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β -catenin/TCF/Lef controls a differentiation-associated transcriptional program in renal epithelial progenitors

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In the embryonic kidney, progenitors in the metanephric mesenchyme differentiate into specialized renal epithelia in a defined sequence characterized by the formation of cellular aggregates, conversion into polarized epithelia and segmentation along a proximal-distal axis. This sequence is reiterated throughout renal development to generate nephrons. Here, we identify global transcriptional programs associated with epithelial differentiation utilizing an organ culture model of rat metanephric mesenchymal differentiation, which recapitulates the hallmarks of epithelialization *in vivo* in a synchronized rather than reiterative fashion. We observe activation of multiple putative targets of β -catenin/TCF/Lef-dependent transcription coinciding with epithelial differentiation. We show in cultured explants that isolated activation of β -catenin signaling in epithelial progenitors induces, in a TCF/Lef-dependent manner, a subset of the transcripts associated with epithelialization, including Pax8, cyclin D1 (Cnd1) and Emx2. This is associated with anti-apoptotic and proliferative effects in epithelial progenitors, whereas cells with impaired TCF/Lef-dependent transcription are progressively depleted from the epithelial lineage. *In vivo*, TCF/Lef-responsive genes comprise a conserved transcriptional program in differentiating renal epithelial progenitors and β -catenin-containing transcriptional complexes directly bind to their promoter regions. Thus, β -catenin/TCF/Lef-mediated transcriptional events control a subset of the differentiation-associated transcriptional program and thereby participate in maintenance, expansion and stage progression of the epithelial lineage.

KEY WORDS: Metanephric mesenchyme, Epithelial differentiation, β -catenin, TCF/Lef-type transcription factors, Neutrophil gelatinase-associated lipocalin, Leukemia inhibitory factor, Wnt4, Emx2, Pax8, Cyclin D1 (Cnd1), Frzb

INTRODUCTION

Development of the metanephric kidney is initiated when the ureteric bud enters the metanephric mesenchyme and induces mesenchymal progenitors to differentiate into renal epithelia. Epithelialization of the metanephric mesenchyme develops in a sequence that is currently defined by morphological criteria and by the expression of signature molecules (Dressler, 2006). Initially, metanephric mesenchymal progenitors organize at the tips of the ureteric bud forming the ‘condensed mesenchyme’. This is followed by translocation relative to the elongating ureteric bud stalk to generate a ‘pretubular aggregate’ characterized by expression of Wnt4 and Pax8 (Stark et al., 1994). Establishment of apical-basal polarity in this aggregate produces the renal vesicle, a structure characterized by the onset of expression of E-cadherin (also known as cadherin 1, Cdh1), a crucial component of epithelial adherens

junctions (Barasch et al., 1999; Cho et al., 1998). Segmentation events lead to the formation of the ‘S-shaped’ body, the direct precursor to mature nephrons, which includes a glomerular pole proximally and a tubular pole distally that connects to the tip of the ureteric bud (Dressler, 2006).

In vivo, epithelial differentiation of mesenchymal progenitors is dependent on the presence of the ureteric bud. This sequence can be modeled *in vitro* by application of defined combinations of growth factors to rat metanephric mesenchyme, which we and others have previously identified by protein chromatography from a ureteric bud cell line (Barasch et al., 1996; Barasch et al., 1999; Karavanova et al., 1996; Plisov et al., 2001; Yang et al., 2002a; Yang et al., 2002b). Application of these growth factors results in the highly reproducible and synchronized appearance of segmented tubules that include glomerular-like structures, proximal tubules and distal tubules (Barasch et al., 1999).

The molecular events that drive stage transitions in the epithelial lineage are subject to ongoing research. Although extracellular factors from the WNT, transforming growth factor β (TGF β), fibroblast growth factor (FGF) and interleukin 6 (IL6) families have been implicated as regulators of different aspects of epithelial differentiation (Barasch et al., 1999; Carroll et al., 2005; Kispert et al., 1998; Oxburgh et al., 2004; Perantoni et al., 1995; Stark et al., 1994), little is known about the transcriptional events involved in these processes. In the current paper, we analyzed genome-wide transcriptional profiles associated with epithelial differentiation using microarray analysis. Activated transcripts included multiple putative target genes of transcription factors of the TCF/Lef family, suggesting that these factors might participate in the control of epithelial differentiation.

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TCF/Lef transcriptional activity is principally regulated through alteration of β -catenin levels in the nucleus (Brembeck et al., 2006; Clevers, 2006). β -catenin, a bifunctional protein that comprises a structural component of adherens junctions and a transcriptional co-activator, converts TCF/Lef from a transcriptional repressor into an activator of specific target genes and thereby regulates crucial transcriptional programs throughout development. Nuclear β -catenin levels are predominantly regulated by a multiprotein complex that targets glycogen synthetase kinase Gsk3 β and casein kinase I (CKI; also known as Csnk1) to mediate amino-terminal serine-threonine phosphorylation of β -catenin and, thereby, promotes proteasomal degradation of β -catenin. This process is inhibited by ligands of the WNT family, which results in stabilization of intracellular β -catenin. However, the process is not specific for WNT proteins as it can be triggered by additional ligands (Brembeck et al., 2006; He, 2006) and, conversely, in many cases the cellular actions of WNT proteins are independent of β -catenin and mediated through alternative pathways, including c-Jun N-terminal kinase (also known as Mapk8 – Mouse Genome Informatics) or calcium signaling (Veeman et al., 2003). Although multiple lines of evidence have established an involvement of WNT signaling in the differentiation of renal epithelial progenitors in both mice and rats (Carroll et al., 2005; Herzlinger et al., 1994; Kispert et al., 1998; Osafune et al., 2006; Plisov et al., 2001; Stark et al., 1994), little is known about TCF/Lef-mediated events as a potential downstream signal.

In the current paper, we show that even in the absence of stimulation by exogenous WNT ligands, epithelial differentiation of metanephric mesenchyme is characterized by the activation of multiple TCF/Lef-dependent targets of β -catenin. We further demonstrate that β -catenin/TCF/Lef signaling is involved in the regulation of survival and proliferation of epithelial progenitors and induces stage progression characterized by the induction of a subset of the tubulogenic transcriptional program. Importantly, cells with impaired TCF/Lef-dependent transcription are progressively depleted during epithelial differentiation, suggesting that this signaling axis controls cellularity in the renal epithelial lineage.

MATERIALS AND METHODS

Mesenchymal organ culture

Fresh metanephric mesenchymes were isolated from E13.5 rat embryos and cultured in basal media supplemented with growth factors (R&D Systems, Minneapolis, MN), or chemicals as indicated according to standard procedures (Barasch et al., 1999). Gsk3 β inhibitors used were lithium chloride (Sigma-Aldrich, St Louis, MO) and 6-bromindirubin-3'-oxime (BIO; EMD Biosciences, San Diego, CA).

Isolation of non-heparin-binding fraction (NHBF)

Approximately 200 l of serum-free media, conditioned by monolayers of ureteric bud cells (Barasch et al., 1996), were concentrated, desalted and applied to heparin-Sepharose (GE Healthcare, Piscataway, NJ) in 10 mM Na₂PO₄ (pH 7.0). Flow-through was reapplied to a fresh heparin-Sepharose column twice to ensure removal of all heparin-binding substances. Following concentration and desalting, the flow-through was applied to ANX Sepharose 4FF columns (GE Healthcare) in 20 mM bis-Tris buffer (pH 9.0) and eluted with a NaCl gradient. The active non-heparin-binding fraction was concentrated, desalted and 100 μ g of total protein was subjected to immunoblotting for LIF (polyclonal anti-mouse LIF; R&D) and NGAL (Lcn2) (Yang et al., 2002b), confirming the absence of these heparin-binding inductive activities.

