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Secreted Wnt antagonist Dickkopf-1 controls kidney papilla development coordinated by Wnt-7b signalling

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ABSTRACT

Wnt signalling regulates several aspects of kidney development such as nephrogenesis, ureteric bud branching and organisation of the collecting duct cells. We addressed the potential involvement of Dickkopf-1 (Dkk1), a secreted Wnt pathway antagonist. Dkk1 is expressed in the developing mouse kidney by pretubular cell aggregates and the nephrons derived from them. Besides the mesenchyme cells, the epithelial ureteric bud and more mature ureteric bud derivatives in the medulla and the papilla tip express the Dkk1 gene. To reveal the potential roles of Dkk1, we generated a floxed allele and used three Cre lines to inactivate Dkk1 function in the developing kidney. Interestingly, Dkk1 deficiency induced by Pax8Cre in the kidneys led in newborn mice to an overgrown papilla that was generated by stimulated proliferation of the collecting duct and loop of Henle cells, implying a role for Dkk1 in the collecting duct and/or loop of Henle development. Since Pax8Cre-induced Dkk1 deficiency reduced marker gene expression, Scnn1b in the collecting duct and Slc12a1 in the loop of Henle, these results together with the extended papilla phenotype are likely reasons for the decreased amount of ions and urine produced by Dkk1-deficient kidneys in the adult. Recombinant Dkk1 protein in cultured cells inhibited Wnt-7b-induced canonical Wnt signalling, which is critical for collecting duct and loop of Henle development. Moreover, Dkk1 deficiency led to an increase in the expression of canonical Wnt signalling of target Lef-1 gene expression in the stromal cells of the developing papilla. Based on the results, we propose that Dkk1 controls the degree of Wnt-7b signalling in the papilla to coordinate kidney organogenesis.

Introduction

Development of the permanent mammalian kidney begins when the epithelial Wolffian duct at its caudal-most end starts to grow dorsally towards the predetermined metanephric mesenchyme and forms the ureteric bud. The ureteric bud branches from its tips during kidney organogenesis, and each of the generated branches induces nephrogenesis in discrete cells of the adjacent mesenchyme, the cap cells, leading subsequently to their mesenchyme-to-epithelium transition. This process is followed by simple morphogenesis steps of the epithelialised renal vesicles and leads to formation of the segmented nephrons via comma and S-shaped stages and fusion of the structures to the branches of the ureteric tree. In association with nephrogenesis, the endothelial cells are attracted to establish the functional glomerular and nephron-associated vasculature network (Vainio and Lin, 2002; Yu et al., 2004; Schedl, 2007; Merkel et al., 2007; Costantini and Kopan, 2010).

The developing kidney expresses a number of Wnt gene family members that encode secreted signalling factors whose function is critical for organogenesis (Merkel et al., 2007; Nusse et al., 2008; Haegebarth and Clevers, 2009). These include *Wnt-2b, -4, -6, -7b, -9b* and *−11* (Stark et al., 1994; Kispert et al., 1996; 1998; Lin et al., 2001a; Itäranta et al., 2002; Yu et al., 2009; Carroll et al., 2005), and knockout studies have so far identified roles for *Wnt-4, Wnt-7b, Wnt-9b* and *Wnt-11* in the control of kidney organogenesis.

The availability of Wnts for signalling is regulated in part by the extracellular matrix (ECM) and certain secreted and ECM-integrated antagonists. The antagonists can bind directly to Wnt proteins and prevent their binding to Frizzled or LRP5/6 co-receptors at the cell surface. Dickkopf (Dkk) proteins represent secreted Wnt antagonists (Glinka et al., 1998; Niehrs, 2006) and they are thought to block Wnt signalling by interacting directly with specific domains of the Frizzled or LRP5/6 co-receptors, preventing formation of a Frizzled-Wnt-LRP6 complex (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001;

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Bourhis et al., 2010). Besides this, *Dkks* can form a complex with Kremen co-receptors, which leads to *Dkk*-regulated caveolae-mediated endocytosis of LRP to inhibit Wnt signalling in a target cell (Mao et al., 2002; Yamamoto et al., 2008; Li et al., 2010a; Sakane et al., 2010). R-Spondin1 is thought to relieve the inhibition that *Dkk1* imposes on the Wnt pathway (Binnerts et al., 2007). *Dkk1* is a critical head inducer in the developing frog and mouse (Niehrs et al., 2001), and evidence is available that in mammals, *Dkks* are involved directly or via modulation of the LRPs in ontogeny of the limb, bone, vertebra, craniofacial, skin and eye morphogenesis and certain diseases such as cancer, blindness and bone disorders (Niehrs, 2006; Li et al., 2010b; Lin et al., 2010).

Given the critical role of Wnt signalling in kidney organogenesis and the presence of *Dkks* in murine kidney (Monaghan et al., 1999) we addressed if *Dkks* would be involved in organogenesis and focused our analysis on *Dkk1*. We demonstrate that *Dkk1* is expressed in the developing kidney, particularly the developing nephrons, and the ureteric bud cells and is later confined to the collecting duct and the kidney papilla epithelial tip cells. By making a floxed *Dkk1* allele and by inactivating *Dkk1* function with *Pax8Cre*, we show that *Dkk1* is important for development of the kidney papilla. *Dkk1* deficiency by *Pax8Cre*-stimulated papilla development by enhancing cell proliferation in the collecting duct and the loop of Henle cells, whose marker gene expression was concurrently reduced. In a cell culture model, *Dkk1* attenuated signalling of *Wnt-7b*, which represents a Wnt that is critical for papilla development and induced expression of *Lef-1* gene encoding a canonical Wnt signalling target. We propose that *Dkk1* coordinates kidney papilla development by taking part in the control over the degree of *Wnt-7b* signalling from the collecting duct cells to the stroma.

Methods

Mouse lines and embryos

Generation of Dkk1flox mice and the Cre lines used for conditional gene inactivation

To generate tissue-specific *Dkk1* mutant mice, we used homologous recombination in ES cells to modify the *Dkk1* allele so that *Dkk1* exons 1 and 2 were flanked by *loxP* sites (Fig. 2). The targeting vector was linearised by NotI digestion prior to electroporation into 129/Ola embryonic stem (ES) cells. To select for correct gene targeting, the electroporated ES cells were cultured in the presence of 300 μg/ml G418. Single G418-resistant ES cell clones were picked and expanded individually. ES cell clones containing a $Dkk1^{floxneo}$ allele were identified by Southern blot (Fig. 2B). Two positive $Dkk1^{floxneo/+}$ ES cells were used to generate chimeric mice by blastocyst injection. Male chimeras were mated with C57BL/6 wild-type females for germ line transmission of the $Dkk1^{floxneo}$ gene. $Dkk1^{floxneo/+}$ progeny were distinguished from wild-type littermates by PCR genotyping of tail samples. To remove the *thymidine kinase* (TK) and *neo* selection cassettes by FLP-mediated recombination, the $Dkk1^{floxneo/+}$ mice were mated with transgenic *Tg(ACTFLPe)9205Dym* mice (Dymecki, 1996) kindly provided by Prof. Günther Schütz (DKFZ Heidelberg). This gave rise to $Dkk1^{flox/+}$ mice that lack the selection cassettes, and in which the exons 1 and 2 are flanked by *LoxP* sites. Recombination was confirmed by PCR and Southern blot analysis. The $Dkk1^{flox/+}$ mice were back-crossed with C57BL/6 mice to obtain a congenic strain.

Generation and genotyping of *Wnt-4Cre*, *Pax3Cre* and *Pax8Cre* mouse lines has been described earlier (Bouchard et al., 2004; Engleka et al., 2005; Shan et al., 2010). *Cre* expression and its function were confirmed by crossing the *Cre* lines with a floxed *Rosa26-LacZ* reporter line (Soriano, 1999). The embryos for the studies were collected from matings between the C57Bl6 and 129SV mice. The embryos were considered to be E0.5 at noon of the day of the appearance of the vaginal plug.

