Towards an understanding of kidney diseases associated with WT1 mutations

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Mutations in Wilms’ tumor 1 (WT1) cause a wide spectrum of renal manifestations, eventually leading to end-stage kidney failure. Insufficient understanding of WT1’s molecular functions in kidney development has hampered efficient therapeutic applications for WT1-associated diseases. Recently, the generation and characterization of mouse models and application of multiple state-of-the-art approaches have significantly expanded our understanding of the molecular mechanisms of how WT1 mutations lead to kidney failure. Here, we discuss the WT1 binding consensus and illustrate the major roles of WT1 in different cell populations in kidney biology. WT1 controls metanephric mesenchyme (MM) self-renewal and proliferation mainly by regulating FGF and BMP-pSMAD signaling pathways as well as Sall1 and Pax2, encoding key transcription factors; WT1 drives MM differentiation and mesenchyme-epithelial transition by targeting Fgf8 and Wnt4; WT1 defines podocyte identity by activation of other podocyte-specific transcription factors, including Mafb, Lmx1b, FoxC2, and Tcf21. These factors potentially cooperate with WT1 regulating the expression of components and regulators of the cytoskeleton for establishing podocyte polarity, slit diaphragm structure, and focal adhesion to the glomerular basement membrane. Understanding of WT1’s function in kidney biology including WT1-regulated pathways will give insights that will eventually help therapeutic applications.

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WT1 and its expression pattern in the mammalian kidney

The human WT1 gene spans 50 kb of genomic DNA and comprises 10 exons, which generate a 3 kb mRNA. It encodes a protein with an amino-terminal proline- and glutamine-rich protein interaction domain and four carboxy-terminal Krüppel-type (Cys2-His2) zinc fingers, major characteristics of a typical transcription factor. Expression of WT1 is complex, with at least 36 isoforms derived from alternative splicing, usage of alternative translation start sites, and RNA editing. There are two major alternative splice sites resulting in four isoforms: proteins with or without the central 17 amino acids from alternative splicing of exon 5 and proteins including or excluding the three amino acids lysine, threonine, and serine (KTS) between the third and fourth zinc finger domain (Figure 1a). The corresponding isoforms are termed WT1+KTS and WT1−KTS and are expressed in a constant ratio of about 2:1 in humans.2 WT1 mutations in human patients are commonly associated with dysfunction of the urogenital system, indicating the essential roles of WT1 for kidney and gonad development. In mammals, the
WT1 ALTERATIONS AND RENAL ANOMALIES
WT1 was initially identified using a positional cloning approach, as it is located in a genomic region, deletion of which results in the WAGR syndrome (Wilms’ tumor, aniridia, genitourinary anomalies, and mental retardation). Since then many WT1 mutations were identified, including missense, nonsense, splice site mutations, as well as deletions. WT1 mutations have been found to be associated with a large spectrum of phenotypes at the clinical level, including Wilms’ tumor, the DDS, the FS, and the isolated steroid-resistant nephrotic syndrome, all of which progress to end-stage kidney disease. Overall, there is a solid genotype-phenotype association of WT1 diseases. Approximately 20% of WT1s, the most commonly found kidney tumor in children, are caused by WT1 mutations. A high number of bilateral tumors are associated with truncation mutations, particularly in the 5’ half of the WT1 gene. Mutations within exon 8 or 9 of WT1 usually lead to the DDS, characterized by diffuse mesangial sclerosis, urogenital abnormalities, and a high risk of developing Wilms’ tumor. Disruption of the KTS splicing, caused by splice site mutations, results in the loss of the WT1+KTS isoform and leads to the FS, characterized by pseudohermaphroditism and focal segmental glomerulosclerosis. In addition, there are idiopathic steroid-resistant glomerulopathies caused by mutations in WT1. Wilms’ tumors or nephroblastoma often contain metanephric blastema, stromal and epithelial derivatives. It is therefore thought to arise by a failure of kidney progenitor cells to differentiate. On the other hand, glomerulopathies as seen in DDS, FS, and steroid-resistant nephrotic syndrome are considered to result from the loss of WT1 function during podocyte development and maintenance. In the following, we will discuss WT1 as a transcription factor and discriminate between its functions in kidney progenitor cells and in podocytes.

