Expansion of the renal capsular stroma, ureteric bud branching defects and cryptorchidism in mice with Wilms tumor 1 gene deletion in the stromal compartment of the developing kidney

Anna-Carina Weiss1, Reginaldo Rivera-Reyes1*, Christoph Englert2 and Andreas Kispert1*©

1 Institut für Molekularbiologie, Medizinische Hochschule Hannover, Hannover, Germany
2 Molecular Genetics, Leibniz Institute on Aging – Fritz Lipmann Institute, Jena, Germany

*Correspondence to: A Kispert, Medizinische Hochschule Hannover, Institut für Molekularbiologie, OES250, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany. E-mail: kispert.andreas@mh-hannover.de

Abstract

Development of the mammalian kidney is orchestrated by reciprocal interactions of stromal and nephrogenic mesenchymal cells with the ureteric bud epithelium. Previous work showed that the transcription factor Wilms tumor 1 (WT1) acts in the nephrogenic lineage to maintain precursor cells, to drive the epithelial transition of aggregating precursors into a renal vesicle and to specify and maintain the podocyte fate. However, WT1 is expressed not only in the nephrogenic lineage but also transiently in stromal progenitors in the renal cortex. Here we report that specific deletion of WT1 in the stromal lineage using the Foxd1cre driver line results at birth in cryptorchidism and hypoplastic kidneys that harbour fewer and enlarged ureteric bud tips and display an expansion of capsular stroma into the cortical region. In vivo and ex vivo analysis at earlier stages revealed that stromal loss of WT1 reduces stromal proliferation and delays and alters branching morphogenesis, resulting in a variant architecture of the collecting duct tree with an increase of single at the expense of bifurcated ureteric bud tips. Molecular analysis identified a transient reduction of Aldh1a2 expression and of retinoic acid signalling activity in stromal progenitors, and of Ret in ureteric bud tips. Administration of retinoic acid partly rescued the branching defects of mutant kidneys in culture. We propose that WT1 maintains retinoic acid signalling in the cortical stroma, which, in turn, assures proper levels and dynamics of Ret expression in the ureteric bud tips, and thus normal ramification of the ureteric tree.

Keywords: Wilms’ tumour; Wt1; kidney; development branching morphogenesis; retinoic acid signalling; Ret

© 2020 The Authors. The Journal of Pathology published by John Wiley & Sons, Ltd. on behalf of The Pathological Society of Great Britain and Ireland.

Introduction

In the permanent kidney of mammals, the metanephros, nephrons, and collecting ducts present as a contiguous network of epithelial tubes, yet they develop by distinct tubulogenic processes from distinct progenitor tissues [1,2]. In the mouse, the primordium of the collecting duct system, the ureteric bud (UB), arises at embryonic day (E) 10.5 as an epithelial diverticulum of the nephric duct at the posterior pole of the intermediate mesoderm. The proximal tip of the UB invades the adjacent metanephric mesenchyme (MM) and engages in numerous rounds of branching and elongation. Nephrons derive from the cap mesenchyme (CM), a SIX2+ subpopulation of the MM that surrounds the UB tips [3]. During each branching step, some cells of the CM aggregate and transit to an epithelial vesicle which develops into the glomerulus and the tubular segments of a nephron [4]. UB and CM development is coupled by reciprocal signalling systems. GDNF secreted from the CM binds to the receptor tyrosine kinase RET in the UB tips and triggers directed cell movements [5,6]. In turn, WNT9B from the UB tips induces the proliferative expansion of the CM and the formation of pretubular aggregates (PTAs) [7]. Development of both CM and UB tips is modulated by signals from a third renal lineage, the stroma [8]. Stromal progenitors are first identified at E11.0 as loosely organised FOXD1-expressing fibrocyte-like cells that ensheath the CM [9,10]. These progenitors are maintained in the cortical region until nephrogenesis ceases. From E12.5, they start to populate the interstitium between the epithelial tubes of the forming nephrons and collecting ducts and differentiate into fibrocytes of the capsular tissue, the cortical and medullary interstitium, and vascular supporting cells [10]. Ablation of FOXD1+ progenitors compromises the stromal endowment of renal epithelial structures, and leads to reduced branching of the UB, expansion of nephron progenitors, abnormalities of the renal capsule, and altered vascular...
patterns [11]. A number of genetic studies indicate that PTA formation and UB branching are controlled by distinct stromal signals [8].

The Wilms tumor 1 (Wt1) gene encodes a Zn-finger-type transcription factor that is expressed in the CM, in PTAs, and in prospective and definitive podocytes within the nephrogenic lineage [12]. Systemic loss of Wt1 leads to renal aplasia due to apoptosis of the MM, defining a role of WT1 in the maintenance and expansion of the CM [13]. Additional in vitro studies and conditional gene targeting approaches correlated the other expression domains with functions of WT1 in the epithelial transition of the PTA and podocyte development [14–19].

Germline mutations of WT1 have been associated with a number of human diseases including Wilms’ tumour, WAGR syndrome, Denys–Drash syndrome, Frasier syndrome, and the steroid-resistant nephrotic syndrome [20–28]. While most of the phenotypic changes described in these syndromes reflect the aforementioned requirements in the nephrogenic lineage, others including the lack of gonadal descent and mesangial defects may indicate additional functions of WT1 in other renal and/or genital lineages. Here, we provide evidence for an independent requirement of Wt1 in the stromal lineage during kidney development.

Materials and methods

Additional details are provided in supplementary material, Supplementary materials and methods.

