WT1 is a key regulator of podocyte function: reduced expression levels cause crescentic glomerulonephritis and mesangial sclerosis

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Glomerular disease is one of the most common causes of end-stage renal failure. Increasing evidence suggests that these glomerulopathies are frequently caused by primary lesions in the renal podocytes. One of the major consequences of podocyte lesions is the accumulation of mesangial matrix in the glomerular basement membrane, a process called glomerulosclerosis. Mesangial sclerosis is one of the most consistent findings in Denys–Drash patients and can be caused by dominant mutations in the Wilms’ tumor 1 gene (WT1). The underlying mechanism, however, is poorly understood. WT1 is expressed in the podocytes throughout life, but its function in this cell type is unknown. Combining Wt1-knockout and inducible yeast artificial chromosome transgenic mouse models, we demonstrate that reduced expression levels of WT1 result in either crescentic glomerulonephritis or mesangial sclerosis depending on the gene dosage. Strikingly, the two podocyte-specific genes nphs1 and podocalyxin are dramatically downregulated in mice with decreased levels of Wt1, suggesting that these two genes act downstream of Wt1. Taken together, our data provide genetic evidence that reduced levels of WT1 are responsible for the pathogenesis of two distinct renal diseases and offer a molecular explanation for the increased occurrence of glomerulosclerosis in patients with WAGR syndrome.

INTRODUCTION

The human Wilms’ tumor 1 gene (WT1) maps to chromosome 11p13 and has been identified as a major player in the development of Wilms’ tumor, an embryonic kidney cancer. WT1 contains 10 exons, which span ~50 kb and encode a transcript of 3 kb (1,2) (Fig. 1). As a result of alternative RNA splicing, RNA editing and the presence of alternative translational initiation sites, each transcript can encode 24 different proteins (3–5). Results from biochemical studies suggest that WT1 may act as a transcription factor, transcriptional cofactor or post-transcriptional regulator, depending on its splice isoform and the cellular context.

In addition to being a tumor suppressor gene, WT1 has been shown to play crucial roles during embryogenesis, in particular during kidney development. The kidney forms through reciprocal interactions of two tissues: the metanephric mesenchyme and the ureteric bud epithelium. As a first step, factors derived from the metanephric mesenchyme induce the ureteric bud to grow out from the mesonephric duct. In turn, the ureteric bud invades the metanephric mesenchyme and induces mesenchymal cells to condense: forming first the renal vesicle, which matures further via the comma- and S-shaped bodies into epithelial cells that form the proximal tubules, the distal tubules and the glomerulus. WT1 expression can already be detected at low levels in the metanephric blastema, but increases in the comma- and S-shaped bodies (6). Upon further differentiation, WT1 expression is downregulated, except in the visceral epithelial cells (podocytes) of the mature glomerulus, where expression can be detected throughout life. In agreement with its expression pattern, WT1 has been shown to play a role during kidney induction (7) and during later steps of nephrogenesis (8). A direct role for WT1 in podocyte function has not been proven so far.

The podocytes line the external surface of the blood vessels in the glomerulus and play an important role in the maintenance of the basement membrane. In addition, they support the capillary tuft and may influence the glomerular filtration rate. Impairment of the podocytes may lead to the development of glomerulosclerosis (9,10). Several lines of evidence suggest that WT1 may indeed play an important role in the maintenance of...
normal podocyte function. Firstly, WT1 is mutated in 94% of all Denys–Drash syndrome (DDS) patients, and the most consistent finding in these patients is the development of glomerular nephropathy involving glomerulosclerosis. Secondly, in support of the above, it has been shown that a common DDS mutation in the WT1 gene can cause the development of glomerulosclerosis in mice (11). Thirdly, WT1 mutations have been found in patients with nephrotic syndrome and isolated cases of glomerulosclerosis (12,13).

We have shown previously that the human WT1 gene could partially rescue nephrogenesis in Wt1-null mice by introducing a 280 kb yeast artificial chromosome (YAC) containing the human WT1 locus (8). Nephrogenesis was arrested after the formation of the comma-shaped bodies. The failure to obtain complete rescue could be explained by too low expression levels of the transgene, which were only 10% of the endogenous Wt1 gene. To further dissect the role of WT1 in nephrogenesis and renal function, we have created mice carrying a 470 kb YAC containing the human WT1 locus. This YAC could rescue the Wt1-null phenotype and, depending on the expression level of WT1, we found that the rescued mice developed crescentic glomerulonephritis or mesangial sclerosis after birth. In this study, we show that reduced expression levels of WT1 alone can result in kidney failure.

