New insights into the function of the Wilms tumor suppressor gene WT1 in podocytes

Avril A. Morrison, Rebecca L. Viney, Moin A. Saleem, and Michael R. Ladomery

Bristol Genomics Research Institute, Centre for Research in Biomedicine, Faculty of Health and Life Sciences, University of the West of England, and Academic and Children’s Renal Unit, Lifeline Building, Southmead Hospital, University of Bristol, Bristol, United Kingdom

Submitted 17 December 2007; accepted in final form 28 March 2008

WILMS TUMOR WAS FIRST DESCRIBED by Max Wilms in 1899. Also known as nephroblastoma, Wilms is a highly malignant, rapidly developing tumor of the kidney. An investigation into the genetic causes of Wilms tumor led to the isolation by positional cloning and characterization of a candidate susceptibility gene called the Wilms tumor suppressor gene WT1 (6, 21). A mouse knockout of WT1 and, more recently, an RNA interference-mediated WT1 knockdown in fetal kidneys (9) have demonstrated that WT1 is required at multiple stages of kidney development. However, there is still no clearly defined function for WT1 in the podocytes of mature glomeruli. Several recent reviews have outlined the role of podocytes in glomerular disease (11, 30, 59), current therapies for Wilms tumor (50, 72), and the role of WT1 in the development of the genitourinary system (23). The aim of this review is to highlight recent studies that have advanced our understanding of WT1 function in mature podocytes.

The WT1 Gene and Its Protein Isoforms

The human WT1 gene spans 50 kb of genomic DNA (6) and comprises 10 exons (19, 22). It encodes a protein with an NH2-terminal proline- and glutamine-rich transactivation domain and four Cys2-His2 zinc fingers of the Krüppel type at the COOH terminus. The proline- and glutamine-rich domain is encoded by the first exon alone, and each zinc finger is encoded by a separate exon, exons 7–10 (22).

Several different isoforms of WT1 are expressed by alternative splicing. Two alternative splicing events give rise to four distinct WT1 isoforms (Fig. 1). Exon 5 is a short cassette exon, which adds 17 amino acids between the trans-activation domain and the zinc finger domain. An alternative splice donor site in exon 9 inserts three additional amino acids: lysine, threonine, and serine (abbreviated as KTS) between the third and fourth zinc finger (22). WT1 splice isoforms that contain exon 5 are only expressed in mammals, whereas isoforms that contain the KTS splice insert are expressed in all vertebrates (32). The ratio of (+/− exon 5) isoforms is regulated in a tissue-specific manner in the genitourinary system (kidney, uterus, ovaries, and testes) and in the hemopoietic system (spleen, fetal liver, and bone marrow), and the ratio of (+/− exon 5) isoforms alters in the kidney during differentiation. In contrast the ratio of (+/− KTS) isoforms is better conserved across tissues, ranging from 1.10 to 1.49 in humans (22, 67). The most prevalent mRNA isoform is +exon 5, +KTS, and the least common is −exon 5/−KTS (22). The four main isoforms are generated from translation initiation at the first initiator codon AUG (5), which gives rise to proteins with a molecular mass of 52–54 kDa depending on whether they include the 17 amino acids encoded by exon 5 (53). However, it should be noted that additional complexity arises from an upstream CUG start codon and a downstream AUG codon, the latter giving rise to an NH2 terminally truncated WT1 isoform termed AWT1 (8). Mouse knockouts of the upstream CUG start codon and of exon 5 develop normally and are fertile (51, 58); however, in contrast, mice with mutations that affect the evolutionarily conserved +/− KTS alternative splice are characterized by severe developmental defects of the genitourinary system (23).
Does WT1 Bind Both DNA and mRNA Targets?

Evidence for the interaction of WT1 with DNA. Given the evolutionary conservation of the +/− KTS alternative splice, and the consistency of isoform levels across tissues, the question arises as to the structural and functional consequence of the insertion of the amino acids KTS. Several studies over the past 15 years have shown that (−KTS) isoforms work best as transcription factors, generally via a GC-rich DNA target (Table 1). An NMR study of the zinc-finger domain demonstrated that the KTS tripeptide abrogates the ability of the fourth zinc finger to bind DNA (40).

