Impaired Glomerular Maturation and Lack of VEGF165b in Denys-Drash Syndrome

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Individuals with Denys-Drash syndrome (DDS) develop diffuse mesangial sclerosis, ultimately leading to renal failure. The disease is caused by mutations that affect the zinc finger structure of the Wilms’ tumor protein (WT1), but the mechanisms whereby these mutations result in glomerulosclerosis remain largely obscure. How WT1 regulates genes is likely to be complex, because it has multiple splice forms, binds both DNA and RNA, and associates with spliceosomes. Herein is described that in DDS podocytes, the ratio of both WT1 +KTS isoforms C to D differs considerably from that of normal child and adult control podocytes and more closely resembles fetal profiles. Aside from the delay in podocyte maturation, DDS glomeruli show swollen endothelial cells, reminiscent of endotheliosis, together with incompletely fused capillary basement membranes; a dramatic decrease in collagen αd(IV) and laminin β2 chains; and the presence of immature or activated mesangial cells that express α-smooth muscle actin. Because appropriate vascular endothelial growth factor A (VEGF-A) expression is known to be essential for the development and maintenance of glomerular architecture and function, this article addresses the question of whether VEGF-A expression is deregulated in DDS. The data presented here show that DDS podocytes express high levels of the proangiogenic isoform VEGF165, but completely lack the inhibitory isoform VEGF165b. The VEGF165/VEGF165b ratio in DDS resembles that of fetal S-shaped bodies, rather than that of normal child or adult control subjects. The alteration in VEGF-A expression presented here may provide a mechanistic insight into the pathogenesis of DDS.

Denys-Drash syndrome (DDS) is a rare urogenital disorder that is associated with male pseudohermaphroditism, a high risk for Wilms’ tumors, and diffuse mesangial sclerosis, which presents before the age of 2 yr and progresses rapidly to end-stage renal failure (1–3). The disease is mainly caused by heterozygous germline missense mutations in exons 8 and 9 of the Wilms’ tumor-1 (WT1) gene, encoding zinc finger 2 and 3 of the protein. Despite this knowledge, the mechanisms whereby these mutations result in glomerulosclerosis are poorly understood. WT1 is a zinc finger transcription factor that is expressed throughout urogenital development and continues to be expressed in mature podocytes (4,5). Alternative splicing of the transcript results in four major isoforms, designated A (−/−), B (+/−), C (−/+), and D (+/+) to indicate the presence or absence of exons 5 and KTS, respectively. Isoforms that lack the three amino acids KTS between zinc finger 3 and 4 (−KTS) bind to DNA and are active in transcriptional regulation, whereas KTS-containing variants (+KTS) preferentially bind to RNA and may be involved in RNA processing (6,7). As shown by Hammes et al. (8), early kidney development in mice occurs despite the lack of either the +KTS or −KTS splice variants. However, glomerular differentiation and the prevention of glomerulosclerosis require both variants. This is in accordance with observations that were made in individuals with Frasier syndrome, who showed a reduction of +KTS variants as a consequence of intronic WT1 mutations and subsequently develop glomerulosclerosis in adolescence (9,10).

The crucial role of WT1 in kidney development is further supported by defects that are seen in various mouse models and organ cultures. Depending on WT1 expression levels,
nephrogenesis is impaired completely (11), arrested at the comma-shaped body stage, or delayed (12,13). It is interesting that mice that express a truncated form of WT1 show abnormal glomerular capillary development, suggesting that WT1 may control the expression of growth factors that affect endothelial cells and capillary structure (14). A similar mechanism may also be involved in early kidney development at the time when the metanephric mesenchyme interacts with angioblasts. As was shown recently by Gao et al. (15), WT1 regulates the expression of VEGF-A in metanephric mesenchyme, which in turn elicits an as-yet-unidentified signal from the angioblast, that is necessary for branching morphogenesis and nephrogenesis.