RESULTS
Generation of transgenic lines carrying an inducible WT1-YAC construct
We have shown previously that the human WT1 gene could partially rescue nephrogenesis in Wt1-null mice by introduction of

Figure 1. Transgenic strategy and identification of the expression pattern. (A) A 10 kb fragment containing En2-SA-IRES-LacZ-Neo-pA-Leu2, flanked by a directed pair of LoxP sites, was targeted into intron 1 of a human WT1 YAC (WT470). Transgenic lines generated with this construct show no leaky expression of the WT1 transgene due to the presence of a strong polyadenylation site (pA) in the loxP-flanked cassette (inactive version). Crossing with a Cre-expressing mouse strain (deleter-Cre) leads to the excision of the loxP-flanked cassette and a concomitant activation of the WT1 transgene. En2-SA, splicing acceptor region of the En2 gene; IRES, internal ribosome-entry site; LacZ, β-galactosidase gene; Neo, Neomycin resistance gene; pA, polyadenylation signal from SV40 large T gene; Leu2, leucine2 gene as positive selection marker in yeast; SVA, short vector arm of the YAC; LVA, long vector arm of the YAC. Exons of the human WT1 locus are shown as green boxes. (B) The presence of the lacZ gene in intron 1 allowed us to monitor the expression pattern of the transgene. LacZ expression is detected in the urogenital ridge at day 11.5 post-coitum (i), and mesenchymal condensations (iii, asterisk), comma-shaped bodies (iii, arrow), S-shaped bodies (iii, arrow head) and the glomeruli (ii, and arrow tail in iii) in the developing kidney.
Fig. 2. BKK, BBKK and K mice express decreased levels of WT1, nephrin and/or podocalyxin. Total kidney RNA isolated from 3-week-old mice was reverse transcribed and subjected to real-time PCR using the LightCycler. Values were standardized using results from real-time amplification with GAPDH primers. Data are shown as means ± SEM, n = 5 for each group. Wt1/WT1 expression levels from wild-type kidneys as a standard (100%), the K, BBKK and BKK mice have a step-wise reduction of Wt1/WT1 expression levels, which fits with the severity of renal dysfunction. Taking nephrin levels from wild-type kidneys as a standard (100%), the K, BBKK and BKK mice show a step-wise decrease in nephrin expression levels as well. It is noteworthy that, K and BBKK mice are histologically indistinguishable from wild-type mice at this age. Podocalyxin levels in K mice are comparable to those in wild-type animals (100%). In contrast, BKK and BBKK mice show a significant decrease in podocalyxin expression levels.

a 280 kb YAC (8). The failure to completely rescue the Wt1-null phenotype could be explained by the low expression level of the transgene. We argued that the low expression level might be due to regulatory sequences downstream of the Wt1 gene, which were not included on the 280 kb YAC construct. We therefore decided to create mice with a 470 kb YAC, containing an additional 190 kb downstream of the human Wt1 gene.

For easier monitoring of transgene expression and to bypass any detrimental effects possibly associated with overexpression of Wt1, we have modified the 470 kb YAC (WT470) (14). An inactivation cassette comprising SA-IREs-LacZ-Neo (SA, splice acceptor; IRES, internal ribosomal entry site) flanked by loxP sites (15) was inserted into intron 1 of Wt1, with the aim of producing a β-galactosidase reporter and to disrupt Wt1 transcription (Fig. 1A). A total of five lines were generated. PCR analysis in lines WT64-B, WT64-C, WT64-D and WT64-E showed the integration of the Wt1 locus plus all of its upstream regions and a varying degree of downstream regions. X-gal staining in these regions demonstrated lacZ expression within the urogenital system in a Wt1-specific pattern. In contrast, line WT64-A was negative for the region >25 kb upstream of Wt1 and lacked renal expression. These data suggest that essential kidney specific regulatory elements are located between −25 and −200 kb upstream of Wt1. Line WT64-B showed full-length integration of a single copy of the transgene (data not shown), and detailed lacZ expression analysis demonstrated that the expression of the transgene followed the pattern of the endogenous Wt1 gene precisely (Fig. 1B) (6). All further analysis was carried out with this line.