The presence of *Cre* and loss of floxed *Dkk1* exons (n, null allele) was analysed by PCR in genomic DNA samples extracted from ear clips. The following primers were used to genotype the *Dkk1* conditional "c" allele; 5′-AGA ACT AAC CCA GCC CCA CAG CAG A-3′, 5′-CTC CTC AGG GAA GAC AAC AAA GCC G-3′ and 5′-GTG CTC AAA CAC AAG CCA GTG ACG A-3′. Inheritance of the *Cre* gene in the embryos and transgenic mice was analysed with the primers 5′-GCA CGT TCA CCG GCA TCA AC-3′ and 5′-CGA TGC AAC GAG TGA TGA GGT TC-3′. To obtain embryos with the conditional *Dkk1* knockout, first the $Dkk1^{n/+}$ mice were crossed with the respective Cre^{+} line, and the littermates that had inherited the Cre^{+}; $Dkk1^{n/+}$ genotype were maintained and crossed with the $Dkk1^{c/c}$ mice to generate Cre^{+};$Dkk1^{n/c}$ mice.

The animal care and procedures in this study were in accordance with the principles and guidelines of the ATBW (officials for animal welfare), German law and Finnish national legislation concerning the use of laboratory animals, the European Convention for the protection of vertebrate animals used for experimental and other scientific purpose (ETS 123), and EU Directive 86/609/EEC. The experiments involving generation of $Dkk1^{flox}$ mice were reviewed by the Internal Animal Protection Commission of the DKFZ and finally approved by the administrative headquarters "Regierungspräsidium Karlsruhe" of the State of Baden-Württemberg. The approval is based on a positive votum of an appointed state governmental ethical commission according to §15 of the German Animal Protection law (approved licence numbers: G-108/05, A-08/05).

RNA isolation and quantitative real-time PCR

RNA from freshly collected wild-type mouse tissue was isolated with Trizol Reagent (Invitrogen). cDNA was synthesised from total RNA using Superscript II Reverse Transcriptase (Invitrogen). The cDNA samples were further diluted and used for quantification of gene expression by real-time PCR. Quantitative real-time PCR (qRT-PCR) was used to analyse and compare *Dkk1* expression levels using cDNA templates from different tissues of newborn mice. Experiments were performed with a Roche LightCycler 480 using the following primer pairs and UPL mono-colour hydrolysis probes: *Actb*: 5′-CTA AGG CCA ACC GTG AAA AG-3′; 5′-ACC AGA GGC ATA CAG GGA CA-3′; *Dkk1*, 5′-CCG GGA ACT ACT GCA AAA AT-3′ and 5′-GGT TTT CAA TGA TGC TTT CCT C-3′, and UPL probes.

For real-time PCR employing the UPL probes, 20-μl reactions containing 10 μl Probes Master mix (2×) (Roche), 1 μl primer mix (10 μM each), 0.2 μl UPL probe, 3.8 μl H_2O, and 5 μl template cDNA were prepared in LightCycler 96 well plates (Roche). PCR was performed with a programme that involved a pre-incubation step followed by 55 amplification cycles, cooling and one-second-long annealing at 60 °C. The samples were analysed in duplicates. Relative expression levels were normalised to the values obtained from the β-actin gene.

Histology, immunohistochemistry and β-galactosidase staining

Kidneys were prepared from E14.5 to E17.5 embryos or newborn mice as indicated in the results section. The organs were fixed in 4% paraformaldehyde (PFA) and processed via routine methods for histology. The serially sectioned paraffin-embedded preparates were either used for *in situ* hybridisation, immunohistochemistry or stained with haematoxylin and eosin to inspect the tissue structures by histology.

Dkk1 protein was detected by immunohistochemistry with an antibody against *Dkk1* (Abcam) and an AlexaFluor 546-conjugated goat-anti-rabbit secondary antibody. An antibody against Aquaporin 2 (AQP2) (Sigma) served to identify the collecting ducts while Lef-1 one (Cell Signalling) was used as a readout for canonical Wnt signalling. Hoechst 33258 (Polysciences, Inc) reagent was applied to

stain the nuclei from the sections. The activity of the LacZ-derived ß-galactosidase was visualised according to Jokela & Vainio (2007).

In situ hybridisation

Kidneys were fixed overnight in 4% PFA at +4 °C and stored in 70% EtOH/PBT at −20 °C before they were processed for *in situ* hybridisation that was performed as described earlier (Lin et al., 2001b; Zhang et al., 2001). Changes in gene expression due to *Dkk1* knockout during kidney development was analysed by whole mount or section *in situ* hybridisation with the aid of Insitupro (Intavis AG Bioanalytical Instruments) or BioLane™ HTI (Hölle & Hüttner AG) robots. Changes in the expression of the *Dkk* genes during kidney development was analysed by using embryonic kidneys obtained from embryos derived from crosses between wild-type CD-1 mice. Probes used to analyse changes in *Podx1, Slc34a1, Slc12a1, Clcnkb* and *Scnn1b* gene expression were obtained as generous gifts, whereas the *Dkk1*, *Dkk2* and *Dkk3* were obtained as sequence tags and confirmed by sequencing.

Cell proliferation assay

Potential changes in cell proliferation induced by *Dkk1* loss of function were analysed in histological sections using a PCNA cell proliferation kit (Zymed Laboratories Inc. Invitrogen) used according to the manufacturer's instructions. Haematoxylin staining was omitted to obtain better resolution of the proliferating cells. Mitotic cells were counted with ImageJ software, using staining intensity as the threshold criterion for assessing proliferating cells, and based on scaling of the nuclear staining. Student's *t*-test was used to estimate the statistical significance of the data.

Collection of urine

The *Wnt-4Cre;Dkk1*$^{n/c}$ and *Pax8Cre;Dkk1*$^{n/c}$ mice and the wild-type littermates at the age of one year were kept separately and individually for 24 h in metabolic cages, and during this period their urine was collected and frozen immediately. The samples were analysed for a panel of constituents of urine by the Mary Lyon Centre's Clinical Pathology Service Laboratory at MRC Harwell in the UK. Biochemical analysis was performed with a Beckman Coulter AU400 clinical chemistry analyser by applying the reagents and protocols recommended by the manufacturer. The amount of ions present in the urine produced by the *Pax8Cre;Dkk1*$^{n/c}$ mice during the 24-h period was normalised to the respective values obtained from the wild-type littermates set at zero. The overall performance of the kidneys was scored by calculating the total volume of urine produced at three time points indicated in the results section. Student's *t*-test was applied to estimate the statistical significance of the results.

Identification of proteins in urine

A total of 400 µl of urine produced by the wild-type or the *Dkk1* mutant mice was centrifuged for 5 min at 10,000 rpm. The supernatant was separated and the pellet was diluted in 30 µl of distilled sterile water and mixed with 10 µl of SDS-PAGE buffer, while 30 µl of the supernatant was diluted to 10 µl of buffer for SDS-PAGE analysis. Proteins present in the urine were separated in a 10% SDS-PAGE gel and identified by staining the gel with a silver staining kit (PageSilver™ Silver Staining Kit, Fermentas). The gels were photographed with a Canon 40D camera. A portion of the urine samples were used to analyse the proteins in it. A MALDI-TOF sequencing of this urine protein component was performed in the protein analysis core facility of Biocenter Oulu (http://www.biocenter.oulu.fi/) using routine methods.

Wnt reporter assay

A Wnt reporter assay was done according to Railo et al. (2008). The following amounts of plasmids were used; 5 ng of *Wnt3a* or *Wnt-7b* expression plasmid, 250 ng of empty *pcDNA3* plasmid that served as the carrier DNA, 100 ng of *SuperTopFlash* canonical Wnt pathway reporter and 5 ng of *CMV-β-gal* plasmid. Recombinant mouse *Dkk-1* protein (R&D systems) was applied 6 h after the transfections as indicated in the results section. Cells were lysed with Cell Culture Lysis Reagent (Promega) 24 h after the transfection, and the respective luciferase signals were monitored using the Luciferase Assay System (Promega) with the aid of the Victor3V Multilabel Counter (Perkin Elmer).

Results

Dkk1 is expressed in the developing ureter and nephrons and later locally in their derivatives

To address the potential functions of *Dkks* during kidney development, we analysed their expression pattern during organogenesis. For this purpose we used data from publicly available kidney atlases (genepaint.org/Frameset.html and gudmap.org/), and based on this we performed further studies with quantitative real-time PCR, RNA *in situ* hybridisation and immunohistochemistry.