Evidence for interaction of WT1 with RNA. In the mid-1990s, it became apparent that +KTS isoforms colocalize and interact with pre-mRNA and splice factors (10, 41). WT1 zinc fingers were also shown to bind RNA in vitro (2, 80), and WT1 was detected in mRNP particles in vivo (38) with a central role fingers were also shown to bind RNA in vitro (2, 80), and WT1 interact with pre-mRNA and splice factors (10, 41). WT1 zinc fingers were also shown to bind RNA in vitro (2, 80), and WT1 interact with pre-mRNA and splice factors (10, 41). WT1 zinc

WT1 Mutations and Developmental Abnormalities

The cells of the glomerulus, proximal, and distal tubules are all derived from the metanephric mesenchyme. Mutations in the WT1 gene can result in the disruption of any of these structures. The nature of the developmental abnormality is dependent on the nature of the disruption of the WT1 gene. Wilms tumor is derived from metanephric mesenchyme cells that fail to differentiate. The WT1 gene is either heterozygously or homozygously mutated in 10–15% of Wilms tumors (18). Other syndromes in which the WT1 gene is disrupted include WAGR (for Wilms tumor, aniridia, genitourinary abnormalities, and mental retardation) (42), Frasier syndrome (FS) (44), and Denys-Drash syndrome (DDS) (7, 29). WAGR results from chromosomal deletions at locus 11p13 that encompass the WT1 and Pax6 genes; the latter accounts for the aniridia phenotype. Reduction of WT1 expression levels in mice also gives rise to crescentic glomerulonephritis and mesangial sclerosis, the implication being that WT1’s target genes are not correctly expressed in podocytes. To confirm this, the expression of the podocyte-specific genes nphs1 and podocalyxin is clearly lower where WT1 levels are reduced (20). This comes as no surprise, as podocalyxin is a transcriptional target of WT1 (61).

WT1 mutations in FS. FS is characterized by unspecific focal segmental glomerular sclerosis (FSGS), male-to-female sex reversal (female external genitals, gonads of reduced size, XY karyotype), and a high risk of gonadoblastoma (52). FS results from a heterozygous point mutation in the splice donor site in intron 9, resulting in the loss of (+KTS) isoform expression from one allele (1, 35). The consequence is a reduction in the amount of WT1(+KTS), and this apparently subtle change in the ratio of WT1 isoforms leads to the severe developmental abnormalities seen in FS patients. The importance of the ratio of WT1 isoforms has been highlighted by the generation of mouse strains in which the ability to express specific isoforms has been removed. Heterozygous mice with a reduction in WT1(+KTS) isoform expression develop glomerulosclerosis and represent a model for FS, and homozygous mice not able to express +KTS isoforms show complete XY sex reversal (23, 39).

WT1 mutations in DDS. DDS is characterized by diffuse mesangial sclerosis. The condition manifests clinically as an early-onset nephrotic syndrome and progresses to renal failure

### Table 1. Proposed WT1 transcriptional targets

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Target Sequence</th>
<th>Repression/Activation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGR-1</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>47</td>
</tr>
<tr>
<td>PDGF-A</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>75</td>
</tr>
<tr>
<td>EGFR</td>
<td>TCC repeats</td>
<td>Repression</td>
<td>16</td>
</tr>
<tr>
<td>IGFR1</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>76</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>WTE</td>
<td>Activation</td>
<td>49</td>
</tr>
<tr>
<td>TGF-B1</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>12</td>
</tr>
<tr>
<td>Insulin-like growth factor 2 (IGF-2)</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>15</td>
</tr>
<tr>
<td>Amphiregulin</td>
<td>WTE</td>
<td>Activation</td>
<td>43</td>
</tr>
<tr>
<td>Podocalyxin</td>
<td>WTE</td>
<td>Activation</td>
<td>61</td>
</tr>
<tr>
<td>Nephrin</td>
<td>WTE</td>
<td>Activation</td>
<td>74</td>
</tr>
<tr>
<td>Pax-2</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>69</td>
</tr>
<tr>
<td>Sry</td>
<td>EGR-1 consensus</td>
<td>Activation</td>
<td>28</td>
</tr>
<tr>
<td>Dax-1</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>33</td>
</tr>
<tr>
<td>α4-integrin</td>
<td>EGR-1 consensus</td>
<td>Activation</td>
<td>34</td>
</tr>
<tr>
<td>c-myc</td>
<td>EGR-1 consensus</td>
<td>Repression</td>
<td>25</td>
</tr>
<tr>
<td>VEGF</td>
<td>EGR-1 consensus</td>
<td>Activation</td>
<td>24</td>
</tr>
</tbody>
</table>