Expression of Wt1 is tightly regulated

Activation of the Wt1 transgene was achieved by crossing line WT64-B with a deleter-Cre strain (16), which resulted in the excision of the SA-IREs-LacZ-Neo cassette. Animals carrying the activated transgene displayed normal survival, indicating that the additional copy of the human Wt1 gene did not have a negative effect on development.

To further analyze Wt1 gene function we crossed the activated transgenic strain WT64-B onto the Wt1-null background (7). To determine the expression level of Wt1, RNA was extracted from kidneys of 20-day-old mice and subjected to real-time RT–PCR analysis choosing primers in exons 9 and 10 with equal binding efficiency between mouse and human (simultaneous amplification of +KTS and −KTS isoforms). Wt1-null mice with one copy of the human transgene (BKK mice) expressed ∼62%, whereas Wt1-null mice with two copies of the transgene (BBKK mice) expressed ∼70% of normal wild-type Wt1 mRNA levels (Fig. 2). Interestingly, halving the gene dosage did not reduce Wt1 expression levels by 50%. Mice heterozygous for the Wt1 mutation (K mice) still expressed ∼95% of wild-type Wt1 mRNA levels (Fig. 2). Moreover, mice carrying one copy of the transgene on a wild-type background (B mice), expressed Wt1 levels comparable with wild-type animals (data not shown). All RT–PCR results were confirmed using northern blot analysis (data not shown). Taken together, these findings suggest the presence of a regulatory feedback mechanism to maintain constant Wt1 expression levels.

Reduced expression of Wt1 causes glomerular disease

Wt1-null mice with one copy of the human transgene (BKK mice) did not develop the embryonic-lethal defects found in homozygous Wt1-knockout animals and survive at least till birth. Of the newborn mice, 26% developed two kidneys, 14% one kidney and 60% no kidneys (Table 1). Mice without kidneys died within 48 h after birth. The remaining mice...
suffered from congenital nephrotic syndrome with severe albuminuria (>1 mg/ml) and died within 3 weeks (Figs 3 and 4; Table 1). Histological analysis of the kidneys at day 10 demonstrated diffuse global mesangial sclerosis, which rapidly developed into severe crescentic glomerulonephritis with numerous tubular cysts, protein and/or cellular casts and severe interstitial inflammation (Fig. 4). Kidneys from 2-day-old BKK mice showed a thinner proliferating zone than their wild-type littermates. Ten days after birth, nephrogenesis should have been completed in the mouse. However, expression patterns of WT1 (Fig. 5) and the paired box protein Pax2 (data not shown) indicated that nephrogenesis was delayed in BKK mice. In addition, the kidneys showed nephrogenic rests, demonstrating that nephrogenesis was not only delayed, but occasionally completely blocked. Immunohistological analysis of the extracellular matrix receptor protein integrin α3 expression (Fig. 5) and actin-associated protein synaptopodin (17 and data not shown) demonstrated a dramatic reduction in the complexity of the glomerular tuft. Similarly, the expression pattern of the podocyte slit specific protein nephrin was also found to be altered reflecting the footprocess retraction (Fig. 5). In contrast, expression of the glomerular marker vimentin remained unchanged (data not shown).

Wt1-null mice with two copies of the human transgene (BBKK mice) showed higher WT1 expression levels compared to BKK mice. In accordance, the observed phenotype was less severe and BBKK mice always developed one (24%) or two kidneys (76%) at birth. From 6 weeks onwards, the BBKK mice also developed albuminuria and 26% of the mice died within 150 days as a result of end-stage renal failure (Fig. 3; Table 1). Histological examination of kidney sections showed diffuse mesangial sclerosis with or without tubular, interstitial damage (Fig. 4). The expression pattern of WT1 (Fig. 5) and Pax2 (data not shown) indicated that, as in the BKK mice, nephrogenesis was delayed albeit less dramatically. The expression pattern of integrin α3 was less complex upon the development of albuminuria, suggesting integrin α3 to be a sensitive marker of podocyte phenotypic changes (Fig. 5).