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) as described previously (Schmidt-Ott et al., 2005). cDNA was synthesized using Omniscript Reverse Transcriptase (Qiagen). Real-time PCR was

carried out using iQ SYBR Green Super Mix and a MyiQ Single-Color Real-Time PCR Detection System (Biorad, Hercules, CA). Primer sequences are available upon request. Specificity of the amplification was checked by melting curve analysis, agarose gel electrophoresis and sequencing of PCR products. Relative levels of mRNA expression were normalized to β -actin mRNA and calculated according to the $\Delta\Delta C_T$ method as described (Schmidt-Ott et al., 2006).

Microarray analysis

Metanephric mesenchymes were cultured in basal media supplemented with Fgf2 (50 ng/ml; R&D Systems) and Tg α (20 ng/ml; R&D Systems) and one of the following inducers: LIF (50 ng/ml; R&D Systems), siderophore-loaded NGAL (100 μ g/ml) (Yang et al., 2002b) or NHBF (100 μ g total protein per ml). Labeled cRNA was prepared from 20 mesenchymes per condition or from freshly dissected E15.5 rat kidneys for two biological replicates per condition as described (Schmidt-Ott et al., 2005) and hybridized to Rat Genome 230 2.0 Arrays (Affymetrix). Raw data are available at Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>, GEO accession GSE5478).

Statistical analysis and classification of microarray data

Image files of microarray data were analyzed by robust multichip analysis as previously described (Schmidt-Ott et al., 2005). To test for significant regulation of gene expression over time, we utilized a recently developed method for significance analysis of time-course microarray experiments (Storey et al., 2005). An average time curve for all inducers was modeled using the software package EDGE. A gene was considered significantly regulated if its false discovery rate (FDR) of differential regulation over time was less than 10% and its expression level was either upregulated or downregulated more than 2-fold by each inducer compared with the baseline. The lists of genes identified thereby are provided in Tables S1 and S2 in the supplementary material. To identify global shifts in gene expression, hierarchical clustering was carried out based on Pearson correlation coefficients between average expression levels at different time points (Montaner et al., 2006).

Identification of putative TCF/Lef binding sites

To identify putative TCF/Lef binding sites in genes activated during epithelial differentiation (classes B and C), sequences 10 kb upstream of the transcriptional start site were extracted from published rat genomic sequences and aligned with the corresponding promoter sequences from the mouse and human genome using ClustalW (Thompson et al., 1994). Alignments were then subjected to an automated search for the TCF/Lef consensus recognition sequence [5'-(A/T)(A/T)CAAAG-3'] on both strands. Only sites conserved between all three species were considered. To test for significant overrepresentation of TCF/Lef consensus sites in classes B and C, the frequency of occurrence of at least one TCF/Lef site in various promoter intervals was compared against a set of 2591 control genes (least significant probe sets in the statistical analysis above). A χ^2 test was used to test for overrepresentation and $P < 0.05$ was assumed to indicate statistical significance. To confirm specificity of overrepresentation of the TCF/Lef consensus sequence in the promoter region of class B and C genes, 4096 nucleotide matrices, equal in dimension but different in sequence to the TCF/Lef matrix, were used to repeat the same type of analysis. An overrepresentation index i was calculated for the TCF/Lef matrix and all control matrices according to the formula $i = f_{BC}^2 / f_{con}$, where f_{BC} is the percentage of 10 kb promoters from class B and C genes that contain at least one occurrence of the nucleotide matrix and f_{con} is the corresponding percentage of promoters from control genes. The calculated value for i for the TCF/Lef matrix was 22.31, which represented the 97.1st percentile of all assayed matrices.

Adenoviral gene transfer

Recombinant adenovirus vectors Ad-GFP, Ad-CTNNB_{WT} and Ad-CTNNB_{S37A} were described previously (Masckauchan et al., 2005). Ad-DN-TCF was prepared from a pEGFP-DN-Tcf4 construct (a kind gift of P. Petzelbauer and W. Holnthoner) (Holnthoner et al., 2002) according to standard methods (Masckauchan et al., 2005). Five freshly dissected metanephric mesenchymes per condition were exposed to 1.5×10^8 plaque

forming units (PFU) of the appropriate adenovirus in 150 μ l basal culture media for 1 hour at 37°C and then plated onto Transwell filters and cultured in media supplemented as indicated.

Reporter assays

HEK293T cells were transfected in 12-well plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) using 190 ng of the TCF/Lef reporter construct Topflash or the mutated control plasmid Fopflash (Korinek et al., 1997) and 10 ng of a plasmid containing *Renilla* luciferase for normalization. A total of 1.5×10^7 PFU of adenovirus was added to the culture media. Cells were harvested after 24 hours of culture and reporter activity was assayed using the Dual Luciferase Assay System (Promega, Madison, WI).

Histology, X-Gal staining, immunofluorescence and in situ hybridization

For histology, metanephric mesenchymes were embedded in Epon, sectioned and stained with Toluidine Blue as described previously (Barasch et al., 1999).

TCF/Lef-*lacZ* embryos (Mohamed et al., 2004) were fixed with 2% paraformaldehyde, cryostat-sectioned and stained in *lacZ* staining solution (1×PBS, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml X-Gal) at 37°C.

For immunofluorescence staining, whole-mount metanephric mesenchymes were fixed in 4% paraformaldehyde and stained using primary antibodies for Pax2 (Invitrogen), goat anti-Cdh1 (R&D Systems), mouse anti-Cdh1 (BD Biosciences, Franklin Lakes, NJ), podocalyxin (R&D Systems), ACTIVE caspase 3 (Promega), phospho-histone H3 (Ser10) (Cell Signaling, Danvers, MA), the HA epitope (Clone 3F10, Roche Applied Sciences, Indianapolis, IN) and the appropriate secondary antibodies labeled with either Cy2, Cy3 or Cy5 (Jackson ImmunoResearch Laboratories, West Grove, PA). For double-staining using Pax2, ACTIVE caspase 3 and phospho-histone H3 antibodies, which are all raised in rabbits, we biotinylated the Pax2 antibody using the EZ-link Solid Phase Biotinylation Kit (Pierce, Rockford, IL) and performed immunostaining for ACTIVE caspase 3 or phospho-histone H3 followed by Pax2, where cross-reactivity of the biotinylated Pax2 antibody was prevented by preincubation with rabbit gamma globulins (Jackson ImmunoResearch) (Wurden and Homberg, 1993). All fluorescent imaging was performed on a Zeiss LSM 510 META scanning confocal microscope.

In situ hybridization on frozen sections was performed using DIG-labeled probes as described previously (Schmidt-Ott et al., 2006). To produce riboprobes, PCR fragments of genes of interest were generated from embryonic kidney cDNA using reverse primers containing a 5' T7 polymerase promoter sequence (primer sequences available upon request). The resulting fragments were purified and sequence-verified, and digoxigenin-labeled cRNA was produced using T7 polymerase.

Chromatin immunoprecipitation (ChIP) assays

In vivo ChIP was performed essentially as described (Chamorro et al., 2005; Lowry et al., 2005), but using cells isolated from kidneys of rat E15.5 embryos. Chromatin was cross-linked for 15 minutes in 1% paraformaldehyde in PBS. Cells were lysed in SDS lysis buffer (ChIP Assay Kit, Upstate USA, Charlottesville, VA), homogenized, and sonicated four times for 15 seconds at an output power of 4 Watts so as to fragment the DNA ('input DNA'). Subsequently, immunoprecipitation was carried out using rabbit anti- β -catenin antibody (H-102, Santa Cruz) and immune complexes pulled down using protein A-agarose beads blocked with salmon sperm DNA (Upstate). Following washes according to manufacturer instructions (Upstate), complexes were eluted in 1% SDS/100 mM NaHCO₃ and cross-links were reversed by adding NaCl to 200 mM and heating to 65°C for 14 hours. DNA was extracted and subjected to PCR using primers flanking the conserved TCF/Lef sites identified by our in silico analysis or off-target control sequences (primer sequences available upon request). DNA fragmentation was monitored by agarose gel electrophoresis of input DNA. Successful immunoprecipitation of β -catenin-containing protein-DNA complexes was confirmed by immunoblotting using a mouse anti- β -catenin antibody (BD Transduction Laboratories, San Jose, CA).