Animals

The experiments were approved by the local Institutional Animal Care and Research Advisory Committee and permitted by the Lower Saxony State Office for Consumer Protection and Food Safety (reference number 42500/1H). R26TmG [Gt(ROSA)26Sortm4(Actb:tdTomato-Egfp)Esca] [29], Foxd1cre [Foxd1tm1(EGFP/cre)Amc] [30], Hoxb7-GFP [Tg(Hoxb7-EGFP);33Cos] [31], and Wt1fl/fl (Wt1tm1.1Ceng) [18] mouse lines were maintained on an NMRI outbred background. For embryo harvest, NMRI females. Littermates without the Foxd1cre allele served as controls.

Organ culture and GFP epifluorescence analysis

Kidney rudiments were explanted onto a 0.4 μm pore size polyester membrane Transwell (#3450; Corning Inc, Lowell, MA, USA) and cultured at the air–liquid interface in FCS-containing medium [32]. Retinoic acid (#0695; Tocris BioScience, Minneapolis, MN, USA) was added at a concentration of 1 μm and the medium was changed every second day of the culture period.

Histological analysis

Urogenital systems were paraffin-embedded and sectioned at 5 μm. Haematoxylin and eosin staining was performed according to standard procedures.

In situ hybridisation analysis

Non-radioactive in situ hybridisation analysis of gene expression was performed on 10-μm-thick midsagittal kidney sections with digoxigenin-labelled antisense riboprobe [33].

Immunofluorescence for detection of antigens

Immunofluorescence analysis was performed on 5-μm-thick paraffin sections as previously described [32].

Apoptosis and cell proliferation assays

Apoptosis was assessed using a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay on 5-μm-thick paraffin kidney sections using the ApoTag Plus Fluorescein Detection Kit (S7111; Merck, Darmstadt, Germany). Cell proliferation rates were analysed on 5-μm-thick midsagittal kidney sections by immunofluorescence detection of Ki-67 (1:50, #M7249; DAKO, Jena, Germany).

RT-PCR analysis

Total RNA of pooled E14.5 kidneys was reverse-transcribed and analysed (see Supplementary materials and methods).

Image analysis

Sections were photographed using a Leica DM5000 microscope with a Leica DFC300FX digital camera or a Leica DM6000 microscope with a Leica DFC350FX digital camera and afterwards processed in Adobe Photoshop CS4.

Results

WT1 is expressed in stromal progenitors in the developing kidney

Previous work described renal expression of Wt1 in the nephrogenic lineage [12] and mentioned weak expression in the cortical stroma at E15.5 [34]. To more carefully characterise tissue-specific expression of Wt1 during kidney development, we performed (co-)immunofluorescence analysis of Wt1, the CM marker SIX2, and ALDH1A2, a marker of the cortical stroma, on midsagittal sections of E12.5, E14.5, and E18.5 wild-type kidneys. Wt1 was expressed in the CM (overlapping SIX2) at all stages analysed. Expression was detected in proximal regions of the renal vesicles and of comma- and S-shaped bodies from E12.5 onwards, and in mature podocytes of the glomerulus at E14.5 and E18.5, as previously reported [12]. From E11.5 to
E14.5, WT1 expression was additionally found in the capsular mesenchyme and overlapping with ALDH1A2 in the cortical stroma (Figure 1A). At E12.5, some WT1+ cells were detected in the medullary stroma (arrows in Figure 1A).

Deletion of Wt1 in the stromal lineage results in hypoplastic kidneys and cryptorchidism

To investigate the specific role of Wt1 in the renal stroma, we used a conditional gene inactivation approach with a floxed allele of Wt1 and a Foxd1<sup>fl/lox</sup> line which mediates recombination in stromal progenitors from E11.0 onwards [18,30]. Absence of WT1 protein in the stromal compartment of E12.5 Foxd1<sup>fl/lox</sup>;Wt1<sup>fl/fl</sup> (Wt1cKO) kidneys confirmed the validity of this approach (Figure 1B). Wt1cKO mice died shortly after birth with reasons unknown. At E18.5, all components of the urogenital system were present in the mutant. However, the kidneys were smaller; the gonads and the sex ducts were stretched due to lateral ligamentous attachment to the kidney; and excessive fibrous tissue covered the upper urinary system (Figure 2A). Histological analysis confirmed a normal cortico-medullary subdivision and the presence of papilla and pelvis in the mutant kidney (Figure 2B, columns 1, 2). In the cortical region, the UB tips presented mostly as strongly dilated ampullae predominantly connected to long unbranched stems compared with the control, where paired small ampullae connected to short stems (Figure 2B, columns 3, 4). The CM in the mutant was of normal thickness and followed the enlarged UB tips. In contrast, the capsular mesenchyme consisted of several layers of fibrocyte-like cells and extended between the UB tips and their CM into the cortex (Figure 2B, columns 3, 4).

We next screened expression of molecular markers indicating regionalisation and differentiation within the nephrogenic, stromal, and UB lineages (Figure 2C and supplementary material, Figure S1 for overviews). In the control, Sfrp1 marked the capsular and medullary stroma; Foxd1 the capsular and cortical stroma; Aldh1a2 the cortical stroma; and Pdgfra the cortical, medullary, and papillary stroma. In Wt1cKO kidneys, Sfrp1 and Foxd1 expression extended from the thickened capsule into the cortical region. All other expression sites of these and other stromal markers were unchanged. The CM markers Eya1, Six2, and Wt1 were expressed at normal levels and surrounded the enlarged UB tips in Wt1cKO kidneys. Expression of Wt1 in renal vesicles and prospective and definitive podocytes in developing and mature glomeruli was unchanged. The PTA marker, Wnt4, was irregularly spatially expressed around the enlarged UB tips including occasional ectopic cortical positions. Expression of Ret and Wnt11 at the widened UB tips was patchy and reduced (Figure 2C and supplementary material, Figure S1). Markers of nephron segments and of the collecting duct epithelium were unchanged, indicating normal nephrogenesis and UB differentiation in Wt1cKO kidneys (supplementary material, Figure S2).