Target sequences responsible for WT1-mediated effects: EGR-1 consensus, GC rich; TCC repeats, TC-rich binding sites; WTE, 10-bp GC-rich sequence. Whether any of the above are also mRNA targets of WT1 remains to be investigated.
during the first 3 yr of life. Patients also develop intersex disorders, the most common of which is gonadal dysgenesis with male pseudohermaphroditism; in addition, they frequently develop Wilms tumors. DDS is generally associated with heterozygous point mutations in the zinc finger region of WT1 (66); the most common occurs within exon 9, where an arginine is substituted for a tryptophan in zinc-finger 3 (4, 65). DDS can also result from mutations that cause the deletion of zinc fingers 3 and 4; this is confirmed in a mouse model of DDS (64). A recent report also describes a patient with DDS with a mutation in exon 1 of WT1 that is predicted to produce a termination signal in codon 90. This produces a severely truncated protein lacking the zinc finger region and part of the NH2-terminal regulatory region (46). Taken together, the different types of mutation indicate that DDS occurs as a result of altered or severely impaired DNA binding activity. This was confirmed using WT1 fusion constructs encompassing the most common DDS mutations (45). Using well-characterized WT1 consensus sequences, including the EGR1-like sequence and the (TCC)n motif from the PDGFA-chain promoter, DNA binding affinity was assessed. All of the DDS mutations abolished DNA binding. However, the possibility that mutations associated with DDS also abolish WT1’s mRNA binding activities remains to be investigated.

The severity of the genitourinary abnormalities that are associated with heterozygous DDS mutations has led to the suggestion that they may arise because of a dominant-negative effect exerted by the mutated forms of WT1. Interestingly, a recent study that examined the posttranscriptional function of (+KTS) isoforms of WT1 found that (−KTS) isoforms, similar to the mutant DDS proteins, are also capable of interacting with +KTS isoforms, abrogating their effect (3). However, there is evidence to suggest that DDS mutants do not always behave in a dominant-negative manner. A paper examining the effect of WT1 on the SRY promoter found that while the most common DDS mutants (R366C, R366H, H377Y, and R394W) were unable to activate SRY transcription, they failed to act in a dominant-negative manner when cotransfected with wild-type WT1 (−KTS) into HeLa cells (28).

WT1 Function in Podocytes: Insights from DDS

The mechanism by which WT1 mutations induce dramatic changes in podocytes (dedifferentiation, abnormal proliferation, morphological alterations) is not well understood; although a number of studies have highlighted the differences between normal podocytes and those found in DDS patients (46, 71, 73, 77). Immunohistochemical analysis of WT1 and proliferation markers in normal and DDS sclerotic glomeruli shows that WT1 expression is significantly reduced in patients with DDS and that the level of proliferation of individual podocytes is inversely related to WT1 expression, consistent with the role of WT1 as a transcriptional repressor of growth factors (77). In agreement with this finding, mutations in WT1 can result in podocyte hyperplasia (64). WT1 is thought to regulate the expression of platelet-derived growth factor-α (PDGF-α) and transforming growth factor-β1 (TGF-β1) (78). WT1 represses TGF-β1 expression via the GC-rich EGR-1 consensus DNA element within the human TGF-β1 promoter (12). The authors found that in the majority of patients examined, both PDGF-α and TGF-β1 are overexpressed in the glomerular podocytes.

