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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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Kidney failure is a worldwide public health problem, with increasing incidence and prevalence, high costs, and poor outcomes.[1] Some of those renal manifestations are caused by *Wilms' tumor 1* (*WT1*) mutations, including Wilms' tumor, the Denys-Drash syndrome (DDS), the Frasier syndrome (FS), and the steroid-resistant nephrotic syndrome. To understand WT1 function in kidney development and homeostasis, multiple mouse models have been generated and characterized. Particularly, the generation of *Wt1* conditional knockout mice crossed to inducible and cell-specific Cre mice allowed to dissect Wt1 function with regard to specific cell populations and the time of development. To decipher the molecular mechanisms of *Wt1* mutations leading to kidney failure, multiple state-of-the-art experimental approaches have been applied, such as chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq) using embryonic and adult kidneys. In this review, we present and summarize our current understanding of WT1's function in kidney biology including WT1-controlled regulatory networks. We also discuss how this knowledge might be translated into therapeutic applications.

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 (Cys_2-His_2) 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

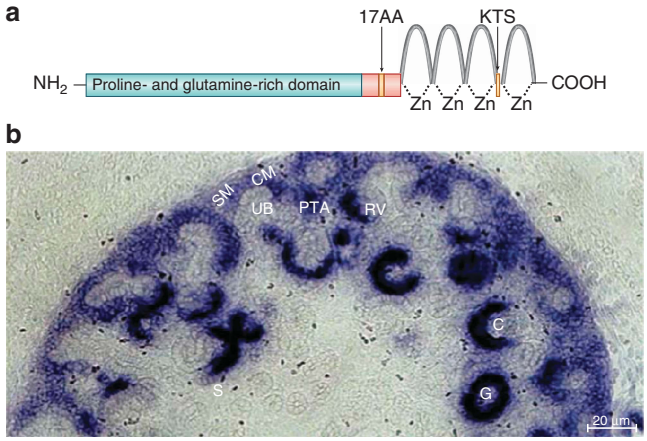


Figure 1 | Wilms' tumor 1 (WT1) protein structure and *Wt1* expression pattern in developing murine kidney. (**a**) Structure of WT1 protein showing alternative splice regions consisting of 17 amino acids encoded by exon 5 and amino acids lysine, threonine, and serine (KTS) encoded by the 3′-end of exon 9 and the different functional domains. (**b**) The expression of *Wt1* in developing kidney (E15.5) detected by *in-situ* hybridization (picture provided by Dagmar Kruspe). *Wt1* is expressed in cap mesenchyme (CM) surrounding the ureteric bud (UB) and at very low level in stromal mesenchyme (SM). *Wt1* expression is increased in pre-tubular aggregates (PTAs) that begin to undergo mesenchymal to epithelial transition to form the renal vesicle (RV). *Wt1* is expressed in the proximal part of renal vesicles and the S-shaped bodies (S), which give rise to podocyte precursors in the capillary loop (C). The highest expression of *Wt1* is found in podocytes within glomeruli (G). Scale bar = 20 µm.

definitive kidney, the metanephros, forms at the end of the posterior intermediate mesoderm through distinct consecutive steps.[3] In brief, first, a specialized region called metanephric mesenchyme (MM) is formed in the posterior intermediate mesoderm; second, the MM induces an outgrowth of the nephric duct called ureteric bud (UB); third, the UB invades the MM and induces adjacent MM cells to condense around the UB tip, forming the cap mesenchyme; the remaining MM cells are called stromal mesenchyme; fourth, the cap mesenchyme cells induce UB to branch, whereas a subset of the cap mesenchyme (pre-tubular aggregate) on the ventral side of the UB tips undergoes mesenchymal–epithelial transition to form a renal vesicle. This fuses with the ureteric stalk, generating a comma-shaped body and subsequently an S-shaped body with a proximal-distal cleft. The most proximal cleft is infiltrated by endothelial cells and forms the glomerular tuft. Finally, reciprocal epithelial–mesenchymal interactions induce UB to branch repeatedly in a highly reproducible manner, and new nephrons form from the mesenchyme adjacent to each new UB tip. The ureteric branches form the collecting duct tree that connects the nephrons to the ureter and drain the urine into the bladder. The earliest WT1 signal is detected in intermediate mesoderm at E9.0, and then the signal increases in cap mesenchyme after UB induction. Higher expression is also detected in pre-tubular aggregates, proximal parts of renal vesicle, and comma- and S-shaped body. The highest expression is in capillary loops and finally restricted to podocytes of the glomerulus, where expression is maintained throughout life (Figure 1b).