Hence, loss of stromal Wt1 leads to an expansion of the capsular stroma into the cortical region. Disturbed morphology of UB tips indicates a non-cell autonomous requirement of stromal Wt1 for UB branching.

Phenotypic changes in Wt1cKO kidneys occur shortly after deletion of stromal Wt1 expression

To define the onset of these phenotypic changes, we analysed Wt1cKO embryos at E12.5, i.e. shortly after conditional deletion of Wt1 in the stroma. On histological sections, the mutant kidney appeared smaller and was poorly detached from the body wall mesenchyme (supplementary material, Figure S3A, columns 1, 2). Histological sections as well as analysis of UB tip markers (Ret, Wnt11) and CM markers (Wt1, Eya1, and Six2) indicated fewer UB tips that were partly ‘encircled’ rather than ‘capped’ by the CM in the mutant (supplementary material, Figure S3A,B). This impression may result from skewed section planes due to the smaller size of the mutant kidney or from an altered architecture of the ureteric tree. Wnt4<sup>+</sup> PTAs were present, but their number was reduced. Expression of Sfrp1, Foxd1, Aldh1a2, and Pdgfra was unchanged (supplementary material, Figure S3B).

Analysis of kidneys at E14.5 recapitulated the findings at E12.5. The mutant kidneys appeared smaller and were poorly detached from the body wall mesenchyme; UB tips were reduced in number. At this stage, Sfrp1 expression expanded from the capsular into the cortical region; Aldh1a2 expression in the cortical domain was clearly reduced. Expression of Ret at the UB tips appeared reduced (supplementary material, Figure S4).

Branching morphogenesis is altered in Wt1cKO kidneys

Our histological and molecular analyses suggested that loss of stromal Wt1 leads to a delay in branching morphogenesis, nephrogenesis, and growth during kidney development. To better appreciate these changes, we determined the number of UB tips and PTAs by counting Ret<sup>+</sup> and Wnt4<sup>+</sup> expression domains, respectively, and estimated kidney size by the area in histological sections at the previously analysed stages, E12.5, E14.5, and E18.5 (Figure 3A). In the control, the number of UB tips rose from 9 to 17 to 50, whereas in Wt1cKO embryos, 5, 10, and 33 UB tips were counted, presenting a significant reduction of 42%, 40%, and 34% at these stages. The number of PTAs was similarly reduced, which resulted in an unchanged ratio of nephrons to UB tips. The reduction in kidney size followed the pattern observed for UB tips and PTAs.

To visualise branching morphogenesis, we introduced the Hoxb7–GFP transgene, which marks the UB epithelium at all stages [31], into the mutant background. We explanted E11.5 kidney rudiments of mutant (Wt1cKO; Hoxb7-GFP+/+) and control (Wt1<sup>fl/fl</sup>;Hoxb7-GFP) embryos, and documented GFP epifluorescence over a
In the mutant, the number of UB tips was significantly reduced to 68% of the control both at day 2 and at day 3 of the culture. After 3 days, the numbers of terminal bifurcations and trifid (a stem with three terminal tips) and bifid (a stem with two terminal tips) branches were reduced, whereas the

Figure 1. WT1 is transiently expressed in the cortical and capsular stroma during metanephric development. (A) Immunofluorescence analysis on midsagittal sections of wild-type kidneys at E12.5, E14.5, and E18.5 determines the cell- and tissue specificity of WT1 expression in embryonic development. WT1 expression on whole kidney sections (column 1) with higher magnifications of the cortical region (column 2), and co-immunofluorescence analysis of WT1, the cap mesenchymal marker SIX2 (column 3) and the marker of the cortical stroma ALDH1A2 (column 4) are shown. The arrows in the first and third images (row 1) indicate WT1+ medullary stromal cells. (B) (Co-)immunofluorescence analysis of WT1 with the CM marker SIX2 on midsagittal sections of control and Wt1cKO kidneys at E12.5. White arrows in the Wt1cKO images indicate that WT1 expression is lost in the cortical and capsular stroma. In the higher magnification images of WT1 expression, the CM is 'stitched out' in white to distinguish it from cortical stroma. n ≥ 3, all staining. cb, comma-shaped body; cm, cap mesenchyme; g, glomerulus; ra, renal aggregate; rv, renal vesicle; sb, S-shaped body; st, stroma; ubt, ureteric bud tip.
Figure 2. *Wt1cKO* embryos exhibit renal hypoplasia, altered branching morphogenesis of the UB epithelium, and cryptorchidism at E18.5. (A) Morphology of the whole urogenital systems of male (columns 1, 2) and female (columns 3, 4) embryos. *n* = 10, all groups. Note the expanded epididymis and uterus tethered to the kidney. (B) Haematoxylin and eosin staining of midsagittal kidney sections. Higher magnification images of the cortex (columns 3, 4): asterisks indicate the widened UB tips, and arrowheads the expanded capsular stroma. *n* ≥ 3. (C) RNA in situ hybridisation analysis on midsagittal kidney sections for markers of the renal stroma (*Sfrp1*, *Foxd1*, *Aldh1a2*, *Pdgfra*), the CM (*Eya1*, *Six2*, *Wt1*), PTAs (*Wnt4*), and UB tips (*Wnt11*, *Ret*). Higher magnification images of the cortical region from whole kidney sections displayed in supplementary material, Figure S1 are shown. *Sfrp1* staining: note the expanded capsular stroma in the mutant (arrowheads). *Wnt11*, *Ret* staining: note the widened UB tips (*). *Wnt4* staining: ectopic *Wnt4*+ domain (arrow). *n* ≥3, all staining. bl, bladder; cb, comma-shaped body; cm, cap mesenchyme; cos, cortical stroma; cps, capsular stroma; e, epididymis; k, kidney; ms, medullary stroma; o, ovary; pa, papilla; pe, pelvis; pta, pretubular aggregate; rv, renal vesicle; t, testis; u, ureter; ubt, ureteric bud tip; ut, uterus.