Misexpression of Pax-2 in DDS. Examination of the expression of the transcription factor Pax-2 in sclerotic DDS glomeruli using in situ hybridization and immunofluorescence revealed that faint and irregular expression of WT1 was associated with persistent Pax-2 expression (79). Pax-2 is expressed during early nephrogenesis in the induced metanephric blastema and the inducing ureteric bud and then is abruptly downregulated in development (13). In transgenic mice, failure to repress Pax-2 in the developing nephrons results in severe developmental abnormalities (14). WT1 has been shown to downregulate the expression of Pax-2 (69). This suggests that in the podocytes of DDS patients, the presence of dominant-negative mutant WT1 proteins or reduced WT1 expression may result in persistent Pax-2 expression, contributing to the podocyte abnormalities observed in DDS. Our own findings examining Pax-2 expression in human podocytes with a DDS mutation show massive upregulation of Pax-2 in mutant cells in culture (Viney R, Ni L, Witherden IR, Morrison AA, Mathieson PW, Ladomery M, van den Heuvel LP, Saleem M, unpublished observations). This indicates an important interaction, and these cells will be a valuable tool in determining the functional aspects of the WT1-Pax-2 interrelationship.

Consequences of truncating WT1’s zinc finger domain. A DDS mutation that truncates zinc finger 3 at codon 396 in heterozygous and chimeric mice produces the characteristic urogenital abnormalities of DDS (62, 64). Further examination of the kidneys of chimeric mice was performed using immunocytochemistry and in situ hybridization. Although Pax-2 expression was a feature of murine DDS, there was no evidence that Pax-2 expression, or a loss of WT1 protein, preceded the development of sclerosis. Pax-2 protein was only detected in a few nonsclerotic kidneys of chimeric mice; however, much higher levels were detected in globally sclerotic kidneys, which suggests that Pax-2 expression in DDS is a result of reexpression due to disease progression. In addition to examining WT1 and Pax-2 expression, Patek et al. also measured the expression of components of the podocyte filtration barrier. Loss of expression of synaptopodin, nephrin, and α-actinin-4 was observed only in globally sclerotic glomeruli, indicating that these changes were a result of disease progression and terminal damage to podocytes.

It is worth noting that the interpretation of protein levels in glomeruli with incipient or active sclerosis is susceptible to several variables; so, the question of reciprocal interaction between these molecules remains open. Furthermore, the consequence of the DDS mutation on the function of specific WT1 isoforms also remains to be clarified.

A point mutation in WT1 results in diffuse mesangial sclerosis in mice. Another mouse strain has been generated that carries the most frequently observed mutation in DDS patients that substitutes a tryptophan for an arginine at amino acid 394 (R394W) and compromises DNA-binding by WT1 (17). The heterozygous mice exhibited no intersex disorders (gonadal dysgenesis and male pseudohermaphroditism); however, the mice did develop proteinuria and diffuse mesangial sclerosis. The mice expressed wild-type WT1 mRNA levels at 50% of that observed in normal kidneys, and there was a 15–20% reduction in total WT1 protein. Electron microscopy of the glomeruli revealed thickening of the glomerular basement
membrane and foot process effacement. Real-time analysis of putative WT1 transcriptional targets demonstrated that TGF-β1 and IGF-1 are significantly upregulated in the kidneys of the R394W DDS mouse. However, no evidence of inappropriate proliferation was observed in the glomeruli of R394W DDS mice compared with wild-type. Analysis of genes that are implicated in nephrosis revealed that CD2AP and podocin expression is elevated in the heterozygous mice at 2 and 4 mo, respectively. However, for all of the changes observed, no direct link with WT1 could be made and therefore the difference in protein levels may have been due to disease progression.