Dkk1-3 genes are expressed in the developing kidney (data not shown). Comparing the relative expression of *Dkk1* in different tissues and organs of newborn mouse embryos, *Dkk1* transcripts are particularly abundant in the kidney (Supplementary Fig. 1), consistent with the presence of a kidney regulatory element in the Dkk1 gene (Lieven et al., 2010). In histology *Dkk1* is expressed from E10.5 onwards in the metanephric mesenchymal cells and later in their derivatives, the assembling nephrons (Fig. 1A–C; genepaint.org/Frameset.html, gudmap.org/; Monaghan et al., 1999). Besides this, *Dkk1* is expressed by cells of the epithelial ureteric bud (Fig. 1B, C, black arrowheads), tubular structures (Fig. 1B, grey arrowhead), in the cortical region at birth where tubules are maturing (Fig. 1B), in the developing ureteric bud-derived collecting duct (Fig. 1C, blue arrowheads), and at the tip of the kidney papilla (Fig.1D, arrowheads). Consistent with the mRNA distribution, *Dkk1* protein is present in the cells of the collecting duct depicted with Aquaporin 2 (AQP2) expression (Fig. 1E, compare F, arrowheads with E) and the loop of Henle (Fig. 1, compare F with E, stars). *Dkk2* is expressed by the interstitial cells around the condensed mesenchymal cells during kidney development, whereas *Dkk3* is expressed weakly throughout the embryonic kidney (data not shown). In addition to *Dkk1* (Fig. 1D), *Dkk2* and *Dkk3* are expressed in the epithelial tip cells of the developing papilla (Supplementary Fig. 2A, C, arrowheads). *Dkk1* expression during kidney development suggests that *Dkk1* may be involved in development of the nephrons and/or ureteric bud, both of which contribute to kidney papilla development.

Dkk1 function is dispensable for early nephrogenesis, but it coordinates tubulogenesis during renal papilla development

To address if *Dkk1* is involved in kidney development, we generated a mouse line that enabled tissue-specific knockout studies and made *Dkk1-floxed* mice by homologous recombination in embryonic stem (ES) cells (Fig. 2). The *Dkk1*$^{flox/flox}$ mice demonstrated normal expression of *Dkk1* in all tissues, including the kidney (data not shown). To address if *Dkk1* plays a role in kidney ontogeny, we used the *Pax3Cre, Wnt-4Cre* and *Pax8Cre* mouse lines, which all target *Cre* from the early developmental stages onward to the kidney (Bouchard et al., 2004; Engleka et al., 2005; Shan et al., 2010). In the *Pax3Cre* and *Wnt-4Cre* mouse lines, recombination of the floxed *Rosa26 LacZ* reporter occurs in kidney mesenchyme. *Pax3Cre* mediates

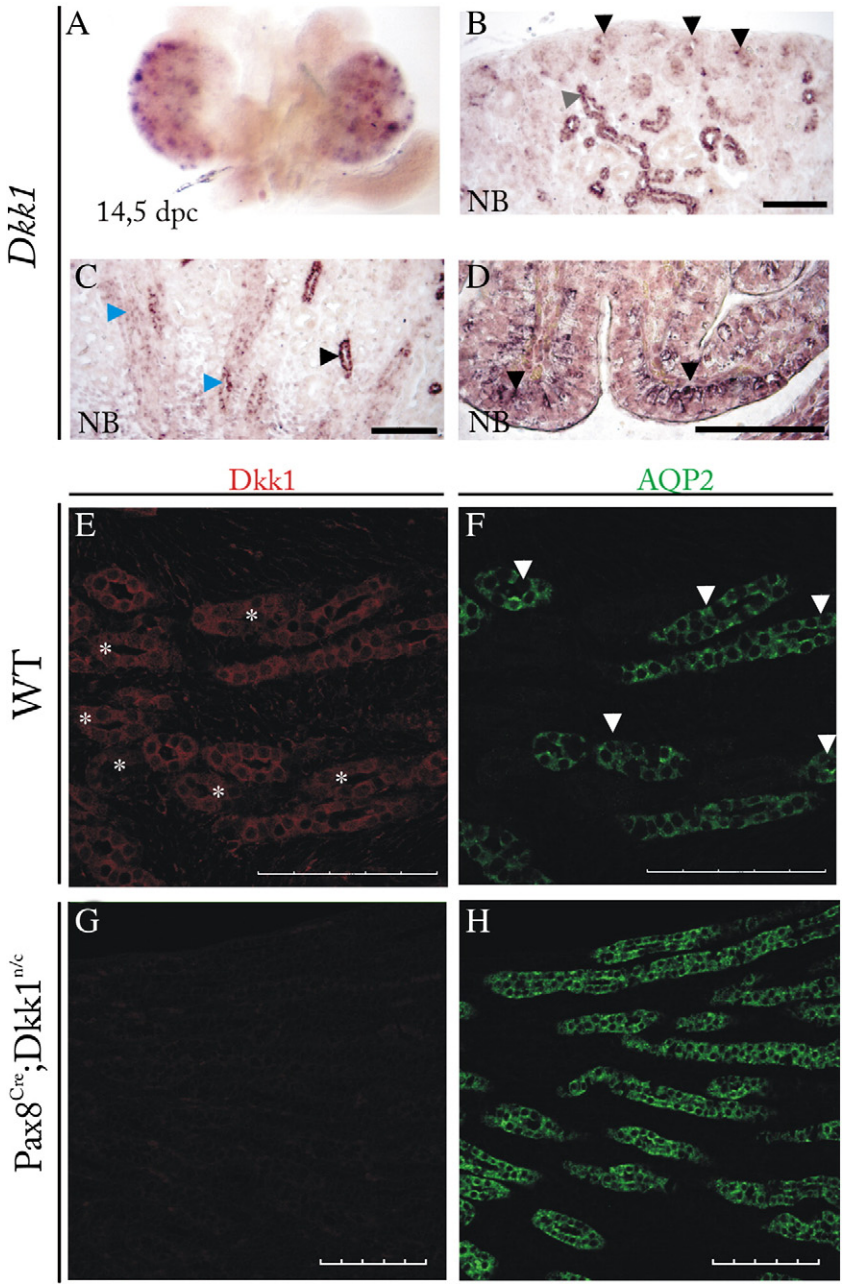

Fig. 1. *Dkk1* is expressed during nephrogenesis as well as in the ureteric bud derivatives and later in epithelial tip cells of the kidney papilla. Expression patterns of *Dkk1* were analysed in the urogenital system by *in situ* hybridisation as whole mount (A), on tissue sections (B–D) or IHC (E–H). *Dkk1* is expressed in defined regions in the embryonic kidney (A), the developing nephrons (B, grey arrowhead), ureteric bud-derived collecting duct cells (B and F, arrowheads), the kidney papilla (E), the loop of Henle (C, blue arrowhead, E, stars) and epithelial tip cells of the kidney papilla (D, arrowheads). Aquaporin 2 (AQP2) immunostaining in F and H depicts the collecting duct cells. *Dkk1* deficiency induced with *Pax8Cre* removes *Dkk1* protein expression from the kidney papilla from both the collecting duct and the loop of Henle cells (compare G with E). (A) E14.5; (B–H) newborn (NB). Scale bar 100 μm.

recombination throughout most of the kidney mesenchyme and the structures derived from it. *Wnt-4Cre* recombines the floxed marker gene specifically in the early pretubular cells that give rise to the nephrons (Supplementary Fig. 3A–F; Shan et al., 2010). Some weak *LacZ*-derived, *Wnt-4Cre*-activated expression is noted also in the epithelial tip cells of the renal papilla (Supplementary Fig. 3E, F, black arrowheads). *Pax8Cre* mediates recombination of the floxed *Rosa26 LacZ* reporter in cells adjacent to the developing nephrons in the ureteric bud (Supplementary Fig. 3G–I, black arrowheads), even though the endogenous *Pax8* transcripts are normally not detected in these cells, and also in the nephron-forming cells (Supplementary Fig. 3G–I, white arrowheads) (Plachov et al., 1990). The introduced *Cre* lines are suitable for addressing if *Dkk1* plays a role in the development of the nephrons and ureteric bud, which are regulated by Wnt signalling (Stark et al., 1994; Karner et al., 2009; Yu et al., 2009).