SEQUENCE AND LOCALIZATION OF WT1 BINDING SITES
WT1 has been suggested to recognize and bind the EGR1-like GC-rich DNA sequence. However, a physiologically relevant WT1 binding sequence cannot only be deduced from in vitro experiments. Recently, chromatin immunoprecipitation (ChIP) followed by microarray hybridization or high-throughput sequencing (ChIP-seq) experiments provided insight into the genomic DNA binding characteristics of WT1 during kidney development and homeostasis. The WT1 binding motif derived from ChIP followed by microarray hybridization shows a high similarity to the EGR1 binding motif, whereas the motif based on ChIP-seq experiments demonstrates a slight variation in the form of an exchange from C to A. Considering all the motifs identified from ChIP-seq experiments, WT1 recognizes the sequence of Gg/tGGGAgGg/t. Interestingly, in many gene regulatory
regions, we found two similar motifs containing $GGGAGg/t$ close to each other, suggesting that WT1 can form and bind as a homodimer.\textsuperscript{12} This could explain the dominant manner of manifestation of diseases caused by WT1 mutations.

In the past, numerous potential target genes of WT1 were identified by characterization of the promoter regions via various approaches, such as reporter assays, electrophoretic mobility shift assay, and co-transfection assays (Supplementary Table S1 online). Examination of genomic WT1 binding sites in kidneys revealed that they were significantly enriched in the promoter region near the transcription start sites. Meanwhile, combining all the WT1 peaks from ChIP-seq results shows around 45\% of WT1 binding sites localized more than 50 kb away from transcription start sites, indicating that WT1 regulates gene expression via both proximal and distal regulatory elements, thus highly extending the WT1 potential target gene list.\textsuperscript{10–13} In addition, the differences of WT1 binding loci between developing and adult kidneys as well as the different gene expression patterns between Wt1 mutant MM and podocytes indicate that the gene sets regulated by WT1 significantly depend on the specific cellular context.

**WT1 IS INVOLVED IN SELF-RENEWAL AND DIFFERENTIATION OF MM**

In view of the important function of WT1 in kidneys, several animal models (e.g., mouse, zebrafish, and Xenopus) have been applied. Particularly, generation and characterization of different mouse models have greatly elucidated the WT1 function.\textsuperscript{14} Wt1 knockout mice have no kidneys with failure of UB outgrowth and MM degeneration by apoptosis at E12. In Wt1$^{-/-}$ MM cells, a lack of FGF and enhanced BMP-pSMAD signaling was observed. Mechanistically, WT1 directly regulates FGF20/16 expression and activates FGF signaling for MM survival.\textsuperscript{11} After the MM responds

**Figure 2 | Wilms’ tumor 1 (WT1) contributes to metanephric mesenchyme self-renewal and its differentiation.** WT1, Sall1, Pax2, Six2, and Hoxd11 are individually required for survival and self-renewal of metanephric mesenchyme (MM). Among them, WT1 is upstream of Sall1 and Pax2 transcription factors. BMP4 induces the apoptosis of MM; however, BMP7 promotes the MM differentiation. In balance of the BMP-pSMAD signaling in MM, WT1 directly regulates Bmper, a BMP-pSMAD inhibitor, and activates the FSG signaling pathway via regulating Fgf20/Fgf16 and Gas1 expression to antagonize BMP-pSMAD signaling for the survival and self-renewal of MM. High expression level of Wnt9b beneath the ureteric tip increases the Wnt signaling activity nearing the MM; there, the $\beta$-catenin-LEF/TCF complex works together with Six2 and WT1 activating the critical differentiation factors, Fgf8 and Wnt4, to form pre-tubular aggregate (PTA). Signals produced by PTA promote the mesenchyme–epithelial transition (MET) to form renal vesicles. The proximal part of the renal vesicle expressing WT1 will develop into the epithelial cells of the glomerulus and the distal part of the renal vesicle expressing Lhx1 will develop into the epithelial cells of the nephron tubule.