RESULTS

Microarray analysis of epithelial differentiation identifies distinct temporal waves of gene expression

In an effort to identify crucial transcriptional programs in renal epithelial progenitors during the processes of expansion and differentiation, we utilized the organ culture model of differentiating rat metanephric mesenchyme. As previously demonstrated, a combination of Fgf2 and Tg α permitted survival of these metanephric mesenchymal explants under defined conditions in organ culture (Fig. 1A-C) (Barasch et al., 1997; Karavanova et al., 1996; Perantoni et al., 1995). These factors induced the formation of clusters of epithelial progenitor cells expressing the characteristic markers Pax2, Wt1 and Wnt4, which were competent to undergo expansion and epithelial differentiation upon addition of an inducer (Fig. 1B-D) (Barasch et al., 1997; Barasch et al., 1999; Karavanova et al., 1996). We and others have previously isolated two such inducers from ureteric bud cell lines by protein chromatography, and identified them as leukemia inhibitory factor (Lif) and neutrophil gelatinase-associated lipocalin (NGAL; also known as Lcn2 – Mouse Genome Informatics) (Barasch et al., 1999; Plisov et al., 2001; Yang et al., 2002b). While these two factors were isolated based on their heparin-binding properties, we also noted an additional, distinct ureteric bud cell-derived inductive activity, which was not retained on heparin-Sepharose columns. We partially purified this activity and provisionally designated it 'non-heparin-binding fraction' (NHBF). In its presence, as in the presence of Lif and NGAL, rat metanephric mesenchymes underwent expansion and converted into organotypic nephron epithelia within 7 days ($n=40$) in stages reminiscent of the morphological sequence in vivo (Barasch et al., 1996; Karavanova et al., 1996) (Fig. 1E-K).

These molecular tools provided the methodological basis of this study and enabled us to monitor transcriptional events in differentiating renal epithelial progenitors in a synchronized organ culture setting (Barasch et al., 1999; Plisov et al., 2001; Yang et al., 2002b), which contrasts with the variety of different stages of epithelial differentiation co-existing at a given time point in vivo. We obtained transcriptional profiles during the course of metanephric mesenchymal differentiation in the presence of either Lif or NGAL or NHBF as indicated in Fig. 2 using Rat Genome 230 2.0 Arrays (Affymetrix), which contain 31,099 probe sets representing a large proportion of the rat transcriptome. In a preliminary analysis, we identified probe sets that were upregulated more than 8-fold by at least one inducer at one or more time points, revealing that a substantial proportion (24.4%) of upregulated probe sets was common to all three inducers (see Fig. S1 in the supplementary material). To relate this finding to gene expression in the embryonic kidney in vivo, we analyzed expression of all upregulated probe sets on a separate set of microarrays prepared from freshly dissected rat E15.5 kidneys. These kidneys display robust epithelial differentiation up the S-shaped body stage. When compared with freshly isolated metanephric mesenchyme, these developing kidneys displayed an enrichment (1.5-fold or more) of 71.2% of the probe sets upregulated by all three inducers. By contrast, only 41.3% of the probe sets that were upregulated by only one individual inducer displayed such enrichment in the developing kidney. Thus, we reasoned that selection of probe sets common to all three inducers would focus the analysis on genes specific to epithelial differentiation in the developing kidney and subtract out inducer-specific effects. To obtain a robust set of genes upregulated by all three inducers, we utilized a statistical approach specifically designed for time-course microarray experiments (Storey et al.,

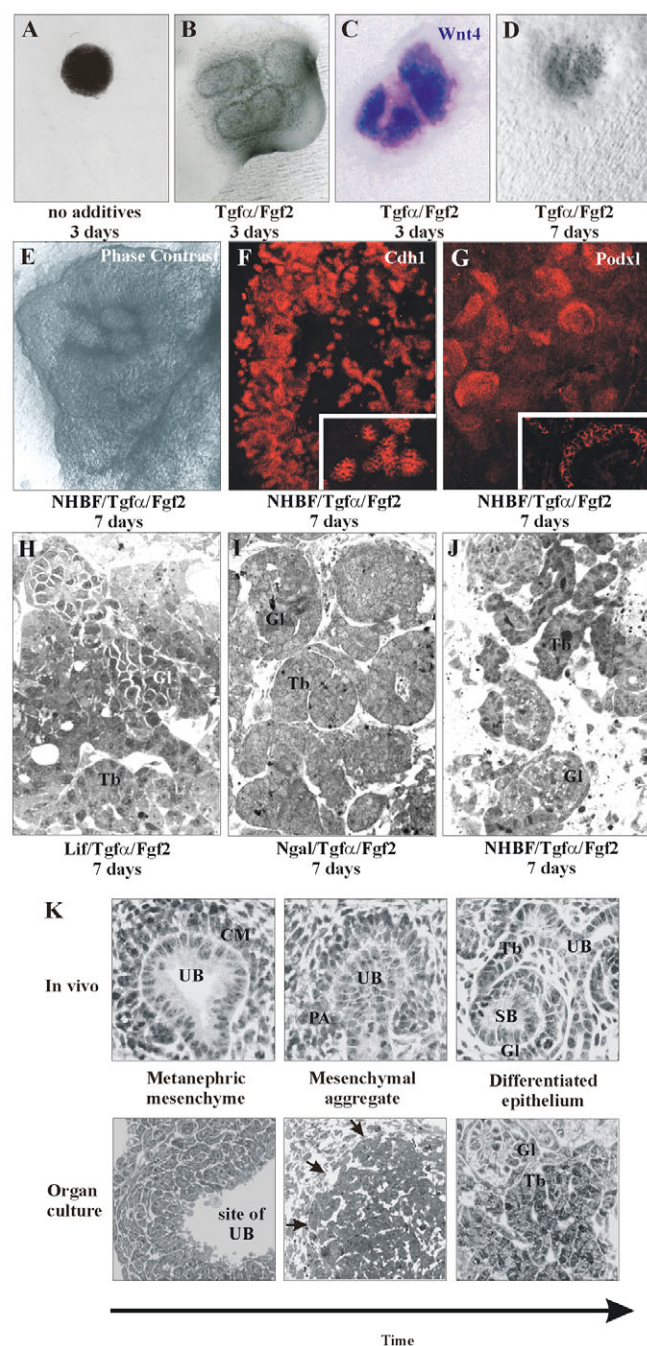


Fig. 1. Rat metanephric mesenchymes recapitulate differentiation of kidney epithelia in vivo under defined culture conditions.

(A) Metanephric mesenchymes cultured in basal media undergo apoptosis. (B) Addition of Fgf2 and Tgfa to the culture media induces survival of clusters of progenitors. (C) These aggregates express *Wnt4* as detected by in situ hybridization. (D) After continued culture with Fgf2 and Tgfa, mesenchymes degenerate without differentiating into epithelia. (E-J) NHBF, Lif or NGAL, when combined with Fgf2 and Tgfa, each induced continued expansion of metanephric mesenchymes and their differentiation into organotypic epithelia within 7 days of organ culture. Tubules stain positive for Cdh1 (E-cadherin; F), whereas glomerular-like structures express podocalyxin-like (Podxl) (G). Histologically, these structures resemble kidney epithelia at and beyond the S-shaped body stage (H-J). (K) The sequence of metanephric mesenchymal differentiation in organ culture recapitulates epithelial differentiation in vivo (in this case in the presence of Lif). Arrows delineate a mesenchymal aggregate, after 3-4 days of differentiation in vitro, reminiscent of pretubular aggregates in vivo. UB, ureteric bud; CM, condensed mesenchyme; PA, pretubular aggregate; SB, S-shaped body; Tb, tubule; Gl, glomerular-like structure.