VEGF-A also exerts its function later in glomerular development and in the maintenance of the glomerular filtration barrier as shown in mice by Eremina et al. (16,17). The most abundant isoform, VEGF165, which in glomeruli is almost exclusively expressed by podocytes, becomes first detectable in S-shaped bodies (18,19) and stimulates endothelial cell migration, proliferation, and differentiation. Recently, a new isoform (VEGF165b) was found to inhibit VEGF165-mediated human umbilical vein endothelial cell proliferation and migration (20), VEGF165-mediated vasodilatation ex vivo (20), and angiogenesis in vivo (21) and to prevent VEGF165-mediated vascular endothelial growth factor receptor 2 (VEGFR2) phosphorylation and signaling in cultured cells (22). It is interesting that the inhibitory isoform is present in differentiated podocytes but absent in dedifferentiated podocytes (23). This splicing switch suggests that the maturation of podocytes, endothelial cells, and hence glomerular basement membrane (GBM) depends on the ratio of both isoforms.

In this report, we compared the maturation states of podocytes, endothelial cells, GBM, and mesangial cells in DDS kidneys with those of normal fetal, child, and adult control kidneys, endothelial cells, GBM, and mesangial cells in DDS kidneys from children and adults were obtained from tumor nephrectomy specimens. The diagnosis of DDS was confirmed by histologic examination and by the presence of WT1 missense mutations. Kidneys from individuals with DDS were obtained at the time of nephrectomy, either because of the presence of a Wilms' tumor or before kidney transplantation at ESRD. The parents of individuals with DDS gave informed consent regarding the study, and kidney tissue was used following the guidelines of the local ethics committee. All tissues that were obtained from individuals with DDS contained at least 50% control glomeruli for histologic evaluation. Table 1 summarizes the details of the analyzed material.

**Materials and Methods**

**Patients and Tissue Samples**

Formalin-fixed, paraffin-embedded or snap-frozen specimens from four fetal (estimated gestational age 16 to 27 wk), 10 child, eight adult, and nine DDS kidneys were included in this study. Normal control kidneys from children and adults were obtained from tumor nephrectomy specimens. The diagnosis of DDS was confirmed by histologic examination and by the presence of WT1 missense mutations. Kidneys from individuals with DDS were obtained at the time of nephrectomy, either because of the presence of a Wilms' tumor or before kidney transplantation at ESRD. The parents of individuals with DDS gave informed consent regarding the study, and kidney tissue was used following the guidelines of the local ethics committee. All tissues that were obtained from individuals with DDS contained at least 50% (usually more than 100) glomeruli for histologic evaluation. Table 1 summarizes the details of the analyzed material.

**WT1 Mutation Analysis**

Of the nine individuals with DDS, four (NS3, NS8, NS9, and NS10) were previously analyzed for WT1 mutations by PCR/single-strand conformational polymorphism and direct sequencing (24). Here, we analyzed five additional patients with the same method.

**Laser Microdissection and RNA Isolation**

Cryosections of 10 μm were stained with hematoxylin under RNase-free conditions, followed by rinses in 70, 80, and 100% ethanol and 100% xylene for 3 min. Human sections were air-dried for 3 min, and glomeruli were laser-microdissected using a PixCell II LCM system (Arcturus, Mountain View, CA) for <1 h per section. Total RNA was extracted with the Absolutely RNA Microprep kit (Stratagene, La Jolla, CA). The numbers of microdissected glomeruli per tissue are given in Table 2.

**Real-Time Reverse Transcriptase–PCR**

cDNA was synthesized by using the TaqMan Reverse Transcription Reagents (Applied Biosystems, Weiterstadt, Germany). Real-time reverse transcriptase–PCR (RT-PCR) was performed in quadruplicate using cDNA transcribed from 50 ng of total RNA, heat-activated TaqDNA polymerase (HotGold Star; Eurogentec, Seraing, Belgium), WT1 oligonucleotides (Table 3; 300 to 900 nM), and a WT1 fluorescence-labeled probe (FAM; 250 nM; 5′-AGCGATAACCAACACAAGCGCCATCC-3′) and run on a TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany). TaqMan reagents for human 18S rRNA were obtained from Applied Biosystems and served as a housekeeping gene. Cycle conditions for WT1 were as follows: 2 min at 50°C, 10 min at 95°C, 50 cycles at 95°C for 15 s, and at 60°C for 1 min. Similar amplification efficiencies for all four WT1 PCR were demonstrated by analysis of serial cDNA dilutions that showed a slope value of log input cDNA amount versus CT of <0.1. The ratio of the four WT1 isoforms within one tissue was calculated using the 2−ΔΔCT value and was expressed in percentage. The expression of VEGF165 was determined by real-time RT-PCR using the SYBR Green JumpStart TaqReadyMix (Sigma, Munich, Germany). Cycle conditions were 10 min at 95°C, 40 cycles at 95°C for 30 s, 68°C for 60 s, and 72°C for 60 s. A formula that took into account the different number of WT1-expressing cells (podocytes) per microdissected area of a glomerulus was used:

\[ E_{P1} = \frac{E_{P2}}{E_{P3}} = \frac{n_1}{n_2} \times \frac{p_2}{p_1} \times 2^{2\Delta CT} \]

where \( E_P \) is the expression of podocytes in a given tissue, \( n \) is the average number of cells per microdissected glomerulus (see Table 4), \( p \) is the average number of podocytes per microdissected glomerulus (see Table 4), and \( \Delta CT \) is \((C_{T \text{ of WT1}}) - (C_{T \text{ of 18S rRNA}})\).
Table 1. Patient data, mutation data, and analyses performed in this studya

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age at NS</th>
<th>Indication</th>
<th>WT1 Mutation</th>
<th>Material</th>
<th>Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>DDS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS3</td>
<td>37 mo</td>
<td>DDS</td>
<td>Cys385Arg</td>
<td>P</td>
<td></td>
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<td>NS8</td>
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<td>DDS</td>
<td>Arg394Tfp</td>
<td>F, P</td>
<td>D&lt;C</td>
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<tr>
<td>NS9</td>
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<td>DDS</td>
<td>Gly379Cys</td>
<td>F, P</td>
<td>No VEGF165b</td>
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<tr>
<td>NS10</td>
<td>14 mo</td>
<td>DDS</td>
<td>Arg394Tfp</td>
<td>F</td>
<td>D&lt;C</td>
</tr>
<tr>
<td>NS18</td>
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<td>DDS</td>
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<td>++</td>
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<tr>
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<td>His373Arg2</td>
<td>F</td>
<td>D&lt;C</td>
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<tr>
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<td>DDS</td>
<td>Arg394Tfp</td>
<td>P</td>
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<tr>
<td>NS21</td>
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<td>Cys388Argb</td>
<td>F, P</td>
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<tr>
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<td>DDS</td>
<td>Arg394Tfpb</td>
<td>EM</td>
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Fetal
NEK1 24 wk Abortion F D<C (C) –(S) –(S) (+) (+)
NEK2 16 wk Abortion F D<C (C) +(+)(C) +(+)(C)
NEK5 20 wk Abortion P + + + +(C) +(C)
NEK6 27 wk Abortion P +(C) +(C)

Child
NAK9 60 mo WT F C>D VEGF165–VEGF165b ++ ++
NAK9 Not known WT F + + ++ ++ + + VEGF165–VEGF165b ++ ++
NAK23 84 mo WT F VEGF165–VEGF165b ++ ++
NAK7 60 mo WT P VEGF165–VEGF165b ++ –
NAK8 24 mo WT P ++ –
NAK18 24 mo WT P ++ –
NAK19 24 mo WT P ++ –
NAK20 96 mo WT P ++ –
NAK21 24 mo WT P ++ –

Adult
NAK4 60 to 70 yr Neoplasm F C>D VEGF165–VEGF165b ++ ++
NAK5 60 to 70 yr Neoplasm F C>D VEGF165–VEGF165b ++ ++
NAK6 25 yr Accident F VEGF165–VEGF165b ++ ++
NAK7 60 to 70 yr Neoplasm F C>D + + ++ ++ + + VEGF165–VEGF165b ++ ++
NAK10 Not known Neoplasm P –
NAK13 Not known Neoplasm P –
NAK14 Not known Neoplasm P –
NAK15 70 yr Neoplasm P –

a(C), capillary loop stage; Coll IV, collagen IV; DDS, Denys-Drash syndrome; EM, material for electron microscopy; F, frozen; Lam, laminin; Ns, nephrectomy; P, paraffin-embedded; (S), S-shaped body stage; SMA, smooth muscle actin; VEGF, vascular endothelial growth factor; WT, Wilms’ tumor; −, absent; (+), focal positivity; +, positive; ++, prominent positivity.

aMutations found in the present study.

aNot suitable as a donor for transplantation because of a bacterial infection.