© 2020 The Authors. *The Journal of Pathology* published by John Wiley & Sons, Ltd. on behalf of The Pathological Society of Great Britain and Ireland. www.pathsoc.org

*J Pathol* 2020; 252: 290–303 www.thejournalofpathology.com
numbers of UB tips connected to long stems (monodifid branches), and branches arising from pre-existing stems were increased, showing that the balance between UB tip branching and stem elongation was shifted towards the latter (Figure 3C). We also measured the length of the stems generated in each generation of branches in
this *ex vivo* culture system. At all the time-points analysed, the parental branch (P1) that arose from the ureter was (significantly) longer in *Wt1cKO* kidneys compared with controls. All daughter generations (filiae F1, F2, and F3) were shorter in the mutant, a finding that reached significance at day 3 of culture (Figure 3D). We conclude that stromal *Wt1* is required to control the branching behaviour of the UB epithelium.

Altered proliferation and lineage contribution in *Wt1cKO* kidneys

We next asked whether the observed changes are preceded and/or accompanied by alterations in apoptosis, proliferation, and/or cell fate. The TUNEL assay invariably detected apoptotic bodies in the medullary stroma of both control and *Wt1cKO* kidneys at E12.5 and E14.5 (Figure 4A). We next performed co-staining for the Ki-67 antigen with ALDH1A2, SIX2, and CDH1 to mark proliferating cells in the cortical stroma, CM, and UB, respectively (Figure 4B). Stromal cell division was significantly reduced in the mutant kidney at both analysed stages. The proliferative index of the CM was significantly elevated in the mutant, whereas proliferation was robustly reduced in the UB tips at E12.5.

To investigate whether the stromal fate is altered upon loss of *Wt1*, we crossed the R26*flm2G* reporter line [29] into the mutant background and performed co-staining of the lineage marker GFP with the regionalisation markers ALDH1A2 (cortical stroma), SIX2 (CM), and SOX9/CDH1 (UB tips) at E14.5 and E18.5 (Figure 4C). At E14.5, the mutant stromal lineage exclusively contributed to the capsular tissue, the cortical interstitium, and the entire medullary stroma as in the control (supplementary material, Figure S5). At E18.5, GFP+ cells were ectopically and variably found in UB tips and stalks (~4.5 GFP+ cells per UB tip, see white arrowheads in Figure 4C) in *Wt1cKO* kidneys. Hence, *Wt1* is required early to increase proliferation in the cortical stroma; at later stages, it may prevent an epithelial transition and a fate shift of stromal cells.

Altered *Spry* expression and RA signalling in the cortical stroma of *Wt1cKO* kidneys

In order to determine the molecular changes that may cause the branching defects in *Wt1cKO* kidneys, we analysed the expression of genes encoding factors previously implicated in the development and crosstalk of the different renal lineages [8] on sections of E12.5, E13.5, and E14.5 kidneys. We did not find changes in the expression of *Axin2* – target of WNT signalling [35,36]; of *Pch1* – target of sonic hedgehog (SHH) signalling [37,38]; of the bone morphogenetic protein (BMP) ligand genes, *Bmp4* and *Bmp7*, of the BMP antagonist *Bmpr1a*; of Id2 and Id4 – targets of BMP signalling [39,40]; of the fibrocyte growth factor (FGF) ligand genes *Fgf7* and *Fgf10*; or of the targets of FGF signalling, *Etv4* and *Etv5* [41–43] (supplementary material, Figures S6 and S7). Other stromal genes and regulators such as *Tcf21*, *Akap12*, *Decorin*, and *Fat4* [8] were not changed at E14.5 either (supplementary material, Figure S8).

In contrast, expression of *Spry1* and *Spry2*, encoding targets and antagonists of FGF/mitogen-activated protein kinase (MAPK) signalling [44], was increased in the cortical stroma at E13.5 and E14.5. Expression of the gene encoding the RA synthesising enzyme, *Aldh1a2*, as well as of the targets of RA signalling, *Ecml* and *Rarb*, was unchanged in this domain at these stages (Figure 5). We concluded that loss of *Wt1* affects RA and FGF/MAPK signalling in the cortical stroma at E13.5 and E14.5.

Reduced RA synthesis contributes to branching defects in *Wt1cKO* kidneys

Since stromal RA acts via *Ret* to maintain branching morphogenesis of the ureteric tree [46–49], we wished to further validate alteration of *Aldh1a2* and *Ret* expression in *Wt1cKO* kidneys. In fact, RT-PCR analysis confirmed the reduction of *Aldh1a2* and *Ret* expression in mutant kidneys at E14.5. Expression of the CM gene *Six2* was unchanged and expression of *Spry1* and *Spry2* was increased in this assay, confirming our *in situ* hybridisation data (Figure 6A and supplementary material, Figure S9).