Pax3Cre-mediated inactivation of *Dkk1* function truncated the anterior portion of the embryos, with the craniofacial region being more poorly developed than in wild-type embryos (Supplementary Fig. 4, compare K with J). This phenotype resembles the head defects of a complete *Dkk1* knockout (Mukhopadhyay et al., 2001) and is the likely reason for the frequent perinatal deaths of *Pax3Cre;Dkk1*$^{n/c}$ embryos. The kidneys of the *Pax3Cre;Dkk1*$^{n/c}$ embryos appeared normal and development of the kidney compartments was indistinguishable from that of the wild-type controls (Supplementary Fig. 4A–F; data not shown). This was also the case if *Dkk1* was conditionally inactivated by *Wnt-4Cre*

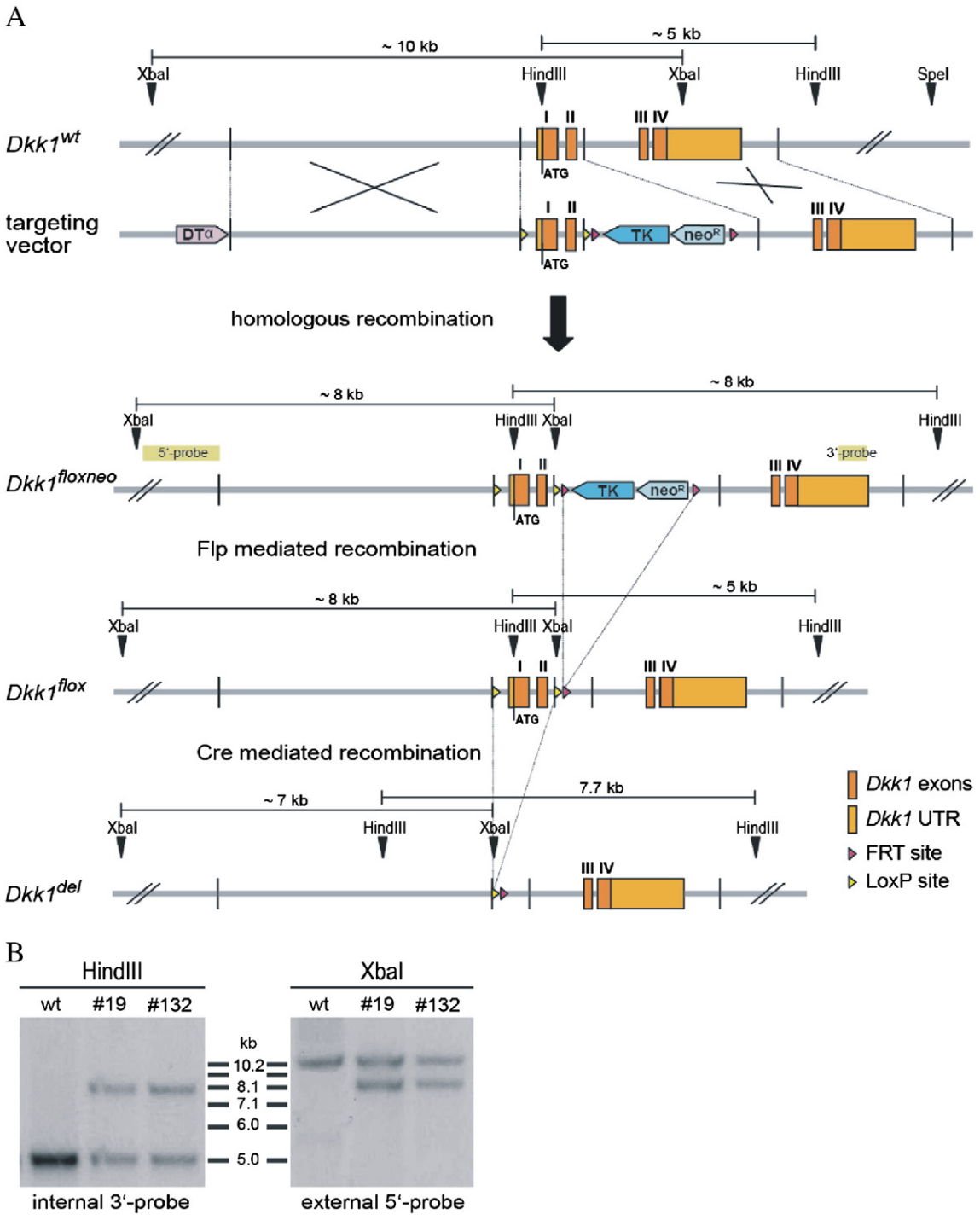

Fig. 2. Generation of a floxed *Dkk1* allele for conditional inactivation of *Dkk1* by *Cre-LoxP* technology. A) *Dkk1*-targeting vector for production of a floxed version of the *Dkk1* gene contains a 5.1 kb 5′ arm for homologous recombination followed by *Dkk1* exons 1 and 2 with *LoxP* sites introduced into the surrounding intronic sequences, a *TK-neo* selection cassette flanked by FRT recombination sites and a 3.3 kb 3′ arm for homologous recombination. Upstream of the 5′ homology arm, the vector contains sequence encoding for the α subunit of diphtheria toxin (DTα) as a negative selection marker. Due to the integration of the *TK-neo* selection cassette into the second intron of the *Dkk1* gene, the size of a HindIII restriction fragment detected with an internal 3′ probe increases from 5 kb to 8 kb (B). Correct targeting introduces a new XbaI restriction site, which gives rise to an 8 kb fragment from the $Dkk1^{floxneo}$ allele after XbaI digestion (B). This fragment is recognised by Southern blot with an external 5′ probe which identifies a 10 kb XbaI fragment from wild-type genomic DNA and an extra 8 kb fragment when correct targeting has taken place.

(Supplementary Fig. 4, compare G–I with A–C), seen also as unchanged expression of *Wnt-11*, *Pax8*, smooth muscle actin markers and parameters of the urine (data not shown).

The efficiency of *Pax8Cre*-mediated *Dkk1* inactivation was estimated by staining wild-type and $Pax8Cre;Dkk1^{n/c}$ embryonic kidneys with an antibody against *Dkk1*. The results revealed those cells where *Dkk1* gene recombination had taken place and had thus inactivated *Dkk1* and the resulting *Dkk1* protein expression. *Pax8Cre* inactivated *Dkk1* expression in the tubules in the emerging medulla, including the papilla (Fig. 1, compare H with G). *Dkk1* expression was lost both from the collecting duct and the loops of Henle cells, as judged by double immunostaining with Aquaporin 2 (AQP2), a collecting duct marker (Fig.1, compare G with F, E, stars). In contrast, *Pax8Cre*-mediated deletion of *Dkk1* did not alter the expression of *Dkk2* and *Dkk3* in the

tip cells of the kidney papilla (Supplementary Fig. 2B, D, arrowheads), and these may compensate *Dkk1* actions to a certain degree, as reported in some other systems (Phillips et al., in press).

In contrast to *Pax3Cre*- and *Wnt-4Cre*-mediated knockouts, *Pax8Cre*-induced inactivation of *Dkk1* function lead to two characteristic phenotypes. The medulla of the kidney, especially the papilla cells, were affected in the *Pax8Cre*-mediated *Dkk1* knockout. Two out of ten kidneys prepared from the *Pax8Cre;Dkk1* $^{n/c}$ newborn mice had developed hydronephrosis (Fig. 3, compare C with A). Besides this condition, a typical phenotype with high penetrance was overgrowth of the renal papilla in proportion to the rest of the kidney (Fig. 3B). In such cases the papilla in the kidney medulla had become extended over the lateral edge of the kidney capsule at the stalk and had reached the epithelial lining of the lumen of the ureter outside the kidney (Fig. 3B). An extended papilla was observed in both kidneys of seven out of ten analysed knockout mice, whereas the kidneys of the stage-matched wild-type mice that served as controls did not show such a phenotype (Fig. 3, compare B with A).

An additional characteristic feature in the kidneys of the *Pax8Cre; Dkk1*$^{n/c}$ mice was hypertrophic collecting duct epithelial cells observed at the tip of the extended renal papilla. The corresponding cells of the kidneys of wild-type controls were cuboidal and arranged in a row with respect to each other in the epithelial lining of the papilla tip (Fig. 3, compare B with A, inserts). The results indicate that *Dkk1* coordinates development of the kidney papilla.

Dkk1 deficiency stimulates cell proliferation in the epithelial tubules of the developing kidney papilla

Elongation of the kidney papilla in the *Pax8Cre;Dkk1*$^{n/c}$ mice is of interest, since morphological changes in the papilla may also have an effect on kidney function. Deletion of *Dkk1* function in the papilla may lead to stimulated Wnt signalling activity that may promote cell proliferation in the papilla region. To address this possibility we analysed the degree of cell proliferation in different proximo-distal areas of the papilla in *Pax8Cre;Dkk1*$^{n/c}$ embryonic kidneys in comparison to wild-type controls.