Our results indicated that the expression level of Wt1/WT1 does not only play an important role during nephrogenesis, but also in the homeostasis of normal kidney function. This hypothesis was strengthened by the analysis of mice, heterozygous for the Wt1 mutation (K mice). Eleven percent of these mice died within 150 days (Fig. 3; Table 1) and all of the diseased K mice showed diffuse mesangial sclerosis with tubular cysts, protein casts and severe interstitial inflammation. No abnormalities could be detected in their wild-type counterparts (Fig. 4). All diseased K mice showed severe albuminuria (data not shown). In contrast to rescued mice, however, the Wt1 and Pax2 expression pattern at day 10 after birth was comparable with that of wild-type animals, indicating that nephrogenesis was not delayed (data not shown). Similar to the BBKK mice, the expression pattern of integrin α3 became more simplified upon the development of albuminuria (data not shown).

Expression levels of nephrin and podocalyxin are significantly decreased in BKK and BBKK mice

The striking phenotype in our rescued mice prompted us to analyze the expression of genes that have been shown to be involved in glomerular disease. Mutations of the NPHS1 gene (encodes nephrin) (9), cd2ap (18) and podocalyxin (19) have been demonstrated to cause glomerular malformation and dysfunction. Semi-quantitative RT–PCR analysis of cd2ap did not show any difference of expression between BKK, BBKK and the wild-type control (data not shown). In contrast, real-time PCR analysis demonstrated that nephrin expression was downregulated to as little as 14% in BKK animals (day 20). Similarly, heterozygous Wt1 knockout mice showed a drop in nephrin expression to 68% of wild-type levels at a time-point well before the onset of glomerular disease (Fig. 2). Moreover, BKK and BBKK mice showed a dramatic decrease in the level of expression of podocalyxin, another podocyte-specific gene (Fig. 2).

Table 1. Summary of the BKK, BBKK, K and B mice

<table>
<thead>
<tr>
<th>Mouse</th>
<th>WT1 level in % (n = 5) day 20, means ± SEM</th>
<th>Kidney number</th>
<th>Albuminuria</th>
<th>Death</th>
<th>Histology in brief</th>
</tr>
</thead>
<tbody>
<tr>
<td>KK</td>
<td>0</td>
<td>100%</td>
<td>0</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>AKK</td>
<td>(10)</td>
<td>Nephrogenesis arrested at comma-shaped body stageb</td>
<td>–</td>
<td>–</td>
<td>100%, within 48 hours after birth Renal dysplasia or agenesis</td>
</tr>
<tr>
<td>BKK</td>
<td>62 ± 11.9</td>
<td>60%</td>
<td>14%</td>
<td>26%</td>
<td>Congenital nephrotic syndrome 100% within 20 days (n = 29) Crescentic glomerulonephritis</td>
</tr>
<tr>
<td>BBKK</td>
<td>70 ± 8.9</td>
<td>–</td>
<td>24%</td>
<td>76%</td>
<td>Adult-onset nephrotic syndrome 26% within 150 days (n = 74) Diffuse mesangial sclerosis</td>
</tr>
<tr>
<td>K</td>
<td>95 ± 15.5</td>
<td>–</td>
<td>–</td>
<td>100%</td>
<td>Adult-onset nephrotic syndrome 11% within 150 days (n = 46) Diffuse mesangial sclerosis</td>
</tr>
<tr>
<td>Wild-type</td>
<td>100 ± 9.26</td>
<td>–</td>
<td>–</td>
<td>100%</td>
<td>No 0% within 150 days (n = 21) Normal</td>
</tr>
</tbody>
</table>

KK, Wt1-null (−/−) mouse (7); AKK, Wt1-null mice carrying the WA280 YAC transgene (8).

aWT1 expression level of WA280 compared to endogenous WT1 level; actual WT1 level in AKK mice could be slightly higher due to autoregulation.

bVariable phenotype, see Moore et al. (8).
Sequences 3′ of \( Wt1 \) are required for high levels of expression

\( Wt1 \) knockout mice have no kidneys due to a failure of the nephric duct to grow out and due to apoptosis of the metanephric blastema. In an earlier study, we have attempted to rescue the null mutation with a 280 kb YAC construct (8). In these experiments, only partial complementation of the null phenotype was achieved and mice died soon after birth with dysplastic kidneys. Functional nephrons never formed, even when the transgene was bred to homozygosity. In contrast, the 470 kb YAC in the present study rescued most of the developmental defects associated with the \( Wt1 \) null mutation and BKK mice survived until 3 weeks after birth. Similarly, transgene expression was significantly higher in BKK mice (62%), compared to animals carrying the 280 kb construct (~10%). Interestingly, the pattern of expression did not seem to differ in mice transgenic for a 280 or 470 kb construct (14). Taken together, these data suggest that elements important for high levels, but not tissue-specific expression are located downstream of the \( WT1 \) gene. Such sequences could have the form of a matrix attachment region or other chromatin organizing elements. Interestingly, \( PAX6 \), a gene mapping only 700 kb downstream of \( WT1 \), also shows a very complex regulation, with regulatory elements mapping 200 kb downstream of the gene (20).