Gene expression changed substantially between day 0 and 1 ($R=0.51$), a minor shift in gene expression was observed between day 2 and 3 ($R=0.77$), and a second major shift of global gene expression occurred between days 5 and 7 ($R=0.20$). Consequently, we grouped significantly induced genes into three different temporal classes based on their maximal expression level (Fig. 2B): genes peaking on day 1 or 2 (class A), genes peaking on day 3, 4 or 5 (class B), and genes peaking on day 7 (class C) (see Table S1 in the supplementary material). To again relate these three classes to stages of epithelial development in the embryonic kidney, we analyzed their expression on microarrays from rat E15.5 kidneys. When compared with freshly isolated metanephric mesenchyme, only 24 of 124 class A genes (19.4%) displayed an enrichment (1.5-fold) in these kidneys, suggesting that most of these genes reflected the introduction of the freshly dissected metanephric mesenchyme into the organ culture setting and were not directly associated with epithelial differentiation. However, 83 of 230 class B genes (36.1%) and 314 of 500 class C genes (62.8%) displayed such enrichment in the embryonic kidney in vivo, revealing a progressive induction in our organ culture model of genes characteristic of epithelial differentiation in maturing kidneys. When correlated with known patterns of gene expression in the developing kidney, class B genes included genes known to be highly expressed in pretubular aggregates in vivo, such as *Pax8* and *Wnt4*, two commonly used markers for this stage of epithelial development in mice (see Fig. S2 in the supplementary material) (Carroll et al., 2005; Stark et al., 1994). In situ hybridization confirmed that these genes and a third class B gene with previously unknown expression pattern, *Frzb*, were expressed in pretubular aggregates in the developing rat kidney (see Fig. S3 in the supplementary material). Class C genes contained multiple transcripts associated with mature polarized kidney epithelia, including those encoding Cdh1, Cdh16 (cadherin 16) and Lama1 (laminin $\alpha 1$) (see Fig. S2 in the supplementary material). In addition, class C genes included transcripts known to be expressed in specific segments of the nephron, such as early glomeruli [podoplanin (*Pdpn*), *CD2ap*, *Mafb*], proximal tubules [jagged 1 (*Jag1*), megalin (also known as *Lrp2* – Mouse Genome

2005) at a 10% false discovery level and selected genes with (1) significantly modulated expression levels and (2) an upregulation of at least 2-fold during the course of differentiation when compared with freshly isolated mesenchyme. This analysis identified 854 probe sets upregulated by each of the three inducers, for which we derived average temporal expression profiles to deduce a common temporal sequence of gene activation (Fig. 2A, see Table S1 in the supplementary material).

To identify global shifts in gene expression over time we performed hierarchical clustering of gene expression signatures at individual time points (0, 1, 2, 3, 4, 5, 7 days) based on matrices of correlation coefficients R between probe set levels (Fig. 2B). This analysis identified three consecutive temporal stages of global gene expression in the organ culture system (Fig. 2B).

Informatics)], the thick ascending limbs of Henle [$\text{Na}^+\text{-K}^+\text{-2Cl}^-$ cotransporter (*Nkcc2*; also known as *Slc12a1*)] and distal tubules (*Hnf1 β* , *AP-2 β* and *Brn1* – also known as *Tcf2*, *Tcfap2b* and *Pou3f3*, respectively) (see Fig. S2 in the supplementary material). Real-time reverse transcriptase (RT)-PCR was used to validate these temporal gene expression patterns in independent biological samples (see Fig. S2 in the supplementary material).

Multiple putative targets of TCF/Lef are activated during epithelial differentiation

Multiple genes in classes B and C have been associated with defects of nephrogenesis both in genetic mouse models and in humans (Dressler, 2006; Yu et al., 2004). In a search for potential key regulators of this transcriptional program associated with epithelial differentiation in our model, we manually screened class B and C genes for characteristic target genes of known developmentally regulated pathways. We noted the presence of multiple transcripts that represent known targets of TCF/Lef signaling in other biological settings, including cyclin D1 (*Ccnd1*) (Tetsu and McCormick, 1999), osteopontin (also known as *Spp1* – Mouse Genome Informatics) (El-Tanani et al., 2004), ecto-5'-nucleotidase (*Nt5e*) (Spychala and Kitajewski, 2004) and *Emx2* (Theil et al., 2002). Real-time RT-PCR confirmed that these transcripts were induced in close temporal correlation with the establishment of segmented epithelia (Fig. 3A).

Given the previous reports of the crucial involvement of WNT proteins in kidney organogenesis (Carroll et al., 2005; Stark et al., 1994) and in epithelial differentiation of the metanephric mesenchyme in response to Lif (Plisov et al., 2001), we hypothesized that β -catenin-induced TCF/Lef-mediated transcriptional programs might constitute a central downstream signal in differentiating epithelial progenitors. To obtain a more comprehensive picture of this putative transcriptional program activated by TCF/Lef, we performed an in silico analysis on transcripts in classes B and C to detect conserved consensus TCF/Lef binding sites in the promoter region of these genes. For this purpose, genomic sequences 10 kb upstream of the transcriptional start were extracted for rat, mouse and human homologs, aligned and analyzed for conserved occurrences of core TCF/Lef binding sites [5'-(A/T)(A/T)CAAAG-3'] of either orientation (for an example, see Fig. 3B). Genomic sequences and ortholog annotations were sufficient to complete this analysis for 71 genes from class B and 171 genes from class C. This approach identified at least one conserved TCF/Lef binding site in the predicted promoter region of 11 genes in class B (15.5%) and 27 genes (15.8%) in class C. Analysis of various intervals of promoter sequences consistently showed that TCF/Lef binding sites were present significantly more frequently in promoters of genes from classes B and C than in a set of 2869 control genes, the expression of which was unchanged during epithelial differentiation (Fig. 3C). Furthermore, when we carried out the same type of analysis using a set of 4096 control nucleotide matrices, equal in dimension but different in sequence to the TCF/Lef matrix, only 2.9% of these sequences displayed an equal or higher degree of overrepresentation in class B/C genes compared with the TCF motif (Fig. 3D). This suggested that TCF/Lef binding sites were specifically enriched in the promoter regions of genes associated with the stages directly preceding and coinciding with epithelial differentiation. The analysis identified conserved TCF/Lef sites in the promoter region of *Ccnd1* and *Emx2*, genes that have already been identified as target genes of TCF/Lef signaling in other biological settings. Importantly, the analysis predicted 36 putative, but as yet unconfirmed TCF/Lef targets,

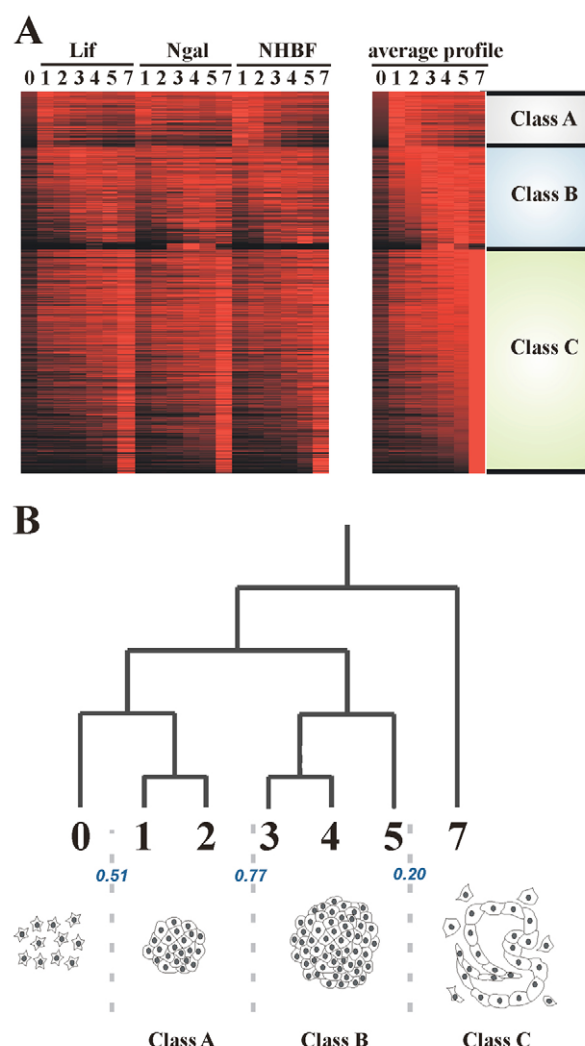


Fig. 2. Microarray analysis of gene expression during epithelial differentiation in rat metanephric mesenchyme. (A) Heatmap representation of all genes upregulated independently by Lif, NGAL and NHBF in a temporal sequence from day 0 (freshly isolated mesenchyme) to day 7 (differentiated renal epithelia). Average profiles (right) were deduced arithmetically and grouped into three classes based on the time point of peak expression (class A, day 1-2; class B, day 3-5; class C, day 7). Note that only genes upregulated by all three inducers were included in this analysis. (B) Correlation matrices comparing global gene expression at individual time points (day 0 to day 7) were calculated and hierarchical clustering identified time points of major shifts in gene expression (tree diagram). Pearson correlation coefficients *R* of neighboring time points (blue) illustrate the degree of change in global gene expression. Epithelial differentiation is depicted at the times of peak expression of each class of genes.