Kidney sections were stained with PCNA antibodies and the amount of cells in mitosis was evaluated. As expected, *Dkk1* deficiency by *Pax8Cre* (Fig. 4B) did not change the rate of cell proliferation in the kidney cortex when the rate was compared with wild-type controls (Fig. 4. compare B with A). However, in the papilla region, *Dkk1* deficiency enhanced the amount of mitotic cells when compared with wild-type controls (Fig. 4, compare D with C).

The papilla of wild-type and *Pax8Cre;Dkk1*$^{n/c}$ kidneys ($N=5$) was divided into three non-overlapping areas: the root, middle and tip regions, depicted in Fig 4E. The number of mitotic cells was evaluated in each of the areas along the proximo-distal axis by defining the mitotic index with the aid of ImageJ software. These studies revealed that the most proximal papilla region contained the highest amount of mitotic cells, in both the wild-type and *Dkk1*-deficient kidneys, but cell proliferation was notably enhanced due to *Dkk1* deficiency in comparison with controls ($p<0.005$). The number of actively proliferating cells decreased towards the papilla tip region in both the *Dkk1* knockout and wild-type kidneys, but the *Dkk1*-deficient papilla still showed enhanced proliferation in each of the analysed papilla segments (Fig. 4G, $p<0.1$ for the middle and $p<0.05$ for the tip region).

To reveal the nature of the cells that were stimulated to proliferate due to *Dkk1* deficiency in the papilla region, we used double staining. Tissue sections were stained simultaneously with PCNA, to monitor changes in cell proliferation, and collecting duct or loop of Henle markers. These studies revealed that actively proliferating *Dkk1*-deficient cells were present in both the collecting ducts and the epithelial cells of the loops of Henle, but the loops of Henle contained more actively dividing cells than the collecting duct (Supplementary Fig. 5, compare B with A, in brown, arrowheads). We conclude that *Dkk1* deficiency promotes development of the kidney papilla by stimulating cell proliferation, possibly due to derepressed Wnt signalling.

Dkk1 antagonises Wnt-7b signalling that is critical for development of the kidney papilla

Given that *Dkk1* functions in some other systems as a secreted Wnt antagonist, it may provide this inhibitory function to coordinate Wnt activities also during kidney papilla development. Of the Wnts, *Wnt-7b* has been implicated in medulla formation, since *Wnt-7b* deficiency in the collecting duct leads to compromised papilla and pelvis development (Yu et al., 2009). We therefore analysed if *Dkk1* would indeed regulate

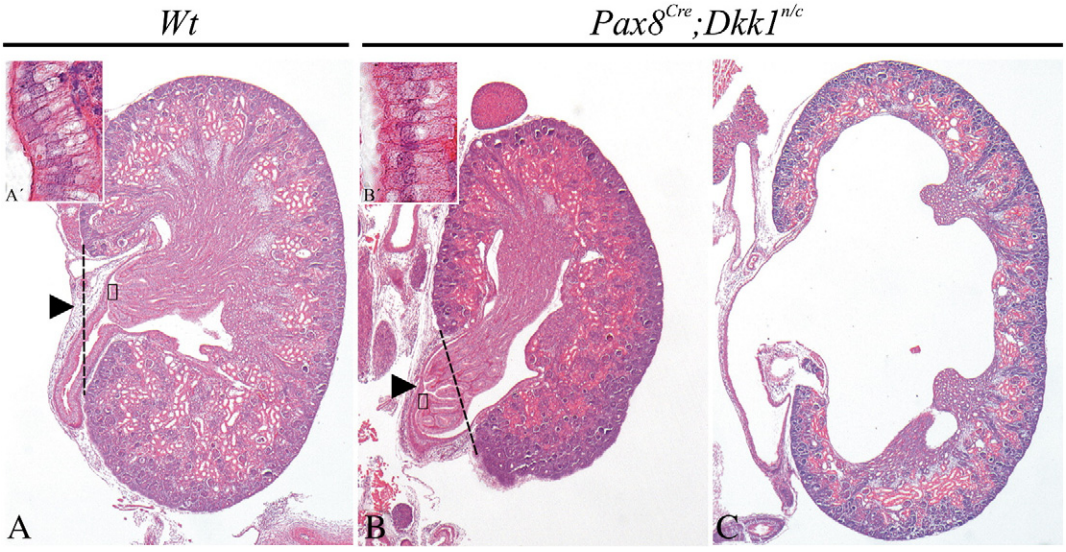

Fig. 3. Conditional knockout of *Dkk1* with *Pax8Cre* stimulates growth of the kidney papilla and can lead to hydronephrosis. Kidneys were prepared from newborn wild-type (Wt) and *Pax8Cre;Dkk1*$^{n/c}$ mice and processed for histology and hematoxylin–eosin staining. A) Section of a wild-type kidney. The lateral edge of the kidney capsule crossing the ureter is depicted by a black dotted line and an arrow indicates the position of the papilla. A') High-power micrograph of the boxed area at the tip region of the papilla, revealing that the papilla tip epithelial cells are cuboidal and project towards the renal pelvis. B) Knockout of *Dkk1* with *Pax8Cre* has led to overgrowth of the whole papilla in proportion to the rest of the kidney. The papilla extends outside the kidney all along the lateral extreme of the lumen of the ureter (arrowhead). The dotted line serves as a reference, indicating the corresponding region depicted in (A). B') Note that the tip cells of the renal papilla appear hypertrophic (compare B' with A'). C) An example of a kidney prepared from a *Pax8Cre; Dkk1*$^{n/c}$ newborn mouse with hydronephrosis. (A–C) Newborn.