**Figure 3 | WT1 defines the podocyte-specific morphology.** (a) Ultrastructural kidney analysis of Wt1 mutant and control adult mice treated with doxycycline for 6 days. Podocytes (arrow) and endothelial cells (arrowheads) were abnormally distant from the GBM (G), which was itself irregularly shaped in the mutant tissue. Black dots indicate negative charges by polyethyleneimine staining. No obvious charge differences between the GBMs of mutant and control glomeruli are detectable. Scale bar = 0.5 $\mu$m. This figure is adapted with permission from Dong et al.\textsuperscript{12} (b) Schematic WT1-controlled networks for establishing podocyte-specific morphology. WT1 regulates the slit diaphragm complex components, including Nephrin (Nphs1), Podocin (Nphs2), Membrane-associated guanylate kinase, WW and PDZ domain–containing protein 2 (Magi2), CD2-associated protein (CD2AP), NCK adaptor protein 2 (NCK2), Kin of IRRE-like protein 1/2/3 (Kirrel) and phospholipase C, and epsilon 1 (Plce1). WT1 controls focal adhesion by regulating integrin α3 (Itga3), Integrin β1 (Itgb1), Laminin α5 (Lama5), and Laminin β2 (Lamb2). WT1 also controls the cytoskeleton components and its regulators to establish the podocyte polarity—namely, Synaptopodin (Synpo), $\alpha$-actinin-4, myosin, heavy polypeptide 9, non-muscle (Myh9), Rho GTPase-activating protein 24 (Arhgap24), and Atypical protein kinase C (aPKC). (c) Multiple WT1 binding sites to cis-regulatory regions of Synpo and Magi2 as viewed in the UCSC browser within the indicated genomic intervals. Conservation denotes placental mammal basewise conservation by phastCons score. The peaks indicate the overlapping WT1 binding sites generated from Kreidberg and colleagues’ and our ChIP-seq data.\textsuperscript{12,13}
to FGF signals, WT1-dependent GAS1 can promote MM proliferation by activation of intracellular FGF-stimulated AKT signaling. Also, WT1 directly activates the expression of BMP modulator Bmper to repress pSMAD signaling for permission of MM survival. In addition, mutation of WT1 in Wilms’ tumor is frequently accompanied by canonical Wnt
WT1 regulates podocyte maturation and homeostasis

After MM mesenchymal–epithelial transition, WT1 expression continues to increase along with the development of podocyte progenitors and is later restricted to adult podocytes. Mature podocytes consist of four morphologically distinct segments: a cell body, major processes, secondary processes, and foot processes (FPs). Between interdigitating FPs of neighboring podocytes, a unique structure, the slit diaphragm (SD), is formed. FPs are functionally defined by three membrane domains: the apical membrane domain, the SD, and the basal membrane domain that is attached to the glomerular basement membrane. The integrity of podocytes and their interaction with the glomerular basement membrane is crucial for maintenance of the intact glomerular filtration barrier. Alteration of the intercellular junctions and cytoskeletal structure of podocytes or their detachment from the membrane results in the development of albuminuria. Investigations in humans, mouse, and fish have revealed a large number of gene products involved in podocyte development and maintenance (Supplementary Table S2 online). WT1 mutations leading to disruption of podocyte development or maintenance are associated with glomerulopathies in DDS, FS, and steroid-resistant nephrotic syndrome. Podocyte-specific WT1 knockout mice display defects in podocyte differentiation and lead to kidney failure and death within 24 h after birth. Inducible adult podocyte WT1 knockout mice show FP effacement, proteinuria, and glomerulosclerosis, thus confirming the essential roles of WT1 in podocyte maturation and maintenance (Figure 3a). Recent ChIP-Seq and transcriptome analyses have shed light on the WT1-controlled transcription network in adult podocytes (Figure 3b).

WT1 controls the expression of SD component encoding genes

The SD is a specialized protein complex structure of podocytes, which serves as a blood filtration unit and constitutes a fundamental component of the glomerular filtration barrier. The SD, as a specialized intercellular junction connecting neighboring FPs, contains elements of tight, adherens, and gap junctions. The SD is also considered as a complex signaling hub for regulating the plasticity of podocyte FPs and the loss of SD integrity leading to proteinuria. Interestingly, WT1 binds to the proximal promoters of many genes encoding proteins that localize to the SD structure of podocytes, such as Nphs1, Nphs2, Magi2, Ptpro, Kirrel, Plce1, Cdhn5, and Nck2. This supports the hypothesis that sharing transcription factor binding sites conserved in distance and orientation can help control the expression of genes that act together in the same biological context. Notably, the expression of SD protein encoding genes is controlled differentially by WT1 variants. Magi2 expression depends on the WT1+KTS isoform, whereas Nphs1 and Nphs2 expressions are regulated by the WT1–KTS isoform.

WT1 controls the polarity of podocytes and cytoskeleton arrangement

Apart from the SD structure, a complex cytoskeleton underlies the delicate architecture of podocytes to build and maintain their apicobasal and planer cell polarity. Interruption of cytoskeleton structure and loss of polarity are among characteristic features of injured podocytes. For example, loss of the actin cytoskeletal proteins, ACTN4, SYNPO, and MYH9, results in autosomal recessive nephrotic syndromes. In addition, mutation of actin regulatory protein, ARHGAP24, a RhoA-activated Rac1 GTPase-activating protein, also causes early or adult onset nephrotic syndrome. Podocyte-specific knockout of the polarity protein aPKC, a central component of Par3-Par6-aPKC complex, in mice also results in the nephrotic syndrome (Supplementary Table S2 online). WT1 binds the cis-regulatory elements of those genes in ChIP-Seq experiments, indicating that WT1 controls the polarity of podocytes and cytoskeleton arrangement.