including the transcription factor Pax8, which is known to be a crucial regulator of specification, survival and proliferation in the nephric lineage (Bouchard et al., 2002).

β -Catenin activates a TCF/Lef-dependent transcriptional program in epithelial progenitors

To test the hypothesis that these putative TCF/Lef targets were activated by β -catenin signaling and to determine the physiological consequences of an activation of β -catenin/TCF/Lef signaling in the metanephric mesenchyme, we used adenoviral gene transfer of

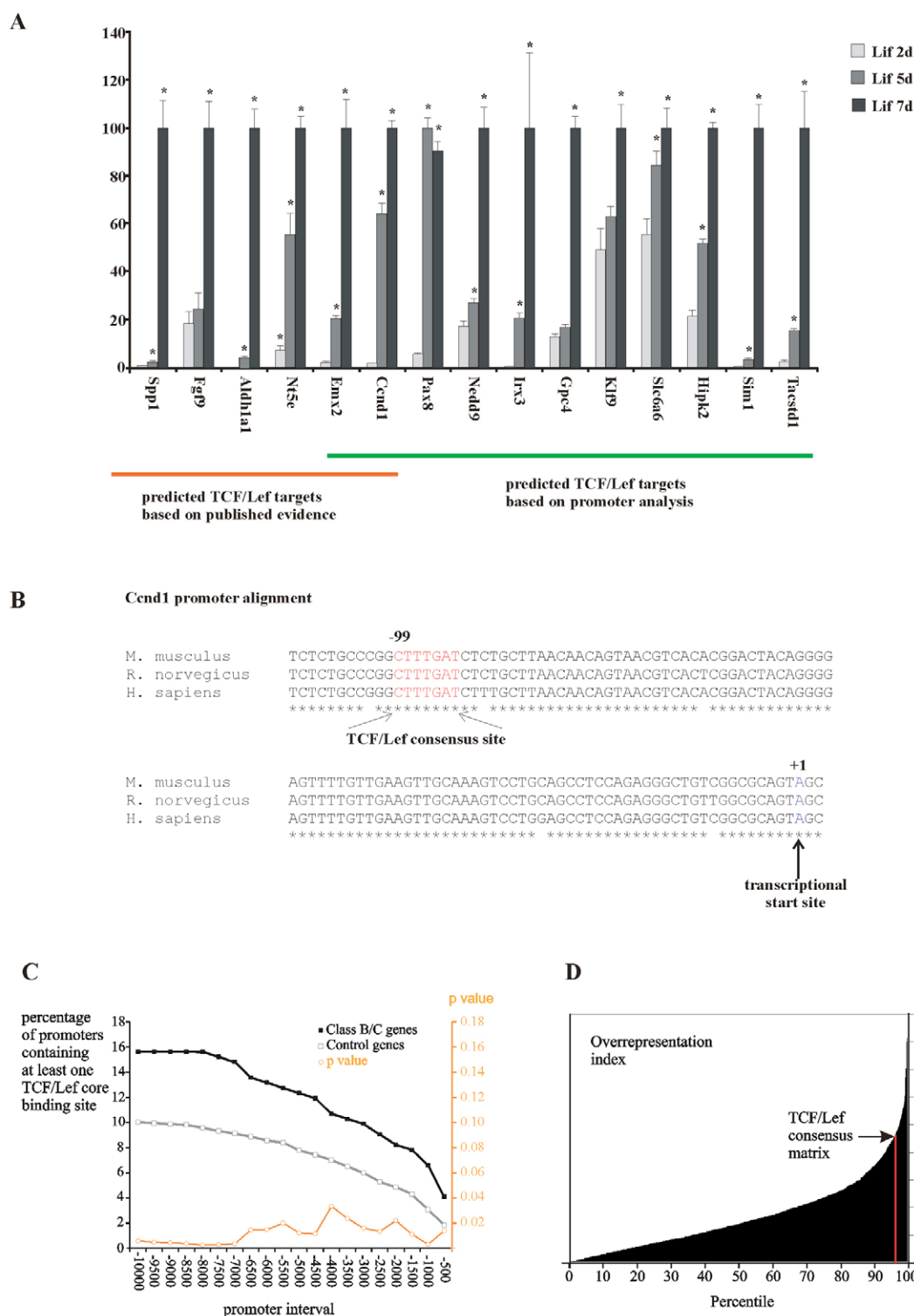


Fig. 3. Prediction of TCF/Lef target genes in differentiating epithelial progenitors. (A) Real-time RT-PCR confirmation of an upregulation of putative TCF/Lef target genes during epithelial differentiation induced by LIF. Expression levels peak on either day 5 (class B genes) or day 7 (class C genes) of organ culture. Maximal expression levels were set to 100%. Prediction is based on either published evidence or computational identification of conserved TCF/Lef consensus sites in the promoter region (see below). *, $P < 0.05$ versus LIF 2 days ($n = 3$). (B) Example of the prediction of a TCF/Lef binding site 99 bp upstream of the transcriptional start site of the cyclin D1 (*Cnd1*) gene. Mouse, rat and human sequences are aligned and reveal a high degree of conservation in the region surrounding the TCF/Lef consensus motif (red). (C) Overrepresentation of conserved TCF/Lef binding sites in promoters of genes induced during epithelial differentiation. Identification of genes containing at least one conserved TCF/Lef binding site in different intervals preceding the transcriptional start site consistently reveals a statistically significant overrepresentation in classes B and C compared with control genes (χ^2 test, P values for different intervals are indicated in orange). (D) Specific overrepresentation of the TCF/Lef core motif as measured by an overrepresentation index (see Materials and methods) is demonstrated by comparison with 4096 control motifs of equal dimension. The TCF/Lef matrix (red) scores in the 97.1st percentile of all examined matrices.

