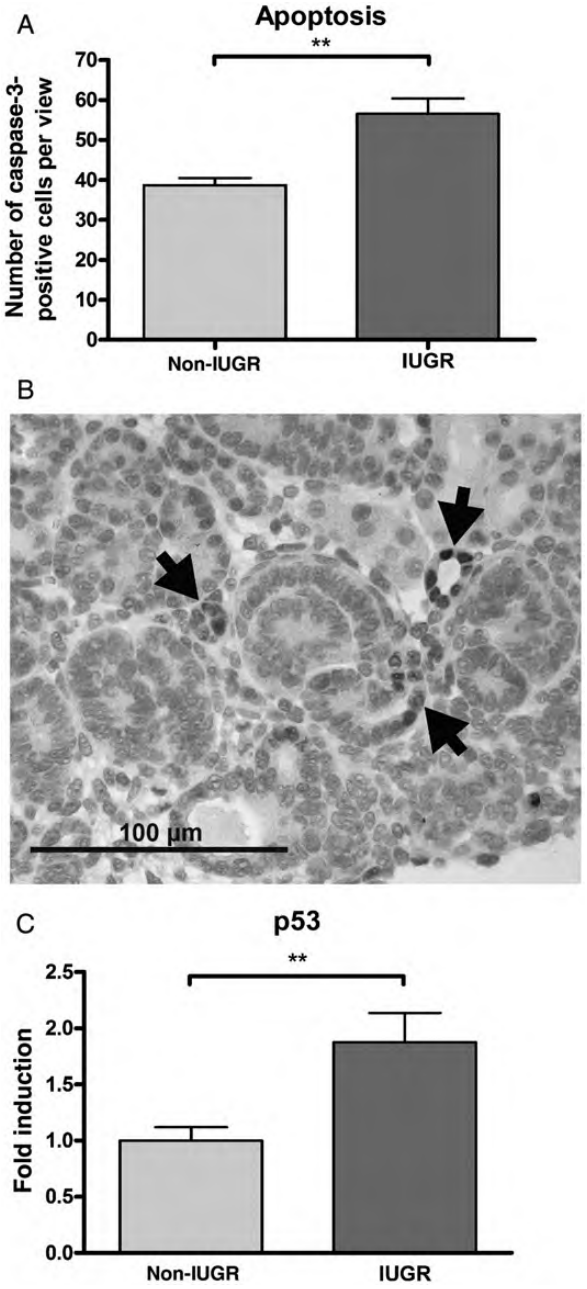

FIGURE 9: Detection of apoptosis in kidneys of neonatal IUGR rats. (**A**) Counts of cells positive for active caspase-3 in the nephrogenic zone. (**B**) Exemplary photomicrograph of active caspase-3 staining in the nephrogenic zone. Black arrows point to apoptotic active caspase-3 positive cells. (**C**) p53 mRNA expression in kidneys of neonatal IUGR compared with non-IUGR rats. **$P < 0.01$.

domain [30]. An altered expression ratio of these WT1 isoforms is associated with renal pathologies: several *in vivo* studies provided evidence that especially an altered ratio of WT1 isoforms +KTS and −KTS is found in congenital glomerular disease like Frasier syndrome [18]. In contrast to our findings, however, Frasier syndrome is associated with a reduced expression of the WT1 +KTS splice variant and consequently a lower ratio of WT1 +KTS/−KTS isoforms [31]. Moreover, it could be shown that the WT1 +KTS isoform plays a crucial role in the maintenance of podocyte structure and functionality [32]. Thus, the observed relative overexpression of the WT1 +KTS isoform after IUGR might also reflect a secondary protective effect as a consequence of primary glomerular alterations.

Hammes *et al.* showed that in mice, the ablation of the WT1 −KTS isoform is associated with a significantly reduced kidney size and glomerular number as well as a smaller width of the nephrogenic zone and small contracted glomeruli. These observations led to the hypothesis that the WT1 −KTS isoform plays a central role during embryonic renal development as antiapoptotic mediator [17].