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).[4,5] Since then many *WT1* mutations were identified, including missense, nonsense, splice site mutations, as well as deletions.[6] *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.[6–8] Approximately 20% of WTs, 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.[9] 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.[10–13] 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/tGGGAGg/t*.[11–13] 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.[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.[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.[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.[11] After the MM responds

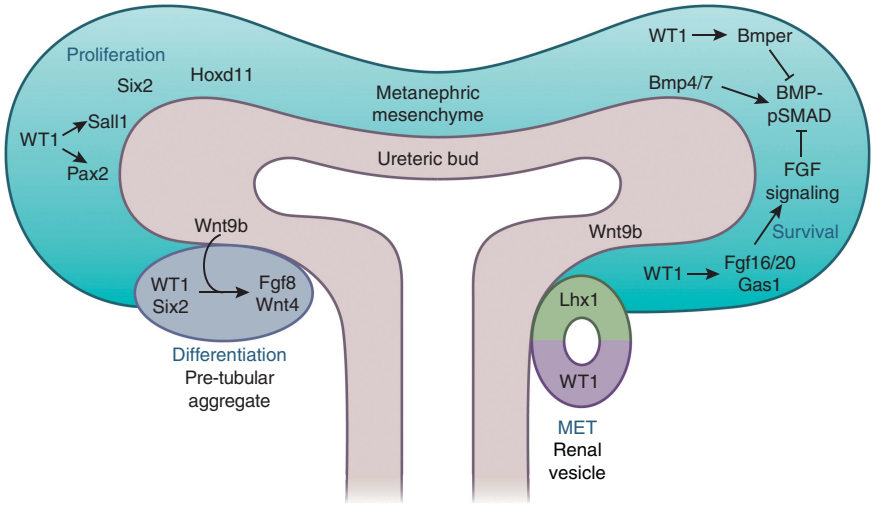
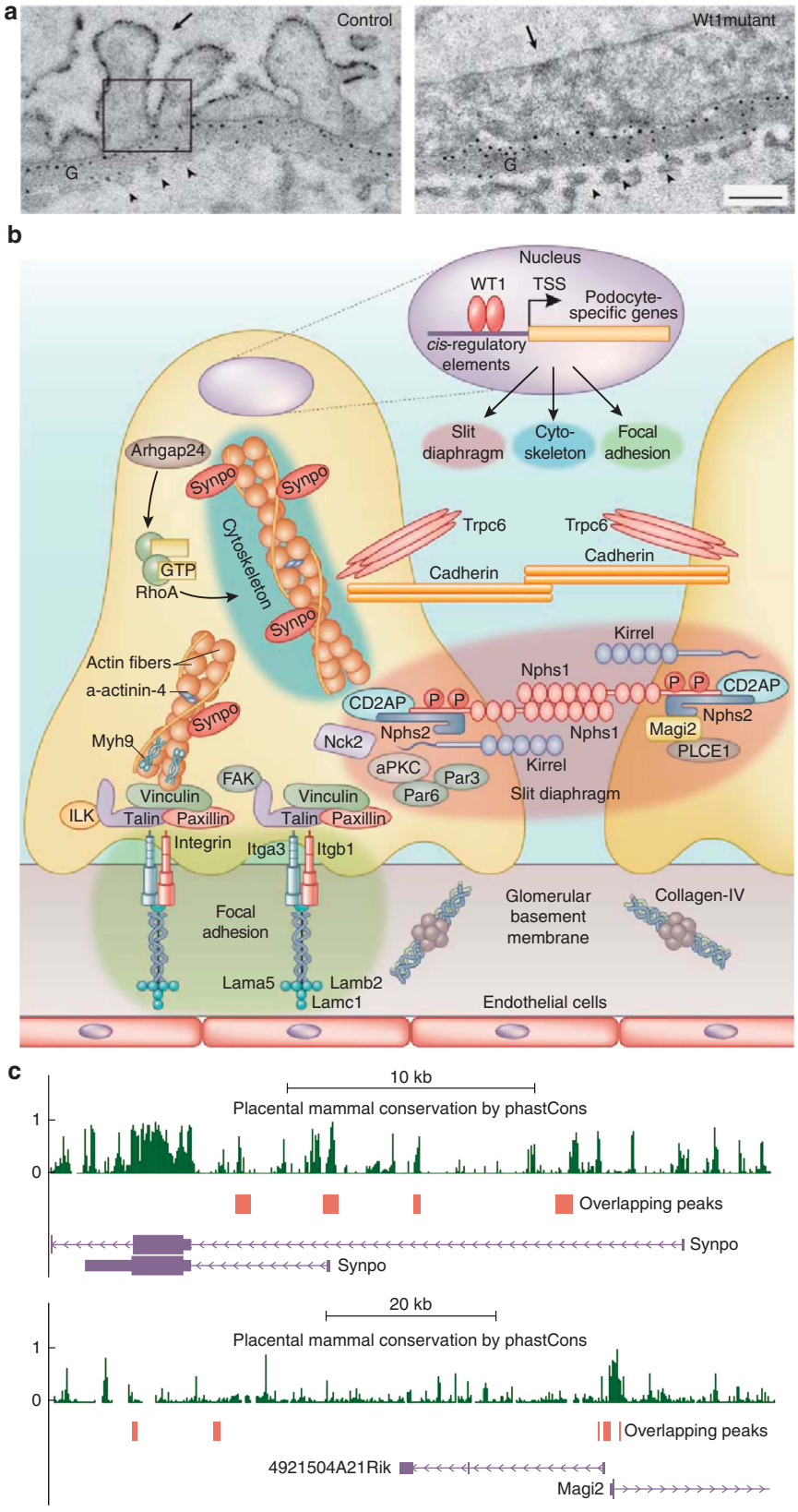