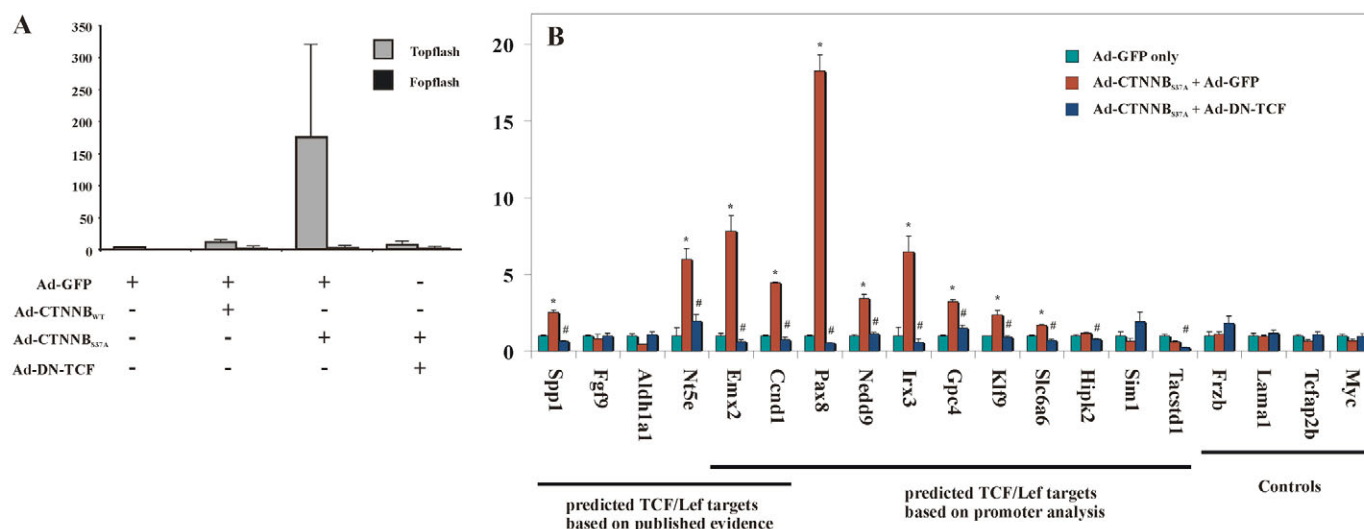


Fig. 4. TCF/Lef-dependent induction of predicted β -catenin targets. (A) Adenoviruses expressing wild-type (Ad-CTNNB_{WT}) or stabilized (Ad-CTNNB_{S37A}) β -catenin induce TCF/Lef-dependent Topflash reporter activity in human embryonic kidney (HEK)293 cells at moderate and high levels, respectively. The effect of Ad-CTNNB_{S37A} is effectively blocked by co-infection of Ad-DN-TCF. (B) As determined by real-time RT-PCR, Ad-CTNNB_{S37A} significantly induces expression of 10/15 (66%) predicted TCF/Lef target genes in metanephric mesenchymes after 14 hours of culture when compared with Ad-GFP only. This induction is consistently blocked by co-infection of Ad-CTNNB_{S37A} and Ad-DN-TCF ($n=3$, values are represented as mean \pm s.e.m.). Expression levels under control conditions (Ad-GFP only) were set to 1. Conversely, stabilization of β -catenin in metanephric mesenchyme does not induce expression of genes associated with epithelial differentiation that lack predicted TCF/Lef sites (*Frzb*, *Lama1*, *Tcfap2b*), or of *Myc*, a TCF/Lef target gene in other cell types, which is not significantly regulated during epithelial differentiation (Controls). *, Significantly upregulated versus Ad-GFP only ($P<0.05$); #, significantly downregulated versus Ad-CTNNB_{S37A}+Ad-GFP ($P<0.05$).

genetic constructs that activate or antagonize the β -catenin/TCF/Lef signaling axis. Although replication-deficient adenovirus has previously been used to achieve efficient gene transfer in cultured kidneys (Leung-Hagesteijn et al., 2005), we initially set out to verify that metanephric mesenchymal progenitors were efficiently targeted by adenovirus without significant pathogenicity. Initial experiments using a replication-deficient adenovirus expressing green fluorescent protein (Ad-GFP) revealed that 14 hours after infection of freshly isolated metanephric mesenchyme with Ad-GFP, followed by organ culture, 30–40% of Pax2-positive epithelial progenitors displayed GFP fluorescence (see Fig. S4 in the supplementary material, also see Fig. 8). GFP fluorescence was maintained at continuously high levels for up to 8 days in organ culture, the longest interval tested. Similar to uninfected metanephric mesenchymes, those infected with Ad-GFP and cultured without addition of growth factors underwent global apoptosis within 3 days of culture (see Fig. 7), indicating that the adenoviral infection itself had no effect on the survival of epithelial progenitors. Conversely, in the presence of inductive growth factors, these GFP-positive cells participated in the epithelialization process, indicating that the adenoviral infection was not inhibitory to epithelial differentiation (see Fig. 8). These observations verified that adenoviral gene transfer could achieve efficient and long-lasting expression of a gene of interest in epithelial progenitor cells without non-specifically perturbing the biological processes relevant to our experimental model.

To constitutively activate β -catenin signaling at high levels in the metanephric mesenchyme, we used a recombinant adenovirus expressing an HA-tagged N-terminally mutant β -catenin (Ad-CTNNB_{S37A}) (Rubinfeld et al., 1997). Conversely, to block β -catenin/TCF/Lef-dependent transcription of target genes, we generated an adenovirus expressing a fusion protein of GFP and an N-terminally truncated, dominant-negative Tcf4 protein (Ad-DN-TCF), which lacks the β -catenin interaction domain and acts as a constitutive

repressor of TCF/Lef target genes (Korinek et al., 1997). To verify the effectiveness of these adenoviruses, we assayed their effect on the expression of a TCF/Lef-dependent reporter, Topflash, in HEK293 cells. As predicted, cells infected with Ad-CTNNB_{S37A} displayed a marked (174-fold) increase in TCF/Lef-dependent reporter activity compared with control conditions (Ad-GFP) (Fig. 4A). Conversely, co-infection of Ad-DN-TCF reduced Ad-CTNNB_{S37A}-induced Topflash activity by approximately 20-fold (Fig. 4A). These initial studies indicated that these adenoviruses provided efficient tools to activate and antagonize β -catenin/TCF/Lef-mediated signaling.

We used Ad-CTNNB_{S37A} and Ad-GFP as a control, to assay the effect of stabilized β -catenin on the expression level of predicted TCF/Lef target genes in the metanephric mesenchyme. We examined the tissue 14 hours after infection, when epithelial progenitors had not yet initiated the default apoptotic program and when cultured explants were morphologically identical under both conditions (see Fig. 7A). We determined by real-time RT-PCR that 10 out of 15 of the predicted target genes were upregulated significantly in response to infection with Ad-CTNNB_{S37A} (Fig. 4B). To test if this response was mediated by TCF/Lef-type transcription factors, we co-infected adenovirus expressing dominant-negative TCF. This consistently antagonized the stimulation of target gene expression by stabilized β -catenin (Fig. 4B). These results established that the majority of putative β -catenin target genes predicted by our analysis were indeed induced by β -catenin in a TCF/Lef-dependent fashion.

Three of these target genes, *Pax8*, *Emx2* and *Cnd1*, particularly attracted our attention because they displayed high levels of induction and had previously been implicated in processes relevant to our model. Transcription factors of the Pax2/8 family are required for the survival and differentiation of progenitor cells in the intermediate mesoderm (Bouchard et al., 2002). *Emx2* is a homeobox gene that is required for normal urogenital tract

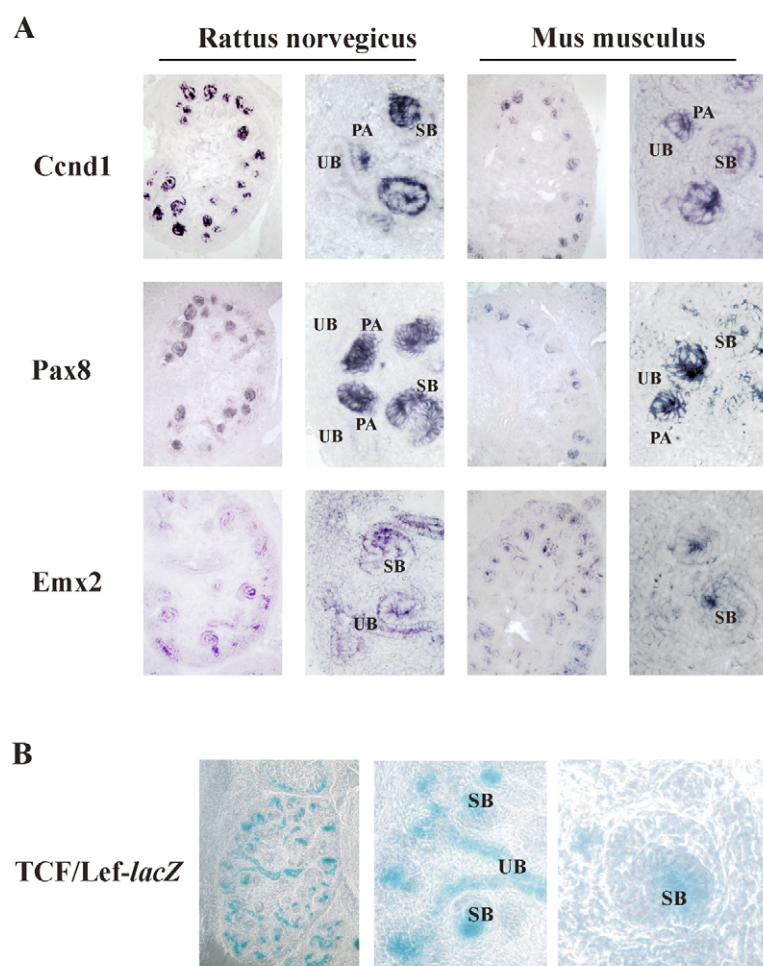


Fig. 5. Conserved co-expression of β -catenin/TCF/Lef targets in the renal epithelial lineage in vivo.