Table 2. Numbers of laser microdissected glomeruli for RT-PCR

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. of Glomeruli</th>
</tr>
</thead>
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<tr>
<td>WT1 RT-PCR</td>
<td></td>
</tr>
<tr>
<td>fetal (16 wk) capillary loop (n = 1)</td>
<td>60</td>
</tr>
<tr>
<td>fetal (24 wk) capillary loop (n = 1)</td>
<td>300</td>
</tr>
<tr>
<td>child (n = 1)</td>
<td>640</td>
</tr>
<tr>
<td>adult (n = 3)</td>
<td>300 to 400</td>
</tr>
<tr>
<td>DDS (n = 3)</td>
<td>200 to 360</td>
</tr>
<tr>
<td>VEGF, laminin, collagen RT-PCR</td>
<td></td>
</tr>
<tr>
<td>fetal (16 wk) s-shaped body (n = 1)</td>
<td>100</td>
</tr>
<tr>
<td>fetal (16 wk) capillary loop (n = 1)</td>
<td>200</td>
</tr>
<tr>
<td>child (n = 1)</td>
<td>720</td>
</tr>
<tr>
<td>adult (n = 1)</td>
<td>300</td>
</tr>
<tr>
<td>DDS (n = 1)</td>
<td>320</td>
</tr>
</tbody>
</table>

aRT-PCR, reverse transcriptase–PCR.

Antibodies and Immunostaining

Immunohistochemistry on formalin-fixed, paraffin-embedded tissues was performed following previously published protocols (26) and on cryosections as described previously (25). Primary antibodies were as follows: For anti-WT1, mAb antibody clone 6F-H2 (DAKO, Hamburg, Germany); for anti-collagen α4(IV), rabbit polyclonal antibody (27); for anti-laminin β2, mouse mAb clone C4 (Hybridoma Bank, Iowa City, IA) (28); and for anti-α-smooth muscle actin (α-SMA), mouse mAb clone 1A4 (DAKO). Morphologic features of the various renal tissues were examined in periodic acid-Schiff–stained sections.

Results

Histopathologic Findings in DDS

DDS glomeruli showed diffuse mesangial sclerosis by light microscopy (Figure 1A). Various stages coexisted in the same specimen with a corticomedullary gradient of involvement, the deepest glomeruli being affected the least: (1) Early stages with mild to moderate increase in the mesangial matrix without mesangial cell proliferation; (2) fully developed lesion with...
thickening of the basement membranes and massive enlargement of mesangial areas and obliteration of capillary lumens; and (3) advanced stages with the typical nodular, “cannon ball” mesangial sclerosis. These findings are in accordance with those from Habib et al. (3,29). Transmission electron microscopy showed massive hyaline deposits with electron-dense strands within sclerosed glomeruli (Figure 2A), and a widespread effacement of podocyte foot processes was found (Figure 2B). Furthermore, endothelial cells were swollen and lacked fenestrae, reminiscent of endotheliosis in eclampsia/pre-eclampsia (Figure 2B). Finally, incompletely fused basement membranes and fibrillar material between two layers were observed. Together, these findings suggest disturbances in podocyte, endothelial cell, and GBM maturation.

**Mutation Analysis and Expression of the WT1 Gene in DDS**

We report the mutation analysis of five novel individuals with DDS (NS18, NS19, NS20, NS21, and NS22; Table 1). The other four investigated here (NS3, NS8, NS9, and NS10) were previously described by us (24).

Because missense mutations are thought to act in a dominant negative manner, the presence of a mutant WT1 transcript/protein is mandatory. By RT-PCR and direct sequencing, it was shown that a mutant transcript is present at similar levels as the wild-type transcript in the analyzed kidneys of patients NS8, NS10, and NS18 (Figure 3 shows transcripts from NS10).