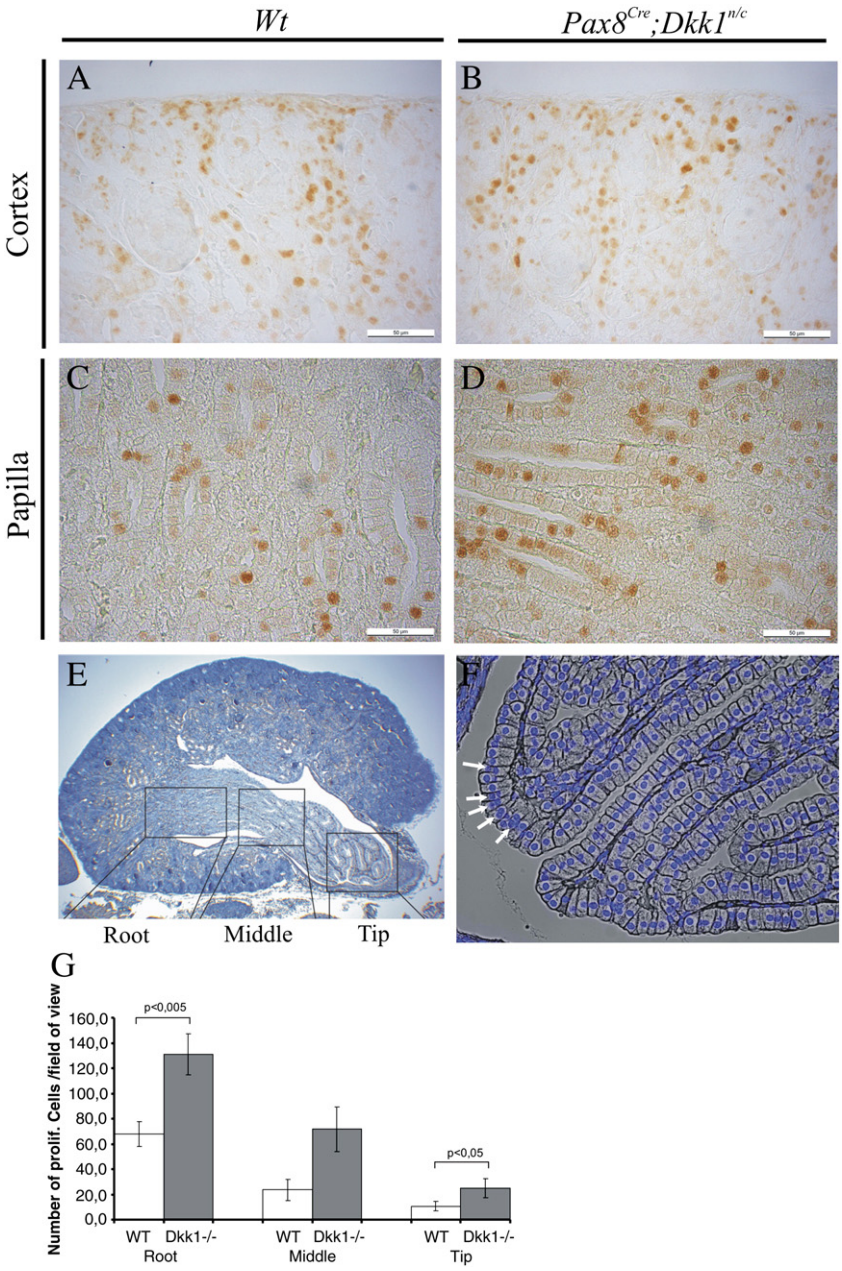

Fig. 4. *Pax8Cre*-mediated *Dkk1* deficiency induces cell proliferation in the kidney papilla. Expression of mitosis marker PCNA is not changed in the cortex of kidneys from *Pax8Cre; Dkk1*$^{n/c}$ newborn mice, when compared with the corresponding region in wild-type kidneys (A, B). Proliferation has been induced in the kidney papilla due to *Dkk1* deficiency, as judged by PCNA expression (compare D with C). (E) All the cells that express the PCNA were counted in the boxed areas (Root, Middle and Tip), and the values are presented in (G). (F) *Dkk1* deficiency stimulates cell proliferation in the papilla, as depicted by Hoechst staining. Note that some epithelial cells at the papilla tips contain two nuclei, indicating mitosis (white arrows). (A–F) Newborn. Scale bar 50 μm.

the degree of *Wnt-7b* signalling. *Wnt-3a* was used as a positive control, since it activates canonical Wnt/β-catenin signalling in most cell lines.

Indeed, transfection of *Wnt-3a cDNA* to both of the model cell lines, the CHO-KI and the embryonic kidney mesenchyme-derived mK4 cells led to activation of the *SuperTopFlash* canonical Wnt pathway reporter (Fig. 5A, C). In contrast to *Wnt-3a*, *Wnt-7b* activated the Wnt signalling reporter only in the CHO-KI cells and the activation was weaker than with *Wnt-3a* (Fig. 5. B, D). Thus, the CHO cells harbor a receptor for *Wnt-7b* and serve as a relevant model for addressing if *Dkk1* regulates the degree of *Wnt-7b* signalling. We found that the recombinant *Dkk1* protein (100 ng/ml) efficiently inhibited both *Wnt-7b*- and *Wnt-3a*-mediated activation of the Wnt reporter (Fig. 5A–C).

We next addressed if *Pax8Cre*-mediated *Dkk1* deficiency would change *Wnt-7b* gene expression, which could be relevant for enhanced growth of the papilla. In wild-type kidneys, *Wnt-7b* is expressed specifically in the collecting duct cells of the papilla (Yu et al., 2009) (Supplementary Fig. 6A). *Wnt-7b* remained expressed in the collecting duct cells irrespective of *Dkk1* deficiency induced by *Pax8Cre* (Supplementary Fig. 6B). However, we found that *Pax8Cre*-mediated deficiency of *Dkk1* increased the number of cells that expressed canonical Wnt signalling of target gene *Lef-1* in the stroma of the medulla (Supplementary Fig. 7A–C). Hence *Dkk1* likely regulates *Wnt-7b* signalling from the collecting duct cells to stromal cells during kidney papilla development.

Pax8Cre-mediated knockout of Dkk1 in the developing nephrons and ureteric bud reduces collecting duct and loop of Henle marker gene expression

Dkk1 is expressed in cells that give rise to the nephrons, the collecting duct and the ureter. *Dkk1* deficiency in the ureteric bud

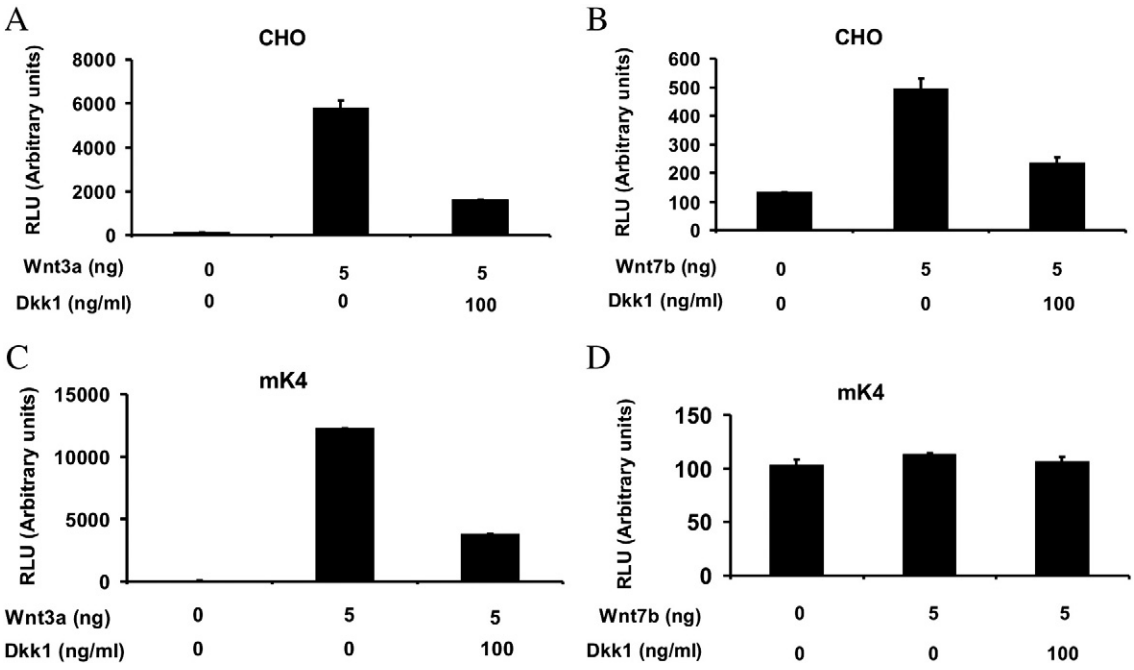

Fig. 5. *Dkk1* inhibits *Wnt-7b* signalling in a model cell line. CHO-KI and embryonic kidney mesenchyme-derived mK4 cells were transfected with canonical Wnt signalling pathway reporter *SuperTopFlash lusiferase (Luc)*, *Wnt-3a* that served as the positive control or *Wnt-7b* cDNAs, CMV-β-Gal and carrier DNA. The capacity of recombinant *Dkk1* protein to regulate Wnt signalling was compared with non-*Dkk1* treated controls. The cells were harvested 18 h after incubation with *Dkk1*, and changes in Luc activity were measured and compared with the controls. Normalised luciferase activity in the control sample was arbitrarily set at 100. A) *Wnt-3a* induces robust activation of the *SuperTopFlash* reporter, which is efficiently inhibited by the presence of recombinant *Dkk1* protein. B) *Dkk1* inhibits signalling of *Wnt-7b*. C) *Dkk1* inhibited *Wnt-3a*-induced activation of the *SuperTopFlash* reporter in the mK4 cells. D) The mK4 cells are not responsive to *Wnt-7b* signalling, suggesting that the kidney mesenchymal cells do not express a proper receptor for *Wnt-7b*.

stimulated cell proliferation, likely via increased *Wnt-7b* activity, and thereby enhanced growth of the papilla. To analyse if *Dkk1* deficiency leads to defects in the structures derived from the *Dkk1*-expressing precursor tissues, we studied segmentation of the nephron, formation of glomeruli and the collecting duct based on *in situ* hybridisation of marker genes. *Podx1* serves as marker for the glomerulus (Takemoto et al., 2006), *Slc34a1* for the proximal tubulus (Raciti et al., 2008), *Clcnkb* for the distal tubulus (Simon et al., 1997), *Slc12a1* for the loop of Henle (Raciti et al. 2008), and *Scnn1b* for the collecting duct (Brooker et al., 1995).

The results indicate that the glomeruli and major segments of the nephron appeared to be correctly specified irrespective of *Pax8Cre*-mediated *Dkk1* deficiency, since no changes in the expression of *Podx*, *Slc34a1* or *Clcnkb* were noted in the kidneys of the *Pax8Cre;Dkk1*$^{n/c}$ embryos when compared with the wild-type controls (Supplementary Fig. 8, compare E, F, G with A, B, C). However, expression of the loop of Henle marker, *Slc12a1*, and the collecting duct marker, *Scnn1b*, were both reduced in their intensity in comparison with the wild-type controls (Fig. 6, compare C, D, F with A, B, E). Hence, *Pax8-Cre*-mediated *Dkk1* deficiency reduces marker gene expression in the loop of Henle and collecting duct, suggesting that besides affecting the tubular organisation in the papilla, *Dkk1* deficiency also affects these structures and may therefore have an impact on the functional performance of the kidney.