(A) *Ccnd1*, *Pax8* and *Emx2* are expressed in epithelia deriving from the metanephric mesenchyme in vivo as determined by in situ hybridization on rat and mouse E15.5 kidneys. *Ccnd1* and *Pax8* are first activated in pretubular aggregates and maintained in nascent early epithelia. *Emx2* is activated slightly later and appears first in early epithelia. Expression patterns are strictly conserved in the two species. (B) TCF/Lef-*lacZ* activity as determined by X-Gal staining is strong in emerging epithelia overlapping with the expression domain of target genes. Note that *Pax8*, *Emx2* and the TCF/Lef reporter are also detected in the ureteric bud. SB, S-shaped body; PA, pretubular aggregate; UB, ureteric bud.

development (Miyamoto et al., 1997), and in the brain cooperates with β -catenin to promote expansion of progenitor cells (Muzio et al., 2005). *Ccnd1*, although uncharacterized in the context of kidney development, is a crucial regulator of cell cycle progression in multiple systems (Stacey, 2003).

We asked whether these genes also represent targets of TCF/Lef signaling in vivo and whether this response is conserved in the rat, the model organism used in this study, and the mouse, the most commonly used genetic model of kidney development. In situ hybridizations for *Pax8*, *Emx2* and *Ccnd1* in rat E15.5 kidneys showed that these genes were expressed in a closely correlating pattern during kidney development in vivo. *Pax8* and *Ccnd1* were first detected in pretubular aggregates and maintained at high levels in the emerging tubular epithelia and in S-shaped bodies (Fig. 5A). *Emx2* appeared slightly later in the nascent tubular epithelia derived from pretubular aggregates (Fig. 5A). Overlapping expression patterns for *Pax8* and *Emx2* in the mouse had previously been published (Miyamoto et al., 1997; Stark et al., 1994) (Fig. 5A) and, also, *Ccnd1* expression was strictly conserved in mouse and rat kidneys (Fig. 5A) indicating that this transcriptional program is conserved in differentiating kidney epithelia in rodents.

To further substantiate that these transcripts were controlled by β -catenin/TCF/Lef in vivo, we used two independent approaches. First, we employed TCF/Lef-*lacZ* transgenic mice that express β -galactosidase under the control of six TCF/Lef response elements, thereby enabling spatial resolution of TCF/Lef-mediated transcription in vivo (Mohamed et al., 2004). In the developing

kidneys of these mice, β -galactosidase activity was detected primarily in nascent epithelial tubules shortly after the establishment of epithelial polarity (Fig. 5B). Thus, consistent with the concept that TCF/Lef control expression of *Emx2*, *Pax8* and *Ccnd1*, the TCF/Lef reporter displayed a close overlap with the expression of these genes. The fact that *Pax8* and *Ccnd1* preceded the expression of *Emx2* and the activation of the TCF/Lef reporter might indicate that additional pathways participate in the transcriptional control of these genes. In the case of the TCF/Lef reporter, the delayed onset of expression might also reflect a limited sensitivity of the construct, which has been previously suggested (Mohamed et al., 2004). Nevertheless, the sensitivity of the TCF/Lef reporter used in this study seems to be improved compared with alternative versions, which display *lacZ* staining only in the ureteric bud, but not in metanephric mesenchymal derivatives (Maretto et al., 2003; Schwab et al., 2007).

In a second approach, we aimed to confirm that β -catenin-containing transcriptional complexes associate with the predicted TCF/Lef binding sites in the promoter region of these genes in vivo. For this purpose, we conducted in vivo chromatin immunoprecipitation (ChIP) assays using an anti- β -catenin antibody that had recently been shown to function in immunoprecipitating chromatin associated with several TCF/Lef target genes (Chamorro et al., 2005; Lowry et al., 2005). We used freshly isolated kidneys from E15.5 rat embryos, cross-linked the protein-DNA complexes, fragmented the DNA to an average size of 400 bp, and then immunoprecipitated β -catenin-containing complexes (Fig. 6A,B). In samples immunoprecipitated with anti- β -catenin antibody, we

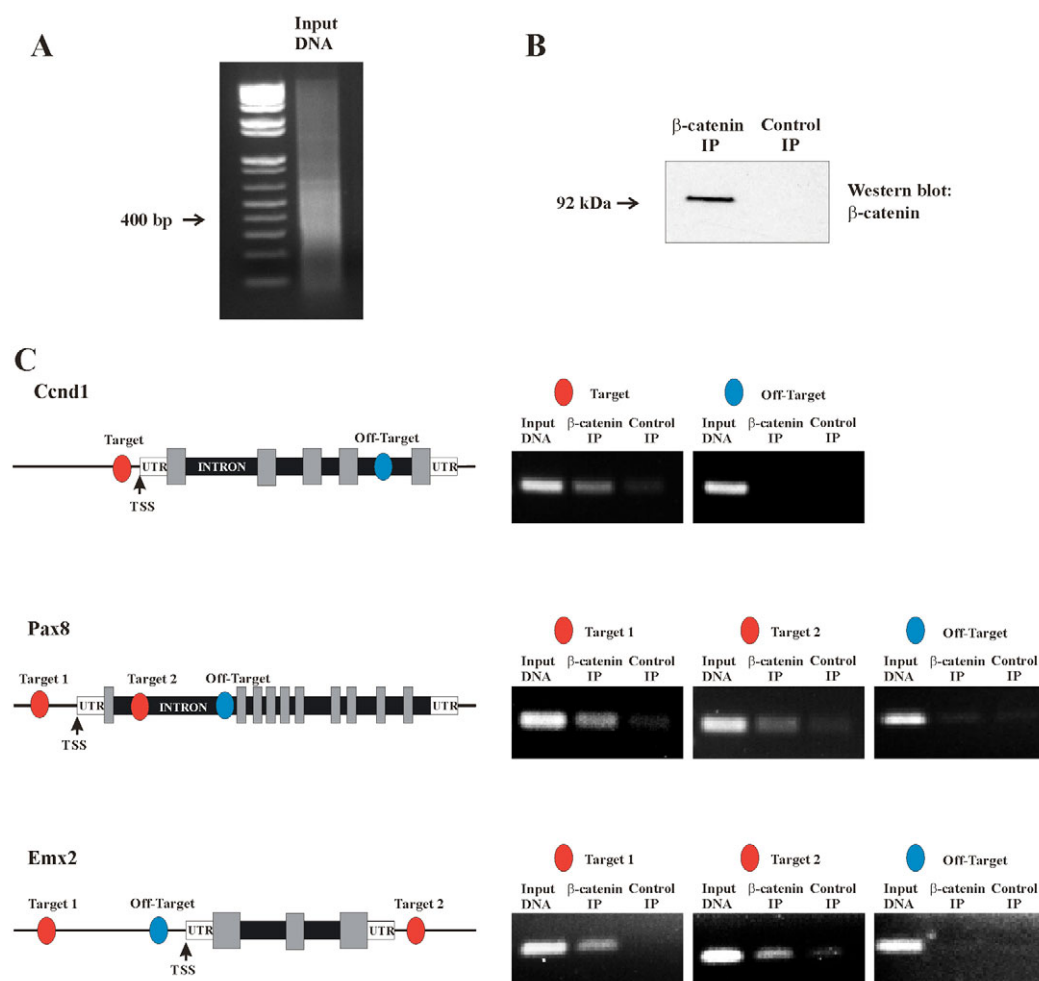


Fig. 6. β -catenin associates with chromatin in promoters of TCF/Lef target genes in vivo. ChIP was performed on rat E15.5 kidneys. **(A)** Chromatin was cross-linked and DNA was fragmented to ~400 bp. **(B)** Subsequently, β -catenin-containing DNA-protein complexes were immunoprecipitated as verified by immunoblotting. **(C)** Following reversal of cross-links, a strong enrichment of promoter sequences containing TCF/Lef sites (targets, indicated in diagrams on the left) was observed in β -catenin immunoprecipitates (β -catenin IP) but not control IP (no antibody). Off-target control sites were not enriched, demonstrating specificity.