Furthermore, by immunohistochemistry, WT1 was found to be restricted to podocyte precursors during glomerulogenesis.
and to podocytes postnataally (Figure 1B), which is in accordance with previous studies (4,5). In DDS, WT1 staining in podocytes was observed in affected glomeruli at all disease stages. However, in advanced stages, a reduced number of WT1-positive nuclei was observed, suggesting a loss of podocytes during progression of the disease (Figure 1B).

**Maturation Status of Podocytes in DDS**

The molecular marker WT1 was used to analyze the maturation status of podocytes in DDS. Mild to moderately affected glomeruli from four individuals with DDS, glomeruli from three adult control kidneys and one child control kidney, and capillary loop/immature glomeruli from two fetal kidneys (16 and 24 wk of gestation) were laser-microdissected and processed for RT-PCR. Figure 4 demonstrates the ratio of the four main WT1 mRNA isoforms within a given sample. During development, most obviously, the ratio of isoform C to D changes by increasing continuously: 0.3 in 16-wk-old fetal glomeruli, 0.7 in 24-wk-old fetal glomeruli, 1.7 in glomeruli of a child, and 2.0 (range 1.6 to 2.4) in adults. Glomeruli from individuals with DDS showed a ratio of 0.5 (range 0.3 to 0.7), resembling fetal capillary loop stages. This demonstrates a delay in podocyte maturation in DDS.

**Maturation Status of the GBM in DDS**

Mild to moderately affected glomeruli from a DDS kidney showed the same distribution of collagen (IV) and laminin β chains as fetal S-shaped bodies, namely high collagen α1(IV) and laminin β1 levels and a lack of collagen α4(IV) and laminin β2 chains (Figure 5). This demonstrates that the developmental switch from collagen α1(IV) to α4(IV) and from laminin β1 to β2 (30) is affected in DDS and that GBM development is arrested at the fetal S-shaped body stage. These data were confirmed in multiple samples by immunohistochemical staining of collagen α4(IV) and laminin β2 (Figure 1; Table 1). Glomerular expression of collagen α4(IV) was first evident within the mesangium and GBM of capillary loop stage glomeruli (Figure 1C). Strong expression persisted in child/adult glomeruli but was significantly reduced in DDS glomeruli. The glomerular deposition of laminin β2 showed a pattern that was similar to that of collagen α4(IV) during the course of glomerular maturation (Figure 1D): Absent in very early glomerular structures,
strongly positive in the mesangium and GBM of capillary loop stage glomeruli, and persistent expression postnatally. In DDS, the expression of laminin β2 was markedly reduced and completely absent in several glomeruli, suggesting a delay in GBM maturation in DDS kidneys.

**Maturation Status of the Mesangium in DDS**

Immunostaining for α-SMA was performed to evaluate the status of mesangial cells in DDS. As demonstrated in Figure 1E, glomerular α-SMA normally first becomes detectable in the cytoplasm of invading mesangial cells in fetal capillary loop stage glomeruli. In mature human glomeruli, mesangial expression of α-SMA is normally absent, but under disease conditions, α-SMA is detected in activated and/or proliferating mesangial cells (31). In cases of DDS (Figure 1E, bottom), α-SMA is detected within glomeruli, indicating the presence of activated and/or proliferating mesangial cells.

**Altered VEGF165 Splicing in DDS**

The data presented here suggest that glomerular maturation in DDS is delayed and affects podocyte, endothelial cell, mesangial cell, and GBM maturation. To address the question of whether this may be caused by disturbed cross-talk between podocytes and endothelial cells, we analyzed VEGF165 as a mediator, which is secreted by podocytes and bound by receptors on endothelial cells. Figure 6A shows the relative amount of the angiogenic stimulatory VEGF165 mRNA in various tissues after normalization to either 18S rRNA alone or 18S rRNA and the number of podocytes. The amount of VEGF165 mRNA increases dramatically between the S-shaped body and capillary loop stage in the fetal kidney and decreases in mature glomeruli from a child and an adult. In contrast to our observations in child and adult glomeruli, DDS glomeruli exhibit a major upregulation of VEGF165 expression, which becomes even more marked when the number of podocytes are taken into account.