WT1 controls the cell-matrix adhesion of podocytes

Tight adherence of podocyte to the glomerular basement membrane is another prerequisite for maintaining the integrity of the glomerular filtration barrier due to its exposure to permanent transcapillary filtration pressure. The major cell-matrix adhesion receptor in podocytes is integrin α3β1 connecting to laminin 521 in the glomerular basement
membrane. Mutations in ITGA3 and LAMB2 in human patients or podocyte-specific knockout of Igfb1 and Lama5 in mice result in FP effacement and proteinuria (Supplementary Table S2 online). All the cis-regulatory regions of these genes have WT1 binding signals, suggesting that WT1 controls the cell-matrix adhesion of podocytes.

WT1 and the concept of super-enhancers

By taking a closer look at the WT1 binding loci in the podocyte-specific genes, it is apparent that, in many cases, several WT1 binding sites localize to intragenic or intergenic regions in the gene’s cis-regulatory domains, such as in Synaptopodin and Magi2 (Figure 3c). Correlation analysis with expression data from FACS-sorted podocytes suggests that genes harboring multiple WT1 binding sites usually have a higher expression level in podocytes than genes having no or only one WT1 binding sequence. The analysis of these enhancer peaks shows a significant enrichment for MAFB, Homeobox, Tcf-family, and Fox-class binding sites, indicating that these factors may cooperate with WT1 to regulate podocyte-specific gene expression. WT1 binds to its own promoter, suggesting an auto-regulatory loop. WT1 also regulates the expression of Mafb, Lmx1b, Foxe2, and Tcf21, which are podocyte-specific transcription factors. Mutation of each of them causes the loss of kidney function (Supplementary Table 2 online). These findings are reminiscent of the concept of super-enhancers, which consist of clusters of enhancers that are densely occupied by master regulators and Mediator for enhancing the cell-specific gene expression pattern. In light of this paradigm, the recent data suggest that WT1 works together with Mafb, Lmx1b, Foxe2, and Tcf21 to define podocyte identity as a master regulator by activating the podocyte-specific super-enhancers, together with Mediator.