Dkk1 deficiency induced by Pax8Cre compromises kidney performance

Inactivation of *Dkk1* with *Pax8Cre* led to overgrowth of the kidney papilla, elongation of the loop of Henle segments of the nephrons and the collecting ducts in this region. In addition to these changes, the expression of specific ion channels in these tissues was reduced. Collectively, these alterations may cause compromised kidney performance. We addressed this possibility by analysing the amount of urine and the presence of urinary proteins and solutes produced by the kidneys of the wild-type and *Pax8Cre;Dkk1*$^{n/c}$ mice.

Indeed, the kidneys of the 1-year-old *Pax8Cre;Dkk1* $^{n/c}$ mice produced clearly less urine than the kidneys of the wild-type control mice during the analysed 24-h time period (Fig. 7A). Moreover, the analysis of the constituents of the urine obtained from the *Pax8Cre;Dkk1*$^{n/c}$ mice revealed changes in the amount of ions in comparison with the wild-type controls. Sodium, potassium, urea, creatinine (CreUr), phosphate, glucose and protein/Uprot amounts were all decreased. In contrast, the amount of chloride and calcium was increased in the urine produced by the *Pax8Cre;Dkk1*$^{n/c}$ mice when compared with the corresponding values of the wild-type mice (data not shown).

Given the changes in urine constituents due to tissue-specific deficiency of *Dkk1*, we finally analysed potential changes in urinary proteins. We found no changes in the amount of albumin in the *Pax8Cre;Dkk1*$^{n/c}$ samples in comparison with the controls. However, protein with a size of around 100 kDa, which was detected in the urine of the wild-type mice, was not present in the urine of the *Pax8Cre;Dkk1*$^{n/c}$ mice (Fig. 7B, arrowheads and data not shown). MALDI-TOF sequencing of this component of urine revealed notable amino acid sequence similarity to the major urinary proteins (MUP; Cavaggioni and Mucignat-Caretta, 2000) that normally are synthesised in the liver, submaxillary, lachrymal and mammary glands, and secreted to serum, being excreted also to urine (Shaw et al., 1983). These results suggest that *Pax8Cre*-mediated *Dkk1* deficiency leads to compromised kidney function.

Discussion

Dkk1 deficiency in the ureteric bud leads to defects in kidney papilla development by impaired Dkk1-mediated antagonism of Wnt-7b signalling

We showed that, of the *Dkks*, *Dkk1* is expressed in the assembling nephrons of the embryonic kidney from the early developmental stages onwards, especially during their epithelialisation, in line with the findings of Monaghan et al. (1999). During maturation of the nephrons, *Dkk1* expression is confined to the medullary junction of the loop of Henle. Besides the nephrons, *Dkk1* is expressed in the

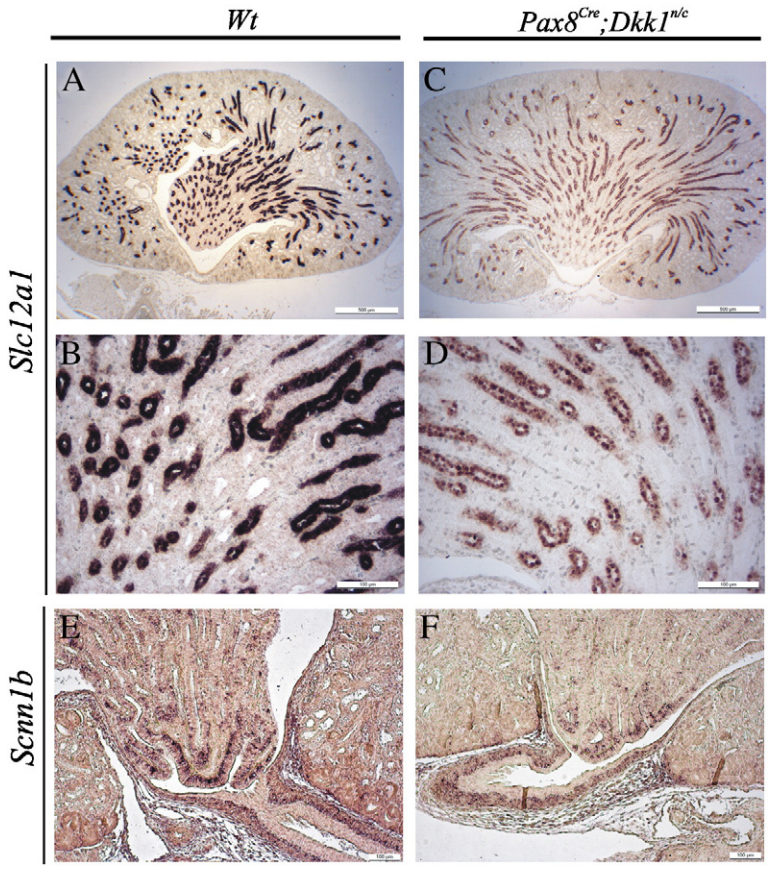

Fig. 6. *Pax8Cre*-mediated *Dkk1* deficiency reduces collecting duct and loop of Henle marker gene expression. Changes in the epithelial ducts of the kidney papilla due to conditional *Dkk1* deficiency were studied by *in situ* hybridisation with *Scnn1b*, a collecting duct marker, and *Slc12a1*, a loop of Henle marker probe. *Slc12a1* is expressed in the loops of Henle (A), but its expression intensity is reduced in the case of *Dkk1* deficiency in the kidney in samples processed identically but excluding the extended papilla tip, depicted in Figs. 3B and 4E (compare C with A, D with B). B, D) High magnification micrographs of A and C, respectively. *Scnn1b* is expressed in the collecting duct epithelial cells of the kidney medulla throughout the papilla, but expression is reduced in this region due to *Dkk1* deficiency (compare F with E). E, F) High magnification of *in situ* hybridisation, depicting the papilla tip region. (A–G), newborn. Scale bars, A and C 500 μm; B, D, E, F100 μm.

ureteric bud, the collecting duct and the papilla epithelial tip cells that derive from it. The expression pattern raised the possibility that *Dkk1* would coordinate Wnt signalling during nephrogenesis and/or formation of the ureteric bud derivatives.

We addressed the potential role of *Dkk1* in kidney development by making a floxed *Dkk1* mouse line. *Pax3Cre*, *Pax8Cre* and *Wnt-4Cre* crossing was used and all these recombine the floxed *Rosa26 LacZ* reporter in developing nephrons (Bouchard et al., 2004; Engleka et al., 2005; Shan et al., 2010). However, all the cases where the embryo had inherited one null and one floxed *Dkk1* allele but no *Cre* demonstrated normal nephrogenesis in a situation where we noted, for example, severe craniofacial defects in the *Pax3Cre;Dkk1*$^{n/c}$ embryos, a phenotype that is typical for *Dkk1*-null embryos (Mukhopadhyay et al., 2001). Of the Wnts, the *Wnt-4* gene is expressed in the pretubular cells but becomes inactivated during transition of mesenchymal cells to tubular epithelial cells (Stark et al., 1994). Hence in principle, of the

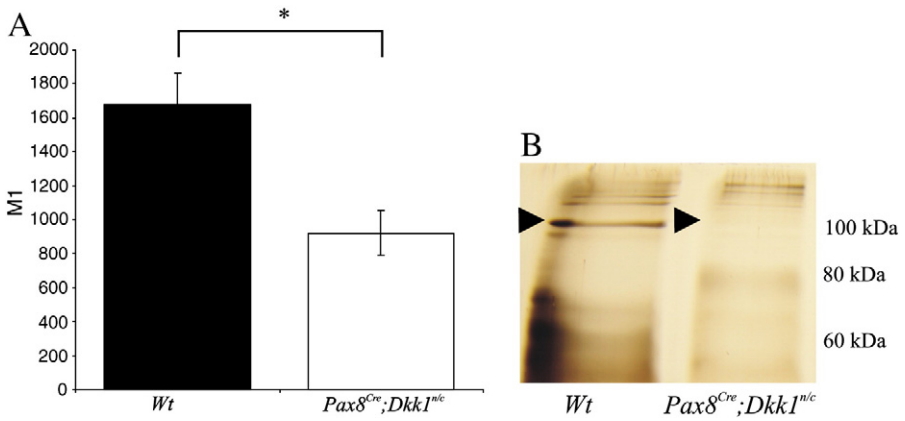

Fig. 7. *Pax8Cre*-mediated inactivation of *Dkk1* reduces urine production. A population of mice ($N=5$) were maintained individually in metabolic cages for 24 h and the urine produced during this time was collected. A) The total amount of urine produced by the kidneys of the *Pax8Cre;Dkk1*$^{n/c}$ mice is reduced considerably when compared with the amount produced by the wild-type mice during the same time ($p<0.0079$). B) Analysis of the protein constituents of the urine produced by the wild-type and *Pax8Cre;Dkk1*$^{n/c}$ mice by SDS-PAGE indicates the loss of protein with around MW 100 kDa.