The ratio of the stimulatory VEGF165 to the inhibitory VEGF165b isoform was determined by semiquantitative RT-PCR. The primers that were used to amplify both forms in a single reaction do not discriminate between the VEGF165 and VEGF189 isoforms. However, it was shown previously that VEGF165 is the most abundant isoform in glomeruli (18,19). During normal development, podocyte maturation is associated with a switch from the stimulatory to the inhibitory isoform (Figure 6B). Therefore, whereas podocytes from fetal S-shaped bodies solely express the stimulatory form, podocytes from the capillary loop stage and onward exhibit increased...
expression of VEGF165b. In contrast, podocytes from six patients with DDS continue to express the stimulatory isoform VEGF165 at high levels and fail to express the inhibitory isoform VEGF165b (Figure 6C).

**Discussion**

Our findings suggest that in DDS, glomerular maturation is delayed and VEGF165 splicing is defective. Kidneys from affected patients exhibit complex glomerular disturbances, including an “endotheliosis-like injury of endothelial cells” with the lack/loss of fenestration, incompletely fused basement membranes with a dramatic decrease in collagen α4(IV) and laminin β2 chains, the presence of immature podocytes showing foot process effacement, and the presence of immature or activated mesangial cells.

**Abnormal WT1 Expression in DDS Glomeruli**

We have demonstrated an overall delay in podocyte maturation in DDS glomeruli, compared with normal child and adult control kidneys. During normal development, isoform D predominates in the fetal capillary loop stage, whereas isoform C becomes more abundant in child and adult glomeruli, suggesting that this latter form may be involved in terminal differentiation processes and in maintaining podocyte function. Remarkably, the ratio of WT1 isoforms C to D in DDS glomeruli most closely resembles that observed in the fetal kidneys at the capillary loop stage. However, the importance of these observations is unclear, because knockout mice that lack exon 5 corresponding to isoform C do not exhibit an obvious renal phenotype (32).

In addition, DDS glomeruli express mutant transcripts,
which are thought to act in a dominant negative manner at the protein level, leading to a dramatic loss of functional WT1 protein. Notably, both mouse in vivo and organ culture models have shown that glomerulogenesis is a WT1-dependent process (11–14,33). Therefore, abnormal glomerular development in DDS is likely due to loss of wild-type WT1.

Abnormal GBM and Mesangial Cell Maturation in DDS Glomeruli

We further demonstrate that the GBM fails to mature appropriately in DDS. DDS glomeruli express abnormally high levels of collagen α1(IV) and laminin β1, a pattern of aberrant collagen and laminin expression that previously was demonstrated in DDS by Yang et al. (34). In addition, DDS glomeruli exhibit dramatic reductions in expression of collagen α4(IV) and laminin β2 chains, factors that normally expressed at high levels in mature glomeruli. Several lines of evidence suggest that collagen α4(IV) and laminin β2 play a critical role in the maintenance of GBM function. Laminin β2 knockout mice (35) and individuals with Pierson syndrome as a result of LAMB2 mutations (which encodes laminin β2) (36) develop proteinuria. Similarly, loss-of-function mutations in COL4A4 (which encodes collagen α4[IV]) in individuals with Alport syndrome result in severe proteinuria (reviewed in reference [37]).

In our analysis of mesangial cell maturation, we show that DDS glomeruli express α-SMA, a marker for fetal mesangial cells, whereas control child and adult mesangial cells do not

Figure 6. Relative expression of total VEGF165 and ratio of VEGF165/VEGF165b mRNA in laser-microdissected glomeruli. S, S-shaped body; c, capillary loop stage. (A) Relative expression of VEGF165 determined by real-time RT-PCR and normalized to 18S rRNA alone (■) or to 18S rRNA and the podocyte number (□). (B) Semiquantitative RT-PCR of VEGF165 and VEGF165b. Aliquots of the PCR products from laser-microdissected material were run on an agarose gel after 30 and 40 cycles. –RT, control without reverse transcriptase; Fetal s, laser-microdissected S-shaped bodies from fetal kidney (16 wk of gestation); Fetal c, laser-microdissected capillary loops from fetal kidney (16 wk of gestation); C1, positive control for VEGF165; C2, positive control for VEGF165b; n/n, not known. (C) Semiquantitative RT-PCR of VEGF165 and VEGF165b. Aliquots of the PCR products from nonmicrodissected material were run on an agarose gel after 30 and 40 cycles. VEGF, vascular endothelial growth factor.
express α-SMA. Together with our previous observations, these results suggest that mesangial cell development is abnormal in DDS glomeruli. However, α-SMA is also expressed in activated mesangial cells after injury, as observed in other diseases (31), alternatively suggesting that mesangial cell activation occurs in DDS glomeruli.