CONCLUSION AND PERSPECTIVE

WT1 mutations result in a large spectrum of phenotypes, either caused by dysfunction of kidney progenitors or podocytes. Significant advances have recently been made in the understanding of WT1 function in kidney biology and kidney pathophysiology associated with WT1 mutations, providing promising therapeutic applications. Recently, application of in utero delivery of a pseudotyped rAAV 2/9 vector provides a possible avenue for the targeting of gene therapies of WT1 mutation–related kidney diseases. In addition, understanding the molecular mechanism of kidney development has fostered the establishment of protocols for the generation of mature kidney cell types and the three-dimensional nephron-like structures from pluripotent cells. Transplantation of the de novo nephrons generated from stem cells under a mouse kidney capsule could integrate the already existing blood stream into the glomerulus, thus enhancing potential treatment of kidney diseases. Although these therapeutic directions point toward a translational relevance, in addition, developing pharmacological agents can help combat kidney disorders. A drug screening platform can be established from reconstituted kidneys in vitro, derived from pluripotent stem cells from WT1 mutant patients. Simultaneously, a better understanding of the complex WT1-dependent molecular mechanisms involved in podocyte biology coupled with directed differentiation protocols will assist in the generation of large quantities of pure podocytes, thus, paving the way for novel clinical applications.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. WT1 target genes.
Table S2. List of genetic mutations or deletion or transgenic animal models that involved in podocyte function.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Eckardt KU, Coresh J, Devuyst O et al. Evolving importance of kidney disease: from subspecialty to global health burden. Lanec 2013; 382: 158–169.
2. Hohenstein P, Hastie ND. The many facets of the Wilms’ tumour gene, WT1. Hum Mol Genet 2006; 2: R196–R201.
3. Little MH, McMahon AP. Mammalian kidney development: principles, progress, and projections. Cold Spring Harb Perspect Biol 2012; 4: a008300.
4. Gessler M, Pousta L, Cawnee E et al. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 1990; 343: 774–778.
5. Call KM, Glaser T, Ito CY et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms’ tumour locus. Cell 1990; 60: 509–520.
6. Lipska BS, Ranchin B, Iatropoulos P et al. Genotype-phenotype associations in WT1 glomerulopathy. Kidney international 2014; 85: 1169–1178.
7. Chernin G, Vega-Warner V, Schoeb DS et al. Genotype/phenotype correlation in nephrotic syndrome caused by WT1 mutations. Clin J Am Soc Nephrol 2010; 5: 1655–1662.
8. Royer-Pokora B, Beier M, Hennler M et al. Twenty-four new cases of WT1 germline mutations and review of the literature: genotype/phenotype correlations for Wilms tumor development. Am J Med Genet Part A 2004; 127A: 249–257.
9. Hashimoto H, Olanrewaju YO, Zheng Y et al. Wilms tumor protein recognizes 5-carboxylcytosine within a specific DNA sequence. Genes Dev 2014; 28: 2304–2313.
10. Hartwig S, Ho J, Pandey P et al. Genomic characterization of Wilms’ tumor suppressor 1 targets in nephron progenitor cells during kidney development. Development 2010; 137: 1189–1203.
11. Motamedi FJ, Badro DA, Clarkson M et al. WT1 controls antagonistic FGF and BMP-pSMAD pathways in early renal progenitors. Nat Commun 2014; 5: 4444.
12. Dong L, Pietsch S, Tan Z et al. Integration of cistromic and transcriptomic analyses identifies Nphs2, Mafb, and Magi2 as Wilms’ Tumor 1 target genes in podocyte differentiation and maintenance. J Am Soc Nephrol 2015.
13. Kann M, Ettou S, Jung YL et al. Genomic-wide analysis of Wilms’ Tumor 1-controlled gene expression in podocytes reveals key regulatory mechanisms. J Am Soc Nephrol 2015.
14. Ozdemir DD, Hohenstein P. WT1 in the kidney–a tale in mouse models. Pediat Nephrol 2014; 29: 687–693.
15. Kann M, Bae E, Lenz MO et al. WT1 targets Gas1 to maintain nephron progenitor cells by modulating FGF signals. Development 2015; 142: 1254–1266.
16. Maiti S, Alam R, Amos CI et al. Frequent association of beta-catenin and WT1 mutations in Wilms tumors. Cancer Res 2000; 60: 6288–6292.
17. Kopan R, Chen S, Little M. Nephron progenitor cells: shifting the balance of self-renewal and differentiation. Curr Top Dev Biol 2014; 107: 293–331.
18. Park JS, Ma W, O’Brien LL et al. Six2 and Wnt regulate self-renewal and commitment of nephron progenitors through shared gene regulatory networks. Dev Cell 2012; 23: 637–651.
19. Essafi A, Webb A, Berry RL et al. A wt1-controlled chromatin switching mechanism underpins tissue-specific wnt4 activation and repression. Dev Cell 2011; 21: 559–574.
20. Hu Q, Gao F, Tian W et al. Wt1 ablation and Igf2 upregulation in mice result in Wilms tumors with elevated ERK1/2 phosphorylation. J Clin Invest 2011; 121: 174–183.
21. Brinkkoetter PT, Ising C, Benzing T. The role of the podocyte in albumin filtration. Nat Rev Nephrol 2013; 9: 328–336.
22. Gebeshuber CA, Kornauth C, Dong L et al. Focal segmental glomerulosclerosis is induced by microRNA-193a and its downregulation of WT1. Nat Med 2013; 19: 481–487.
23. Chau YY, Brownstein D, Mjoseng H et al. Acute multiple organ failure in adult mice deleted for the developmental regulator Wt1. PLoS Genet 2011; 7: e1002404.
24. Grahammer F, Schell C, Huber TB. The podocyte slit diaphragm—from a thin grey line to a complex signalling hub. Nat Rev Nephrol 2013; 9: 587–596.
25. Cohen CD, Klingenhoff A, Boucherot A et al. Comparative promoter analysis allows de novo identification of specialized cell junction-associated proteins. Proc Natl Acad Sci USA 2006; 103: 5682–5687.
26. Lefebvre J, Clarkson M, Massa F et al. Alternatively spliced isoforms of WT1 control podocyte specific gene expression. Kidney Int 2015.
27. Welsh GI, Saleem MA. The podocyte cytoskeleton–key to a functioning glomerulus in health and disease. Nat Rev Nephrol 2012; 8: 14–21.
28. Sachs N, Sonnenberg A. Cell-matrix adhesion of podocytes in physiology and disease. Nat Rev Nephrol 2013; 9: 200–210.
29. Whyte WA, Orlando DA, Hnisz D et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 2013; 153: 307–319.
30. Picconi JL, Muff-Luett M, Wu D et al. Kidney-specific expression of GFP by in-utero delivery of pseudotyped adeno-associated virus 9. Mol Ther Methods Clin Dev 2014; 1: 14014.
31. Humphreys BD. Kidney structures differentiated from stem cells. Nat Cell Biol 2014; 16: 19–21.