Figure 3. Branching morphogenesis is severely altered in *Wt1cKO* kidneys in *vivo* and in *vitro*. (A) Branching morphogenesis of *Wt1cKO* kidneys at E12.5, E14.5, and E18.5 *in vivo*. Quantification of UB tips by counting *Ret*+ domains (first panel) and of PTAs by counting *Wnt4*+ domains (second panel) on midsagittal kidney sections subjected to *in situ* hybridisation analysis. Neprhin induction (ratio of *Ret*+ UB tips to *Wnt4*+ PTAs for each section analysed by double *in situ* hybridisation) was unchanged (third panel). Estimation of the size of the developing kidney was done by analysis of the area (in μm²) in midsagittal histological sections. All quantifications were performed on six to eight mid-sagittal sections of three control and mutant kidneys each at E12.5, E14.5, and E18.5 as shown. (B, C) Branching morphogenesis of explants of E11.5 kidney rudiments of control (*Wt1fl*/*fl, *Hoxb7-GFP/+*) and mutant (*Wt1cKO; *Hoxb7-GFP/+*) embryos cultured over a 4-day period. (B) Epifluorescence analysis of GFP expression 1-day intervals over the culture period. (C) Quantification of *in vitro* branching morphogenesis by counting the number of UB tips at days 1, 2, and 3 of the culture (first panel), and by counting terminal bifurcations (second panel), stem branches (third panel), trifid (fourth panel), bifid (fifth panel), and single tips connected to a stem (monofid branches, sixth panel) after 3 days in culture. Insets and arrows therein indicate the branching event counted. All quantifications were performed on six control and mutant kidneys each at the indicated time-points. (D) Quantification of the length of each branch generation of the UB tree at day 1 to day 3 of the culture. Coloured skeletons in the upper right inset (row 5) serve as examples of branch generations. P, parental; F, daughter branches. Values are displayed as mean ± SD; ns: *p > 0.05*, *p ≤ 0.05*, **p ≤ 0.01**, ***p ≤ 0.001* by two-tailed Student’s *t*-test. For statistical values see supplementary material, Table S1.
To explore whether reduced RA synthesis contributes to the observed branching defects, we explanted Wt1cKO kidneys at E11.5 and treated them with 1 μM RA. After 3 days in culture, we detected a significant increase in UB tips, terminal bifurcations, and stem and bifid branches, and a decrease of trifid and monoid branches in Wt1cKO kidneys treated with RA compared with mutant kidneys treated with solvent. Importantly, monoid branches were reduced to the level of the untreated control (Figure 6B and supplementary material, Figure S10). The length of the parental branch that arose from the ureter was reduced to the untreated control level in Wt1cKO kidneys treated with RA (Figure 6C). We conclude that stromal Wt1 maintains branching morphogenesis of the ureteric tree at least partly by maintaining Aldh1a2 expression and RA signalling.

Figure 4. Changes of proliferation and cell fate in Wt1cKO kidneys. (A) The TUNEL assay detects cell death in the medullary stroma both in control and in Wt1cKO kidneys at E12.5 and E14.5 (n = 3 each). Nuclei are counterstained with DAPI. (B) Determination of cellular proliferation by Ki-67 antigen detection on midsagittal sections of control and Wt1cKO kidneys at E12.5 and E14.5. The ratio of Ki-67-positive nuclei to DAPI-positive nuclei in the cortical stroma, the CM and the UB tips in control (grey bars) and Wt1cKO embryos (blue bars) is shown. Values are shown as mean ± SD. ns, non-significant (p > 0.05), *Significant (p < 0.05); two-tailed Student’s t-test. For statistical values see supplementary material, Table S1. (C) Lineage tracing of Foxd1+ stromal progenitors in control and Wt1cKO kidneys at E18.5. Co-immunofluorescence analyses on midsagittal kidney sections of the lineage marker GFP and the marker for cortical stroma ALDH1A2, the CM marker SIX2, the UB tip marker SOX9, and the epithelial marker CDH1 of control (Foxd1cre+/R26mTmG/+; Wt1fl/fl) and mutant (Foxd1cre+/R26mTmG/+; Wt1fl/fl; R26mTmG) embryos are shown. Nuclei are counterstained with DAPI. White arrows point to GFP+ cells in UB tips. n ≥ 3, each staining. csb, comma-shaped body; cm, cap mesenchyme; cos, cortical stroma; g, glomerulus; ms, medullary stroma; u, ureter; ubt, ureteric bud tip.
Figure 5. RA signalling is transiently reduced in \textit{Wt1cKO} kidneys. \textit{In situ} hybridisation analysis of expression of the genes encoding the FGF/MAPK antagonists, \textit{Spry1} and \textit{Spry2}; the genes encoding the RA synthesising enzyme, \textit{Aldh1a2}; and the targets of RA signalling \textit{Ecm1} and \textit{Rarb} on midsagittal sections of control and \textit{Wt1cKO} kidneys at E12.5, E13.5, and E14.5. Expression of \textit{Spry1} and \textit{Spry2} is enhanced in the cortical stroma; expression of RA pathway genes is reduced in \textit{Wt1cKO} kidneys at E13.5 and E14.5. At least three embryos of each genotype were used for each analysis.
Discussion

Previous work described that WT1 controls multiple cellular programs in the nephrogenic lineage. Here, we revealed an independent requirement of Wt1 in the FOXD1+ stromal lineage to maintain the proliferative expansion of cortical stromal progenitors; to restrict differentiation of capsular stromal cells; and to control branching morphogenesis of the UB tips. The latter function may at least partly be mediated by maintaining Aldh1a2 expression, hence RA signalling.