**Altered VEGF Expression in DDS Glomeruli**

Podocyte–endothelial cell interactions are crucial for the development and maintenance of glomerular architecture and hence the glomerular filtration barrier. Eremina et al. (16) elegantly showed that VEGF-A is a key signal that mediates these podocyte–endothelial cell interactions. Indeed, podocyte-specific heterozygosity for VEGF-A results in glomerular maturation defects, glomerulosclerosis (17,16), and swollen endothelial cells (endotheliosis), the latter being part of the renal lesion that is seen in preeclampsia (38). The defects in mice ultimately lead to nephrotic syndrome and ESRD.

There are striking similarities between the phenotype of VEGF-A heterozygous mice and DDS. Together with the previous demonstration that WT1 controls expression of VEGF-A during normal renal development (15), we asked whether loss-of-function mutations in the podocyte-specific WT1 gene in DDS result in dysregulation of VEGF-A in the podocyte, leading to the DDS phenotype. To address this issue, we focused on the analysis of VEGF165, the predominant VEGF-A isoform in podocytes (18–20).

Our data show that during normal fetal development, angiogenic stimulatory VEGF165 expression is highest in the capillary loop stage and diminishes thereafter with an isoform switch in favor of the inhibitory VEGF165b isoform. VEGF165b first becomes detectable at the capillary loop stage and increases continuously, showing the highest expression in adult control glomeruli. It is interesting that the isoform switch of VEGF165 to VEGF165b is paralleled by a change in the ratio from the +KTS WT1 isoforms C to D, suggesting that the +KTS isoforms may be involved in the alternative splicing of VEGF165. In contrast, VEGF165 expression is elevated in DDS, whereas VEGF165b expression is completely abrogated, demonstrating that isoform switching is affected in DDS. Taken together, these results suggest that the complex pathologic changes that are seen in DDS are driven at least in part by a lack of VEGF165b, resulting in disturbed signaling among podocytes, endothelial cells, and mesangial cells. Such a disruption of signaling may be central to the genesis of endotheliosis, GBM, and mesangial cell alterations and may ultimately lead to glomerulosclerosis. Podocytes may respond to these secondary defects by upregulating the stimulatory VEGF165 isoform, which in turn may enhance glomerular permeability, leading to albuminuria and accumulation of mesangial matrix, as has been postulated for diabetic nephropathy (39).

Our results suggest that VEGF165 splicing is altered in WT1 mutated podocytes, resulting in an overall delay in glomerular maturation and glomerulosclerosis. Dysregulated VEGF165 splicing may reflect a novel pathogenic mechanism that is involved in other diseases. Bates et al. (40) showed that inhibitory VEGF isoforms are downregulated, whereas angiogenic stimulatory isoforms are moderately upregulated, in preeclamptic placentas. Complementary studies on diabetic retinopathy demonstrate a switch in splicing from inhibitory to stimulatory isoforms in this disease (41), and in renal cell carcinoma, the inhibitory VEGF165b isoform is downregulated (20). These examples illustrate the importance of maintaining the correct balance between stimulatory and inhibitory VEGF-A isoforms. These observations, together with the similarity of phenotype between DDS and VEGF-A heterozygous mice, suggest that at least a portion of the mouse phenotype may be due to an imbalance in the ratio of VEGF isoforms and reduction of VEGF164b expression.

**Conclusion**

The data presented here illustrate the importance of assessing the ratio of expression between stimulatory and inhibitory VEGF-A isoforms in disease, in contrast to conventional approaches that measured only overall VEGF-A expression. In the future, measuring both stimulatory and inhibitory isoforms may help to resolve conflicting reports concerning the role of VEGF in glomerular disease. We present here the first mechanistic insight into how aberrant expression of WT1 may affect VEGF165 splicing and result in glomerulosclerosis in DDS.

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**Disclosures**

None.

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