Figure 2 | Wilms' tumor 1 (WT1) contributes to metanephric mesenchyme self-renewal and its differentiation. WT1, Sall1, Pax2, Six2, and Hoxd11 are individually required for survival and self-renewal of metanephric mesenchyme (MM). Among them, WT1 is upstream of Sall1 and Pax2 transcription factors. BMP4 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 FGF 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 singling activity nearing the MM; there, the β-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 polyethylenimine staining. No obvious charge differences between the GBMs of mutant and control glomeruli are detectable. Scale bar = 0.5 μm. This figure is adapted with permission from Dong *et al.*[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), α-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.[12,13]

to FGF signals, WT1-dependent GAS1 can promote MM proliferation by activation of intracellular FGF-stimulated AKT signaling.[15] Also, WT1 directly activates the expression of BMP modulator Bmper to repress pSMAD signaling for permission of MM survival.[11] In addition, mutation of *WT1* in Wilms' tumor is frequently accompanied by canonical Wnt



activation.[16] WT1 is indicated to inhibit Wnt signaling in the MM by activating *CXXC5* expression, encoding a Wnt/β-catenin inhibitor.[10] The interaction of BMP, FGF, and Wnt signaling has been implicated for the survival of MM.[17] Interestingly, WT1 can affect all key signaling pathways in MM by transcriptional regulation of respective modulators. As shown for *Wt1*, inactivation of other transcription factors encoding genes like *Sall1/4, Six1/2, Eya1, Pax2,* and *Hoxd11* also results in kidney agenesis.[17] Among them, WT1 directly regulates *Sall1* and *Pax2* in MM (Figure 2).[10]

In addition to self-renewal and expansion of nephron progenitors, signals for initiation of nephrogenesis are required in order for kidney development to occur. One such signal is Wnt9b, which is expressed at a high level beneath the ureteric tip and induces the adjacent nephron progenitor cells to differentiate and to form pre-tubular aggregates. *WT1* expression level is increased in the pre-tubular aggregate domain (Figure 1b). *Fgf8* and *Wnt4* are expressed in pre-tubular aggregates and are required for mesenchymal–epithelial transition to develop the renal vesicle.[17] On the basis of direct evidence from ChIP experiments, β-catenin and LEF/TCF complexes together with Six2 regulate *Fgf8* and *Wnt4* transcription in pre-tubular aggregate.[18] Interestingly, WT1 also binds to the cis-regulatory regions of *Fgf8* and *Wnt4* and activates their expression.[11,19] Somatic deletion of *Wt1* at E13.5 disrupts MM epithelialization and results in the lack of nephrons at birth.[20] Taken together, WT1 controls MM differentiation and mesenchymal–epithelial transition mainly by regulation of *Fgf8* and *Wnt4*, probably cooperating with β-catenin, LEF/TCF, and Six2 complexes (Figure 2).

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.[21] 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.[12] 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).[22,23] Recent ChIP-Seq and transcriptome analyses have shed light on the WT1-controlled transcription network in adult podocytes (Figure 3b).[12,13]

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.[24] 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*, *Cldn5*, and *Nck2*.[12,13] 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.[25] 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.[26]

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.[27] 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.[12,13]

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.[28] Mutations in ITGA3 and LAMB2 in human patients or podocyte-specific knockout of *Itgb1* 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.[12,13]

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 S*ynaptopodin* 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.[13] WT1 binds to its own promoter, suggesting an auto-regulatory loop.[11–13] WT1 also regulates the expression of *Mafb*, *Lmx1b*, *Foxc2*, 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.[29] In light of this paradigm, the recent data suggest that WT1 works together with Mafb, Lmx1b, FoxC2, 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.[30] 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.[31] 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.[31] 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

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