Figure 6. Ectopic RA ameliorates branching defects in Wt1cKO kidneys in culture. (A) RT-PCR analysis shows that expression of Aldh1a2 and Ret is strongly reduced in mRNA derived from pools of kidneys from male (m) and female (f) Wt1cKO embryos at E14.5. Note that the CM marker Six2 is unchanged. Expression is normalised to Gapdh. (B, C) Branching morphogenesis of explants of E11.5 kidney rudiments of control (Wt1fl/fl; Hoxb7-GFP/+; Six2-GFP/-) and mutant (Wt1cKO; Hoxb7-GFP/+; Six2-GFP/-) embryos cultured over a 3-day period in the presence of solvent (DMSO) or 1 μM RA. Quantification of in vitro branching morphogenesis by counting the number of UB tips at days 1, 2, and 3 of the culture (first panel), and by counting terminal bifurcations (second panel), stem branches (third panel), trifid (fourth panel), bifid (fifth panel), and single tips connected to a stem (monoid branches, sixth panel) after 3 days in culture. All quantifications were performed on six control and mutant kidneys each at the indicated time-points (B). Quantification of the length of each branch generation of the UB tree at day 1 to day 3 of the culture. P, parental; F, daughter branches (C). All values are displayed as mean ± SD; ns: p > 0.05; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 by two-tailed Student’s t-test. For statistical values see supplementary material, Table S1.
WT1 acts downstream of FOXD1 to mediate some of the stromal functions

Our immunofluorescence analysis detected expression of WT1 in the cortical stroma at E12.5 and E14.5. Weak expression may persist until E18.5 according to recent scRNA-seq data [50]. Conditional deletion in the stromal lineage left WT1 expression in the nephrogenic lineage unchanged, indicating that both domains are independent of each other. Wt1cko kidneys exhibited reduced proliferation of the cortical stroma and an expansion of the capsular stroma into the cortical region. Maintenance or expansion of capsular fibrocytes may also account for tethering of the gonads and the sex ducts to the kidneys, which we observed in Wt1cko embryos. Interestingly, a significant number of patients with germline mutations in WT1 display cryptorchidism or a non-scrotal testis position. This phenotype was initially explained by genital defects including failed masculinisation [51]. More recently, a Retcre line was used to conditionally delete Wt1 in the gubernacula, the caudal genital ligaments important for testicular descent. About 40% of adult males exhibited unilateral, always left-sided cryptorchidism which was explained by a failed differentiation of the gubernaculum from mesenchymal precursors [52]. Given the complete penetrance of cryptorchidism in our conditional model and the fact that Ret is expressed in stromal cells, it is likely that the ligaments important for gonadal descent are derivatives of FOXD1+ stromal cells, the correct differentiation of which depends on Wt1. In any case, it shows that cryptorchidism is unrelated to a genital function but reflects a novel stromal requirement for Wt1.

Besides these stromal differentiation defects, we observed delayed and altered branching morphogenesis of the ureteric tree in Wt1cko kidneys. The total nephron number was reduced but correlated with the number of UB tips, indicating that the nephron-inducing capacity of the UB tips in the mutant (at least until E18.5) was not affected. Kidney hypoplasia is therefore likely to relate to the delay in branching morphogenesis. Occasional ectopic cortical expression of Wnt4 may be due to altered architecture of the ureteric tree rather than premature differentiation of the CM.

The phenotypic changes observed in Wt1cko kidneys are much less severe than those observed in mice with loss of Foxd1 or the FOXD1 lineage [9,11], and do not comprise an expansion of SIX2+ CM observed in mice with conditional gene targeting of Pbx1, Tcf21, Sall1, and Fat4 in the stroma [53–57]. This confirms that stromal control of CM and UB tip development are molecularly distinct and shows that WT1 acts downstream of FOXD1 in a subprogram of stromal proliferation and differentiation, and in signal-mediated control of branching morphogenesis of the UB epithelium.

Reduced RA signalling accounts for defects in branching morphogenesis in Wt1cko kidneys

A number of studies provided compelling evidence that stromal RA acts via Ret to maintain branching morphogenesis. Deletion of Aldh1a2 resulted in smaller kidneys, decreased branching morphogenesis, and reduced expression of Ret. Conversely, isolated UB tips treated with RA upregulated Ret expression [49]. Misexpression of a dominant-negative RARa in the UB lineage also led to reduced Ret expression and branching, as did the combined stromal loss of Rara and Rarb [47,49]. Induced expression of Ret in UB tips rescued UB branching in RA signalling mutants [48]. More recently, evidence was provided that RA-induced extracellular matrix 1 (ECM1) reduces RET in the UB cleft, where bifurcation normally occurs [46].

Our expression analysis showed that expression of the genes encoding the RA synthesising enzyme, Aldh1a2, and the targets of RA signalling, Rarb and Ecm1, was downregulated in the cortical stroma of Wt1cko kidneys at E13.5 and E14.5. This correlated with reduced expression of Ret at the UB tips at this and later stages, and an irregular patchy expression at the widened UB tips at E18.5. Importantly, we found that administration of RA to explant cultures of Wt1cko kidneys ameliorated the branching defects, mainly by favouring UB tip bifurcation at the expense of stem elongation. Together, these findings argue that stromal Wt1 is transiently required to maintain or increase Aldh1a2 expression and, hence, RA signalling, which in turn, possibly via ECM1, modulates Ret expression and RET dynamics at the UB tips. At later stages, when Wt1 expression is downregulated, Aldh1a2 expression becomes independent of WT1. It is important to note that the UB branching defects in Wt1cko kidneys are much less severe than those observed in kidneys with complete loss of Aldh1a2 or of stromal and epithelial RA signalling. This is in line with our observation that Aldh1a2 expression and RA signalling, as well as Ret expression, are transiently and weakly reduced in WT1cko kidneys. Interestingly, dependence of Aldh1a2 expression on WT1 function has been observed in other developmental contexts as well [58–62].

Our analysis detected increased expression of the genes encoding the FGF/MAPK signalling inhibitors, Spry1 and Spry2 [44], in the cortical stroma of Wt1cko kidneys. Although our in situ hybridisation analysis did not find changes of the FGF signalling targets Etv4 and Etv5 [41], it remains possible that enhanced SPRY expression interferes with FGF receptor activities and leads to transcription-independent changes in the activity of downstream kinases. Possibly, this contributes to reduced proliferation of the cortical stroma in WT1cko kidneys.