Dkks, *Dkk1* could regulate *Wnt-4* signalling to control nephrogenesis. However, besides *Dkk1*, embryonic kidney mesenchyme expresses other Wnt antagonists, namely secreted Frizzled-related proteins (sFRPs) 1 and 2, and these either inhibit or stimulate *Wnt-4* signalling (Yoshino et al., 2001). Hence, the FRPs may compensate for the loss of *Dkk1* in the control of early stages of nephrogenesis. It is also worth noting that we detected some *Dkk1* protein in the cortical regions of the developing kidney in the case of *Pax8Cre*-mediated inactivation of *Dkk1* in a situation where kidney papilla development was disturbed. In the hypomorphic *Dkk1* allele, *doubleridge,* where only 10% of the normal *Dkk1* remain expressed, for example, head development is still normal (MacDonald et al., 2004). Hence, greatly reduced *Dkk1* amounts can still be physiologically effective and therefore, residual cortical *Dkk1* in the *Pax8Cre;Dkk1*$^{n/c}$ embryonic kidney may still be sufficient to drive nephrogenesis. Deficiency in Lrp6 that is bound by *Dkk1* perturbs kidney development, with a certain resemblance to the phenotype caused by *Wnt-4* loss of function (Stark et al., 1994; Pinson et al., 2000). This may be taken as indirect support for the premise that *Dkk1* may indeed have a role in kidney mesenchyme development. *Dkk1* deficiency in the adult protects against diabetes-induced renal dysfunction and indicates that *Dkk1* has an intrinsic role in the kidney later (Lin et al., 2010). Hence, new and more effective kidney-targeting *Cre* lines may be required to obtain complete inactivation of *Dkk1* in the nephron precursor cells to reveal potential roles in early nephrogenesis stages.

Besides the cells that undergo nephrogenesis, *Pax8Cre* recombines the floxed reporter gene in the ureteric bud and its derivatives. The staining revealed that *Pax8Cre* inactivated *Dkk1* function in the ureteric bud derivatives, the collecting duct, the cells of the renal pelvis epithelial cells, the papilla tip cells and the ureter. Indeed, an extended papilla developed in the kidneys of seven out of ten of the analysed *Pax8Cre; Dkk1*$^{n/c}$ mice, and some kidneys were hydronephrotic. The extended papilla phenotype is likely caused by enhanced proliferation of the kidney papilla cells, the collecting duct and the loop of Henle cells. Typically, reduction of *Dkk* function is connected to enhanced proliferation of cancer cells (Niehrs, 2006; Hirata et al., 2010; Zhou et al., 2010), and this is in line with our findings. Hence, we conclude that *Dkk1* function is connected to maturation of the papillary epithelial cells, the ureteric bud-derived collecting duct, the loops of Henle cells or both.

Kidney medulla expresses several Wnts, namely *Wnt-2b, -4, -6, -7b, -9b* and *−11* (Stark et al., 1994; Kispert et al., 1996; 1998; Lin et al., 2001a; Itäranta et al., 2002; Yu et al., 2009; Carroll et al., 2005), but out of these, *Wnt*-7b is critical for papilla development (Yu et al., 2009), a process that is influenced by *Dkk1* deficiency. *Wnt-7b* is expressed in the collecting duct epithelial cells in the developing papilla region, but the knockout also influences the loop of Henle development, making *Wnt-7b* a signal that also coordinates loop of Henle development via the stromal cells. Since *Pax8Cre;Dkk1*$^{n/c}$ kidneys exhibit an extended papilla and inhibition of both collecting duct and loop of Henle marker gene expression took place at the same time, we propose that *Dkk1* in the kidney papilla regulates kidney development by controlling the activity of *Wnt-7b* in the stromal cells. Thus, by binding to the Frizzled/LRP complex in the stromal cells, *Dkk1* may fine-tune the activity of *Wnt-7b*, thereby also influencing loop of Henle growth and maturation (Glinka et al., 1998; Niehrs, 2006) (Supplementary Fig. 9). As support to this proposal, we demonstrated that *Dkk1* protein indeed inhibits *Wnt-7b*-induced activity of a canonical Wnt signalling pathway reporter when the receptor for *Wnt-7b* is expressed in the target cell. Also, expression of canonical Wnt signalling of targeted gene *Lef-1* was increased due to *Dkk1* deficiency. We cannot exclude at present that *Dkk1* may also have an independent role in the embryonic loop of Henle cells.

Dkk1 deficiency in the ureteric bud leads to changes in the performance of the kidney

The length of tubules in the kidney papilla was extended in the kidneys of the *Pax8Cre;Dkk1*$^{n/c}$ mice, and especially the loop of Henle cells, but also the collecting duct contained more proliferating cells when compared with the controls. We showed that *Dkk1* knock out by *Pax8Cre* reduced expression of loop of Henle and collecting duct marker genes. Of these, the *Slc12a1* gene encodes a Na^+–K^+–$2Cl^-$ pump in the loop of Henle, and the *Scnn1b* gene encodes a sodium channel non-voltage-gated 1, β in the collecting duct. The respective proteins take part in the tubules to control reabsorption of ions, calcium and magnesium from the urine into the body and maintenance of the volume of extracellular fluid (Mount et al., 1999). Thus, the reduction in *Slc12a1* expression is in line with the increase in calcium that was observed in the urine of the *Dkk1*-deficient mice. The reason for the reduced overall urine production noted in the *Dkk1* mutant mice may be the elongated papilla with an extended loop of Henle, the collecting duct tubules and a defect in their maturation. These changes likely alter the balance of the ion channels that are involved in absorption of ions and fluids from primary urine. Hence, by controlling development of the papilla and expression of the associated genes, *Dkk1* is involved in setting up the functional capacity of the mature kidney.

Dkk function and the papilla tip

Based on long-term tracing of bromodeoxy uridine-labelled cells in the kidney papilla, the papilla has been shown to contain slowly cycling cells (Al-Awqati and Oliver, 2006). We found that the proximal papilla tip had less proliferating cells than the more distal papilla region. Moreover, fate mapping experiments with the aid of *Pax8Cre* and *Wnt-4Cre* crossed with the *floxed Rosa26 LacZ* reporter line revealed LacZ-positive cells in the tip of the papilla. We noted LacZ-positive cells in both the epithelial lining of the papilla and the adjacent mesenchymal zone. These data indicate that in cells expressing *Wnt-4* and *Pax8,* their established cell lineages give rise to cells that contribute to papilla development. Even though *Wnt-4Cre* line-based fate mapping is not a direct indication of Wnt activity in the papilla tip region, the presence of *Dkk1* and other *Dkk* gene transcripts suggests that the Wnt signalling system operates in the tip region to coordinate generation of cells from the possible stem cell niche. Whether the Wnt signals indeed also control the papilla tip and promote generation of progenitor cells in these regions warrants further investigations.

In summary, we showed that *Pax8Cre*-mediated *Dkk1* knockout leads to enhanced cell proliferation in the kidney papilla, especially in the collecting duct and loop of Henle, being the likely reason for extended papilla growth. The overgrown papilla is expected to be behind the more efficient water absorption from primary urine due to the extended Henle's loops, which may cause the observed decrease in urine volume in the *Pax8Cre;Dkk1*$^{n/c}$ mice. In the case of *Dkk1* deficiency we propose that *Wnt-7b* signalling to the stromal cells is enhanced and stimulates growth of the papilla, including its loops of Henle and collecting duct. Hence, *Dkk1* may function during papilla development by fine-tuning *Wnt-7b* signalling to coordinate the canonical Wnt signalling connected to collecting duct and loop of Henle development critical for kidney function (Supplementary Fig. 9; Yu et al., 2009).

Supplementary materials related to this article can be found online at doi: 10.1016/j.ydbio.2011.02.019.

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