Consistent with these observations, we detected a relative underexpression of the WT1 −KTS isoform in kidneys of former IUGR rats already at Day 1 of life in our study. This is accompanied by a significantly reduced kidney weight, a reduced glomerular number and a smaller nephrogenic zone. According to the hypothesis of Hammes *et al.* [17], one might postulate that IUGR leads to this specific renal phenotype by an increased degree of apoptotic processes during embryonic renal development, which could be confirmed in our model of IUGR by assessing apoptotic markers in neonatal kidneys. During nephrogenesis, WT1 is a member of an entity of developmental genes, balanced among each other by complex feedback control mechanisms [15]. In this context, we could also show altered expression patterns of WT1-associated nephrogenic factors Pax2 and Six2 in IUGR, indicating that the functional alteration of WT1 also leads to a dysregulation of nephrogenesis which primarily results in a significantly reduced nephron number. Extrapolation of the proapoptotic character of the relative underexpression of the −KTS isoform detected in IUGR animals until Day 70 of life, one might suppose that IUGR impairs glomerular homeostasis and regeneration later in life and thus makes the kidney more susceptible for glomerular injury as observed in 70-day-old IUGR animals [8]. Moreover, according to Hammes *et al.* [17], the −KTS isoform is important for podocyte integrity as well. For this reason, the ratio of +KTS and −KTS isoforms is very conserved [30]. In view of the consistently increased expression of total WT1 and higher ratio of WT1 +KTS/−KTS splice variants already from the time of birth on, one might speculate that these alterations of WT1 expression patterns in 70-day-old IUGR animals are not secondary effects but have been existing since birth according to the theory of fetal programming [4].

This is also supported by the findings in a model of severe glomerular damage: analysing the ratios of WT1 +KTS/−KTS isoforms in rats with DOCA-salt induced hypertension revealed no significant differences between the intervention group and controls. In this context, it is necessary to keep in mind that in this model, we see a manifest podocyte damage which is accompanied by severe proteinuria and a significant down-regulation of total WT1 [19, 33], while in our IUGR model, only minor podocyte alterations and moderate albuminuria were detected. The WT1 down-regulation and the conserved ratio of WT1 isoforms in the DOCA model of induced severe glomerular changes on the one hand and WT1 up-regulation with a significantly increased expression of its isoforms in IUGR rats on the other hand could be due to different and independent underlying pathomechanisms.

At present, it is not clear, whether deficits in nephron endowment during renal development and alterations in podocyte structure and function in the mature glomerulus are pathophysiologically linked or are merely epiphenomena. A relatively reduced expression of the WT1−KTS isoform could contribute to a nephron deficit during renal development [17]. Reduced nephron endowment usually leads to hyperfiltration of the remaining glomeruli which could result in podocyte stress [34]. This would render the podocytes more vulnerable to secondary injury. In view of the fact that in the present study, only two time points were investigated and a time course of albuminuria was not assessed, we cannot exclude that an early onset of albuminuria in our model could lead to the podocyte changes observed at Day 70 of life. On the other hand, the fact that a dysregulation of genes important for the maintenance of the podocyte phenotype was observed already at the time of birth argues for a primary podocyte damage. Using two-way ANOVA, we could show that 'age' and 'diet (IUGR)' both have a significant impact on +KTS/−KTS ratio and the number of WT1-positive glomerular cells but without a significant interaction. This result suggests that these are independent changes.

Taken together, we speculate that IUGR leads to a dysregulation of WT1 accompanied by compromised renal development and premature damage of podocytes later in life. This might render the kidney more vulnerable to injury after IUGR.

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CONFLICT OF INTEREST STATEMENT

None declared.

(See related article by Rookmaaker and Joles. The nephron number counts—from womb to tomb. *Nephrol Dial Transplant* 2013; 28: 1325–1328.)

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