A detailed molecular analysis previously reported that WT1 acts in the CM by maintaining FGF signalling and inhibiting BMP signalling via induction of Bmpr [63]. We did not observe changes of these pathways in the mutant kidney, indicating that WT1 regulates distinct sets of target genes in the two tissue contexts.

Our lineage tracing analysis identified that the bulk of FOXD1+ stromal progenitors contributed to capsular tissue, and interstitial fibrocytes of cortex, medulla, and papilla in Wt1cko kidneys. However, expression of the GFP reporter was also detected in some cells of the
Stromal WT1 and Wilms’ tumour

Wilms’ tumour, the most frequent renal paediatric cancer, classically exhibits a triphasic histology with epithelial duct-like structures, blastemal elements of an undifferentiated, strongly proliferative character, and a stromal fibrous component that ensheaths the blastema [65]. Given the finding that WT1 is mutated in almost 15% of all presented Wilms’ tumour cases [22], it is conceivable that a stromal loss of WT1 may somehow contribute to tumour initiation or progression. However, a recent study that deleted Wt1 in the stromal lineage with or without Ctnnb1 or Igf2 stabilisation did not detect tumour development in these mice [66]. We did not detect tumours in W1cKO embryos but either observed Sfrp1-positive strands of capsular stroma invading the cortex of the mutant kidneys after E14.5. This is reminiscent of the stromal appearance in Wilms’ tumours and may suggest a contribution of stromal WT1 loss to the cytoarchitecture of this childhood tumour.

Acknowledgements

We thank Rolf Kemler for the anti-CDH1 antiserum; Rolf Zeller and Frank Costantini for mice; and Marc-Oliver Trowe, Timo H-W Lüdtke, and Marc-Jens Kleppa for technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG KI1728/7-2) to AK. Open access funding enabled and organized by Projekt DEAL.

Author contributions statement

ACW, CE and AK conceived and designed the study. ACW and RRR performed experiments. CE provided essential material. ACW performed statistical analyses. All the authors contributed to data interpretation. ACW and AK drafted the paper. All the authors approved the final version of the paper.

References

1. McMahon AP. Development of the mammalian kidney. Curr Top Dev Biol 2016; 117: 31–64.
2. Little MH, McMahon AP. Mammalian kidney development: principles, progress, and projections. Cold Spring Harb Perspect Biol 2012; 4: a008300.
3. Kobayashi A, Valerius MT, Mugford JW, et al. Six2 defines and regulates a multipotent self-renewing nephron progenitor population throughout mammalian kidney development. Cell Stem Cell 2008; 3: 169–181.
4. Oxburgh L. Kidney nephron determination. Annu Rev Cell Dev Biol 2018; 34: 427–450.
5. Costantini F, Shakya R. GDNF/Ret signaling and the development of the kidney. Bioessays 2006; 28: 117–127.
6. Riccio P, Cebrian C, Zong H, et al. Ret and Evt4 promote directed movements of progenitor cells during renal branching morphogenesis. PLoS Biol 2016; 14: e1002382.
7. Carroll TJ, Park JS, Hayashi S, et al. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. Dev Cell 2005; 9: 283–292.
8. Rowan CJ, Sheybani-Deloui S, Rosenblum ND. Origin and function of the renal stroma in health and disease. Results Probl Cell Differ 2017; 60: 205–229.
9. Hatmi V, Huh SO, Herzlinger D, et al. Essential role of stromal mesenchyme in kidney morphogenesis revealed by targeted disruption of Winged Helix transcription factor BF-2. Genes Dev 1996; 10: 1467–1478.
10. Kobayashi A, Mugford JW, Krautzberger AM, et al. Identification of a multipotent self-renewing stromal progenitor population during mammalian kidney organogenesis. Stem Cell Rep 2014; 3: 650–662.
11. Hum S, Rymer C, Schaefer C, et al. Ablation of the renal stroma defines its critical role in nephron progenitor and vasculature patterning. PLoS One 2014; 9: e88400.
12. Armstrong JF, Pritchard-Jones K, Bickmore WA, et al. The expression of the Wilms’ tumour gene, WT1, in the developing mammalian embryo. Mech Dev 1993; 45: 85–97.
13. Kreidberg JA, Sariola H, Loring JM, et al. Foxd1 shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. Development 1999; 126: 1845–1857.
14. Armstrong JF, Pritchard-Jones K, Bickmore WA, et al. The expression of the Wilms’ tumour gene, WT1, in the developing mammalian embryo. Mech Dev 1993; 4: 85–97.
15. Davis JA, Ladomery M, Hohenstein P, et al. Development of an siRNA-based method for repressing specific genes in renal organ culture and its use to show that the W1 tumour suppressor is required for nephron differentiation. Hum Mol Genet 2004; 13: 235–246.
16. Moore AW, McHness L, Kreidberg J, et al. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. Development 1999; 126: 1845–1857.
17. Chua YY, Brownstein D, Mjoseg H, et al. Acute multiple organ failure in adult mice deleted for the developmental regulator Wt1. PLoS Genet 2011; 7: e1002404.
18. 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.
19. Berry RL, Ozdemir DD, Aronow B, et al. Deducing the stage of origin of Wilms’ tumours from a developmental series of Wt1-mutant mice. Dis Model Mech 2015; 8: 903–917.
20. Call KM, Glaser T, Ito CY, et al. Isolation and characterization of a zinc finger polypeptide gene at the human chromosome 11 Wilms’ tumor locus. Cell 1990; 60: 509–520.
21. Gessler M, Poustka A, Cavenee W, et al. Homozygous deletion in Wilms tumours of a zinc-finger gene identified by chromosome jumping. Nature 1990; 343: 774–778.
22. Charlton J, Pritchard-Jones K. WT1 mutation in childhood cancer. Methods Mol Biol 2016; 1467: 1–14.
23. Haber DA, Buckler AJ, Glaser T, et al. An internal deletion within an 11p13 zinc finger gene contributes to the development of Wilms’ tumor. Cell 1990; 61: 1257–1269.
24. Rose EA, Glaser T, Jones C, et al. Complete physical map of the WAGR region of 11p13 localizes a candidate Wilms’ tumor gene. Cell 1990; 60: 495–508.