WT1 acts at multiple stages during kidney formation

Our rescue experiments have demonstrated several crucial functions for \( Wt1 \) throughout kidney development. Depending on \( Wt1/WT1 \) expression levels, nephrogenesis is impaired

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Figure 3. Survival of BKK, BBKK, K and B mice. (A) Protein analysis of urine collected from BBKK mice. Severe albuminuria is detected from week 6 of life. Lanes 1–9, urine samples from 90–120-day-old BBKK mice. Lanes 1, 2, 3 and 6 are positive for albumin, with lane 6 at a nephrotic level. (B) x-axis, age (days); y-axis, percentage survival. B mice survive without any abnormalities or disease for >500 days, which were plotted on a curve identical to wild-type mice; in contrast, 11% of K mice and 26% of BBKK mice die within 150 days due to renal failure. More strikingly, all BKK mice die within 20 days of birth.
completely (Wt1-null mice) (7), arrested at the comma-shaped body stage (WA280 mice) or delayed (BKK and BBKK mice) (8). In this paper, we have shown that sufficient expression levels of \textit{Wt1}/\textit{WT1} are required not only for successful completion of nephrogenesis, but also for the maintenance of renal function in adult life. Reduced expression levels resulted in the development of glomerular diseases such as crescentic glomerulonephritis (BKK mice) and mesangial sclerosis (BBKK mice).

A single copy of the human transgene was not sufficient in all cases to rescue all embryonic defects, and 60% of the BKK mice failed to develop kidneys. Embryonic analysis of rescued animals clearly demonstrated a complete lack of ureters (data not shown), hence supporting the idea that a certain threshold of \textit{Wt1}/\textit{WT1} expression is required for ureteric bud induction from the mesonephric duct. Since our transgene covers the human locus, we cannot directly compare expression levels between the transgenic and wild-type animals. Moreover, the human protein may be slightly less active in its functions in the mouse, although \textit{Wt1}/\textit{WT1} amino acid sequences are >99% conserved between mouse and human (21). The observed variability in the number of kidneys formed is likely to be due to genetic modifiers as our experiments were carried out on a mixed genetic background. Breeding onto a defined background may allow us to confirm and possibly identify such modifiers.

In contrast to the clear developmental abnormalities, such as renal aplasia and nephrogenic rests, the observed kidney diseases are unlikely to be caused by developmental defects. Detailed histological analysis of mice heterozygous for the \textit{Wt1} knockout mutation (K mice) did not reveal any delay of nephrogenesis and glomeruli seemed normal. The very similar phenotype of \textit{K} and BBKK animals demonstrates that the kidney disease in rescued animals is not due to an incompatibility of the human \textit{WT1} transgene with mouse development, but rather the cause of insufficient functional \textit{WT1} protein. However, we cannot exclude that the human protein works...
slightly less efficiently within the mouse, which may contribute to the severity of the phenotype.

**Wt1/WT1 expression levels and regulatory feedback mechanisms**

We have also analyzed Wt1/WT1 expression levels and correlated them with the observed phenotype. To our surprise, changes of expression levels in the various models were relatively small. Halving the gene dosage did not reduce Wt1/WT1 expression levels by 50% and mice heterozygous for Wt1 (K mice) still expressed ~95% of normal wild-type Wt1 mRNA levels 20 days after birth. Similarly, expression levels in BKK animals (62% of endogenous levels) were 89% of those in BBKK mice. These findings demonstrate the presence of a strong autoregulatory feedback mechanism, which maintains Wt1/WT1 expression levels close to the wild-type situation. The feedback mechanism may be either direct, with Wt1 acting as a repressor, or may be realized through other regulatory factors. In support of the first hypothesis, Wt1 has been shown to repress its own promoter through other regulatory factors. In support of the first hypothesis, Wt1 has been shown to repress its own promoter at least in vitro (22,23).