**WT1 Function: Insights from Conditionally Immortalized Podocytes**

Early attempts to assess the biological function of glomerular cells used a combination of microdissection and sieving to isolate individual glomeruli from small pieces of kidney cortex (27). Type I cells are identifiable as podocytes by their large size (100–150 μm in diameter), branched structure, and stellate appearance. Initially, in culture the cells possess primary, secondary, and tertiary branching typical of podocytes in vivo; however, this differentiated structure is progressively lost in tissue culture. After 2–3 wk, withdrawal and fusion of the cytoplasmic branches give the cells the appearance of large, round, vacuolated cells. This observation has also been reported in another study published in 1978 which observed that epithelial processes showed abnormalities after just 8 h in culture (60).

**In vitro culture of podocytes.** More recently, human and rat podocyte cultures have been established by isolating outgrowing podocytes from glomeruli in culture (56). Immunocytochemical analysis of WT1 and O-acetylated ganglioside revealed that the cells are podocyte in origin. Problems associated with cell culturing are resolved by using conditionally immortalized podocyte cell lines that exploit the temperature sensitive SV-40 antigen. Activation at the permissive temperature induces the cells to proliferate. Inactivation at the non-permissive temperature and no subcultivation result in the cultured podocytes undergoing a phenotypic conversion to arborized podocytes. This system facilitates the growth of large numbers of podocytes in culture, overcoming the problems associated with culturing differentiated podocytes which show very little proliferative activity.

**Development of murine and human podocyte cell lines.** The first conditionally immortalized podocyte cell line was generated by Mundel et al. (57). It was derived from a transgenic mouse that expressed a temperature-sensitive SV-40 large T-antigen (tsA58Tag). Cells grown at 33°C display the characteristics of cobblestone-type podocytes. In contrast, podocytes cultured at 37°C appear arborized, cease to proliferate, and acquire features of mature differentiated podocytes (57). More recently, conditionally immortalized human podocyte cell lines were derived by Saleem et al. (70) from nephrectomy specimens transfected with a temperature-sensitive SV-40 large T-antigen. Cells maintained at the nonpermissive temperature of 37°C expressed markers of differentiated podocytes including nephrin, podocin, synaptopodin, and P-cadherin (Fig. 2).

Wild-type and DDS conditionally immortalized human podocytes were recently compared. Differentiated DDS podocytes did not acquire an arborized morphology but instead appeared more elongated and fibroblastic in appearance, and inappropriately expressed Pax2 and smooth muscle actin. These changes were not the result of excessive passaging, and the upregulation of fibroblastic markers was observed in several independent clones. A multiplex difference gel electrophoresis proteomic comparison of podocytes showed that DDS podocytes misexpress proteins associated with cytoskeletal architecture, including cofilin, calponin, elfin, hsp27, and vinculin; in addition, total levels of filamentous actin were also reduced in DDS podocytes (73). Taken together, these findings suggest that in addition to its more traditional roles as a regulator of cell proliferation, WT1 can influence cytoskeletal architecture. This
represents a novel aspect of WT1 function, recently observed in another context when WT1 isoforms were overexpressed in human cancer cell lines, resulting in misexpression of cofillin, α-actinin, and altered cell invasiveness and migration (31).

Conclusions

WT1 has been intensively studied for the past 18 years, but despite recent progress, much remains to be understood about its role in podocytes. It is now clear that the ratio of WT1 splice isoforms needs to be tightly controlled to prevent the development of FS and that specific mutations in WT1 can lead to DDS. Changes in the expression of growth factors TGFB-1 and PDGF-α, the transcription factor Pax-2, and proteins that affect cytoskeletal architecture have all been implicated. The complete set of WT1’s DNA and mRNA targets in podocytes remains to be defined. Recent developments including the generation of transgenic mice that express the most frequently observed WT1 mutations (17, 62), together with the development of both rodent and human conditionally immortalized podocyte cell lines (57, 70), mean that we now have the tools necessary to determine the precise role of WT1 in podocyte differentiation and physiology.

GRANTS

The authors acknowledge support from the University of the West of England, the University of Bristol, and The Royal Society UK.

REFERENCES