25. Polletier J, Brunovnek W, Kashtan CE, et al. Germline mutations in the Wilms’ tumor suppressor gene are associated with abnormal urogenital development in Denys-Drash syndrome. Cell 1991; 67: 137–47.

26. Barbaux S, Niaudet P, Gubler MC, et al. Donor splice-site mutations in WT1 are responsible for Frasier syndrome. Nat Genet 1997; 17: 467–470.

27. Narahara K, Kikkawa K, Kimira S, et al. WT1 promotes nephrogenesis by controlling the expression of pronephric genes in the kidney. Nat Genet 1997; 17: 471–474.

28. Hulfler V. Wilms tumor genetics. Am J Med Genet 1998; 79: 260–267.

29. Muzumdar MD, Tasie B, Miyamichi K, et al. A global double-fluorescent Cre reporter mouse. Genesis 2007; 45: 593–605.

30. Humphreys BD, Lin SL, Kobayashi A, et al. WT1 expression is restricted to the nephron progenitor cells in the developing mouse kidney. J Am Soc Nephrol 2010; 19: 85–97.

31. Srinivas S, Goldberg MR, Watanabe T, et al. Expression of green fluorescent protein in the ureretic bud of transgenic mice: a new tool for the analysis of ureteric bud morphogenesis. Dev Genet 1999; 24: 241–251.

32. Bohnenpoll T, Ferraric S, Nattkemper M, et al. Diversification of cell lineages in urerter development. J Am Soc Nephrol 2017; 28: 1792–1801.

33. Moorman AF, Houweling AC, de Boer PA, et al. Sensitive nonradioactive detection of mRNA in tissue sections: novel application of the whole-mount in situ hybridization protocol. J Histochem Cytochem 2001; 49: 1–8.

34. Dong L, Pietsch S, Engert C. Towards an understanding of kidney diseases associated with WT1 mutations. Kidney Int 2015; 88: 684–690.

35. Jho EH, Zhang T, Domon C, et al. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. Mol Cell Biol 2002; 22: 1172–1183.

36. Boivin FJ, Bridgewater D, β-Catenin in stromal progenitors controls medullary stromal development. Am J Physiol Renal Physiol 2018; 314: F1177–F1187.

37. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. Genes Dev 2001; 15: 3059–3087.

38. Rowan CJ, Li W, Martirosyan H, et al. Hedgehog/GLI signaling in Fzd1-positive stromal cells promotes murine nephrogenesis via TGFB signaling. Development 2018; 145: dev159947.

39. Hoffmann A, Ohlmann V, Heymer J, et al. Igf1 genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. J Biol Chem 1999; 274: 19838–19845.

40. Nishinakamura R, Sakaguchi M. BMP signaling and its modifiers in kidney development. Pediatr Nephrol 2014; 29: 681–686.

41. Zhang Z, Verheyden JM, Hassell JA, et al. FGF-regulated Etv genes are essential for repressing Shh expression in mouse limb buds. Dev Cell 2009; 16: 607–613.

42. Qiao J, Uzzo R, Obarah-Ishihara T, et al. FGF-7 modulates ureteric bud growth and nephron number in the developing kidney. Development 1999; 126: 547–554.

43. Obuchi H, Hori Y, Yamasaki M, et al. FGF10 acts as a major ligand for FGF receptor 2 IIb in mouse multi-organ development. Biochem Biophys Res Commun 2000; 277: 643–649.

44. Hanafusa H, Torri S, Yasunaga T, et al. Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. Nat Cell Biol 2002; 4: 850–858.

45. Mendelsohn C, Ruberte E, LeMeur M, et al. Developmental analysis of the retinoic acid-inducible RAR-beta 2 promoter in transgenic animals. Development 1991; 113: 723–734.
SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Wt1cKO kidneys show a normal cortico-medullary subdivision but exhibit subtle changes in the organisation of the cortical region at E18.5
Figure S2. Nephron development and collecting duct differentiation are not affected in WT1cKO kidneys at E18.5
Figure S3. Phenotypic changes in Wt1cKO kidneys are present at E12.5
Figure S4. Phenotypic changes in Wt1cKO kidneys are present at E14.5
Figure S5. The fate of stromal cells is not changed in Wt1cKO kidneys at E14.5
Figure S6. Loss of Wt1 in the cortical stroma does not affect WNT, SHH, or BMP signalling in the developing kidney
Figure S7. Loss of Wt1 in the cortical stroma does not affect FGF signalling in the developing kidney
Figure S8. Loss of Wt1 in the cortical stroma does not affect expression of genes encoding regulators of stromal development in the E14.5 kidney
Figure S9. Expression of Spry1 and Spry2 is increased in Wt1cKO at E14.5
Figure S10. Ectopic RA ameliorates branching defects in Wt1cKO kidneys in culture

Table S1. Statistical analyses of all quantitative assays performed in this study

50 Years ago in The Journal of Pathology...

Fine structure of the skin in angiokeratoma corporis diffusum (Fabry’s disease)
P. J. Van Mullem, M. Ruiter

Changes in liver lysosomes and cell junctions close to an invasive tumour
S. T. G. Butterworth

Evidence for a posible endogenous antigen in chronic inflammation
D. A. Willoughby, G. B. Ryan

To view these articles, and more, please visit: www.thejournalofpathology.com
Click 'BROWSE' and select 'All issues', to read articles going right back to Volume 1, Issue 1 published in 1892.