The reduced amount of Wt1/WT1 expression in BKK (62%) and BBKK (70%) mice may be due to several reasons. Firstly, the Wt1/WT1 locus in mouse and man may be different, and mouse specific regulatory factors may act less efficiently on the human transgene. Alternatively, cis-elements essential for full expression may not be included on our 470 kb YAC construct. Also, there may be competition between the mouse and human Wt1/WT1 promoter for a limiting transcription factor(s) resulting in less efficient activation of the transgene. Finally, the reduction may be due to the remaining loxP site inserted into intron 1 in our construct. Interestingly, an antisense transcript with a start site within intron 1 has been reported (24), which has been suggested to be involved in the control of WT1 expression. A deregulation of antisense transcription caused by the insertion of the loxP sequence, may therefore have consequences on the expression of our transgene.

**Reduced expression levels of WT1 are responsible for several renal diseases**

The consistent expression of Wt1/WT1 in mature podocytes has suggested an important function within this cell type throughout life. Indeed, recent reports have shown that mutations in WT1 can be associated with isolated cases of diffuse mesangial sclerosis (IDMS) or incomplete DDS in human patients (12,13,25–27). Mesangial sclerosis observed in DDS patients can be caused by dominant mutations in the WT1 gene (28), and a DDS mouse model with a truncation of the WT1 gene develops typical glomerular defects (11). Our results suggest that the phenotype is the result of reduced expression rather than that of gain-of-function mutations. DDS is therefore likely caused through the formation of WT1 heterodimers between wild-type and mutant proteins leading to a reduction of functional protein, as has been proposed previously (11,29,30). Interestingly, human mutations leading to truncated proteins are rarely associated with renal disease, possibly indicating that in humans the truncated proteins are quickly degraded and replaced by proteins from the wild-type allele.

Frasier patients suffer from focal segmental glomerular sclerosis and also carry WT1 mutations. Interestingly, all of the mutations detected so far are intronic and interfere with the production of +KTS splice variants from the mutant allele. Our group has recently reported a mouse model for the human Frasier syndrome (31). Surprisingly, mice carrying the heterozygous Frasier mutations develop diffuse mesangial sclerosis, which is typically associated with DDS patients. These data together with a recent report by Kohsaka et al. (32) suggest that Frasier and DDS syndrome are overlapping disease entities and may therefore share the same molecular mechanism. Moreover, since mutations in Frasier syndrome are caused by a reduction of +KTS variants, we can conclude that these isoforms are crucial for normal podocyte function.

Progressive renal damage has also been reported in children nephrectomized for Wilms` tumor (33,34), but it was argued that this could be the result of the nephrectomy itself and/or radio- and chemotherapy (35). Our results may suggest that at least a proportion of these patients develop glomerulosclerosis due to reduced WT1 expression levels, although only 10–15% of Wilms` tumors are associated with mutations in the WT1 gene. Certainly, WAGR patients, in which one allele of WT1 is deleted (36,37), are likely to develop progressive renal damage and should therefore be closely monitored.

**Mechanistic aspects of glomerulosclerosis**

We have begun to dissect the molecular mechanisms leading to the glomerular diseases caused by WT1. Using a candidate target approach we could show that both podocalyxin and nephrin are downregulated in animals with reduced Wt1/WT1 expression. Both proteins are expressed at high levels in the podocyte, and reduced levels of them may well be the primary cause of the glomerulosclerosis in our animal models. Podocalyxin is the major sialoprotein of glomerular epithelial cells and helps to maintain the characteristic architecture of the foot processes (38). Interestingly, podocalyxin has been shown to be a downstream target of WT1 (39). Homozygous podocalyxin knockout mice fail to develop foot processes and slit diaphragms and die soon after birth due to kidney failure (19). Nephrin is a transmembrane protein, which is believed to be a major component of the slit diaphragm, bridging the gap between two adjacent foot processes (40). Mutations in NPHS1 are associated with the Finnish type congenital nephrotic syndrome (41). Similarly, nephrin (encoded by nphs1)-null animals show effacement of foot processes and lack slit diaphragms, resulting in neonatal death (42). The relatively small drop in podocalyxin levels even in BKK animals is unlikely to be solely responsible for the glomerular phenotype, in particular since podocalyxin splice variants are normal. In contrast, nephrin levels are dramatically decreased in BKK, BBKK and even K mice. The absence of a glomerular phenotype in nphs1 dom animals may be due to differences in the genetic background, or maybe of a rather late onset, which may have been missed in the initial analysis of nephrin knockout animals. Alternatively, a combination of podocalyxin and nephrin and possibly other factors may be responsible for the observed phenotype. Crossbreeding of Wt1 heterozygous mice with either nephrin or podocalyxin knockout animals should allow us to clarify the involvement of these proteins in the pathogenesis of glomerulosclerosis.
MATERIALS AND METHODS

YAC targeting

Using a homologous recombination strategy in yeast, the En2-SA-IREs-βgeo-pA fragment from pGT1.8 (15) and a yeast selection marker gene Leu2 were inserted into intron 1 of the human WT1 YAC, WT470 (Fig. 1A). The targeting event in yeast was confirmed by Southern hybridization with a human WT1 cDNA probe (8) and genomic DNA probe covering exon 1 and intron 1. The modified WT1 YAC was named WT64 thereafter.

Microinjection, identification of the transgene and genotyping

YAC DNA for microinjection was gel-purified, quantified and microinjected into fertilized oocytes (FVB/NxC57/B6 mating; mice purchased from Charles River) as described previously (43). Founder mice were first screened by human WT1-specific primer pairs located in intron 9 (5′-ACT TCA CTC GGG CCT TGA TAG-3′) and exon 10 (5′-GTG GAG GTG CAG ACT TGA AAG-3′). Full-length integration was confirmed using primer pairs specific for vector arms (long vector arm specific primers, 5′-TTT CAC AGG TAG TTC TGG TC-3′ and 5′-TGG GAA GCT GCA CTG AGT AG-3′; short vector arm specific primers, 5′-TAT ATC GCC GAC ATC ACC G-3′ and 5′-TTA GGA AGC AGC CCA GTA G-3′), upstream region, downstream region, the loxP-flanked cassette region (further primer information available on request) and by Southern hybridization with the human WT1 cDNA (8) as a probe. founders were genotyped using various primer pairs to distinguish the WT64 transgene (see above) and the WT1 knockout allele: L272, 5′-GGG GAG GAG TAG AA-3′; L273, 5′-TGA CTA GGG GAG GAG TAG AA-3′; L274, 5′-GCA GCT CGT AGT AG-3′; short vector arm specific primers, 5′-CAT ATG GCC GAC ATC ACC G-3′ and 5′-TAT GGA AGC AGC CCA GTA G-3′). Full-length integration was confirmed using primer pairs specific for vector arms (long vector arm specific primers, 5′-TTT CAC AGG TAG TTC TGG TC-3′ and 5′-TGG GAA GCT GCA CTG AGT AG-3′; short vector arm specific primers, 5′-TAT ATC GCC GAC ATC ACC G-3′ and 5′-TTA GGA AGC AGC CCA GTA G-3′), upstream region, downstream region, the loxP-flanked cassette region (further primer information available on request) and by Southern hybridization with the human WT1 cDNA (8) as a probe. Primers and probes for nphs1 (encodes nephrin): 5′-AGG GTC GGA GGA GGA TCG AA-3′; 5′-GGG AAG ACT GGA ACT GAA GT-3′; 5′-CTA ACC GTG GAG CTT CTG TTG GCC C-3′; 5′-GC Red640-TTT GCC CGG TCA GCT GTG ACT CAT A-3′; 5′-LC Red640-TTT GCC CGG TCA GCT GTG ACT CAT G-3′; 5′-GC Red640-GCC CCT ATT CTA GAT AAC CCG GGG-3′. Individual samples were standardized by measuring the amount of Gapdh RNA using quantitative PCR as described (31).

Histology and immunohistochemistry

For histological analysis, kidneys were fixed overnight in 4% paraformaldehyde (PFA) at 4°C and embedded in paraffin. Sections were cut at 1–2 µm and stained by PAS (periodic acid and Schiff staining plus hematoxylin counterstain).

Kidneys for immunohistochemical analysis were fixed for 15 min in 4% PFA on ice, rinsed once in 1× PBS for 10 min and embedded in Tissue-Tek® O.C.T. compound (Sakura Finetek Europe B.V.). Cryosections were cut at 8 µm. Immunostaining was carried out as described (14). Antibodies against WT1 (C-19), Integrin α3 (C-18) and Vimentin (C-20) were purchased from Santa Cruz. Pax2 antibody was purchased from BabCo, Inc. Fluorescent conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. Fluorescent images were produced on Zeiss Axioplan2 imaging microscope with a SPOT-RT camera and Metamorph (v.4.1.2) software.

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