detected 'target sequences' by PCR using primers that encompassed one of the putative TCF/Lef binding sites in the promoters of *Pax8*, *Cnd1* and *Emx2*, and these displayed strong enrichment compared with control immunoprecipitates (no antibody) (Fig. 6C). We also found enrichment of a conserved TCF/Lef motif in intron 1 of *Pax8* and of a previously published TCF/Lef site 3' to the *Emx2* gene (Theil et al., 2002) (Fig. 6C). Conversely, when using primers amplifying 'off-target' sequences that lacked conserved TCF/Lef sites within the same genes, we did not observe such enrichment (Fig. 6C). These results confirmed that β -catenin-containing transcriptional complexes specifically associated with those promoter regions containing TCF/Lef binding sites and, to our knowledge, provide the first in vivo ChIP data on embryonic kidneys. Overall, these results revealed a good correlation between the in vivo expression pattern of predicted β -catenin/TCF/Lef target genes, the binding of β -catenin-containing protein complexes to their predicted TCF/Lef consensus binding motifs, and the expression of TCF/Lef reporter activity.

Activation of β -catenin/TCF/Lef signaling in epithelial progenitors induces survival and proliferation but not epithelial conversion

The known functions of the TCF/Lef target genes in development and their expression at critical stages during the process of stage progression in the epithelial lineage suggested an involvement in the maintenance and differentiation of epithelial progenitor cells. In

addition, previous reports indicated that WNT-expressing cell lines and Gsk3 β inhibitors, both of which converge on β -catenin signaling as well as other pathways, trigger survival, cellular proliferation and epithelial differentiation in the metanephric mesenchyme (Davies and Garrod, 1995; Kispert et al., 1998). To determine whether β -catenin signaling was sufficient to phenocopy these responses, we induced expression of stabilized β -catenin in the metanephric mesenchyme by adenoviral gene transfer. As expected, metanephric mesenchyme infected with the control virus Ad-GFP and cultured in basal media in the absence of growth factors underwent apoptosis (Fig. 7A). After 3 days of culture, the entire Pax2-expressing pool of epithelial progenitors stained positive for activated caspase 3, a marker of apoptotic cells (Fig. 7A). By contrast, metanephric mesenchymes infected with Ad-CTNNB_{S37A} were salvaged from apoptosis. This was evident after 2-3 days of culture, when aggregates of progenitor cells expanded and formed clusters (100% of infected metanephric mesenchymes, $n > 20$, Fig. 7A). These cell aggregates were positive for Pax2 indicating that they represented the nephric epithelial lineage ($n = 5$, Fig. 7A). The absence of activated caspase 3 in these nodules demonstrated that these Pax2-positive cells were selectively spared from apoptosis ($n = 5$, Fig. 7A). By contrast, cells outside of the Pax2-positive cell population, despite efficient infection (see Fig. 7C), were not rescued and underwent apoptosis (Fig. 7A). These data indicated that an activation of β -catenin signaling exerted anti-apoptotic effects specifically in epithelial progenitors.

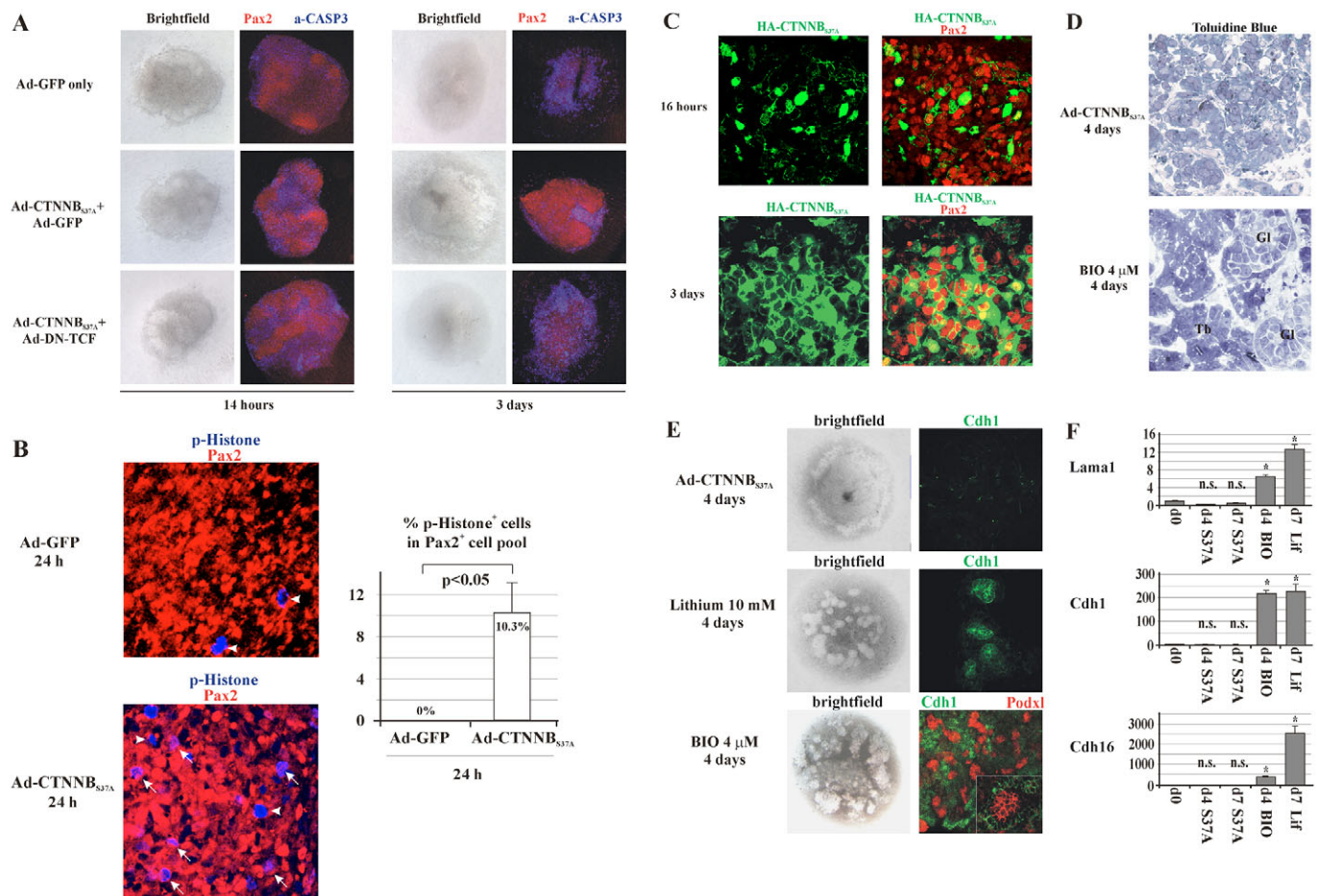
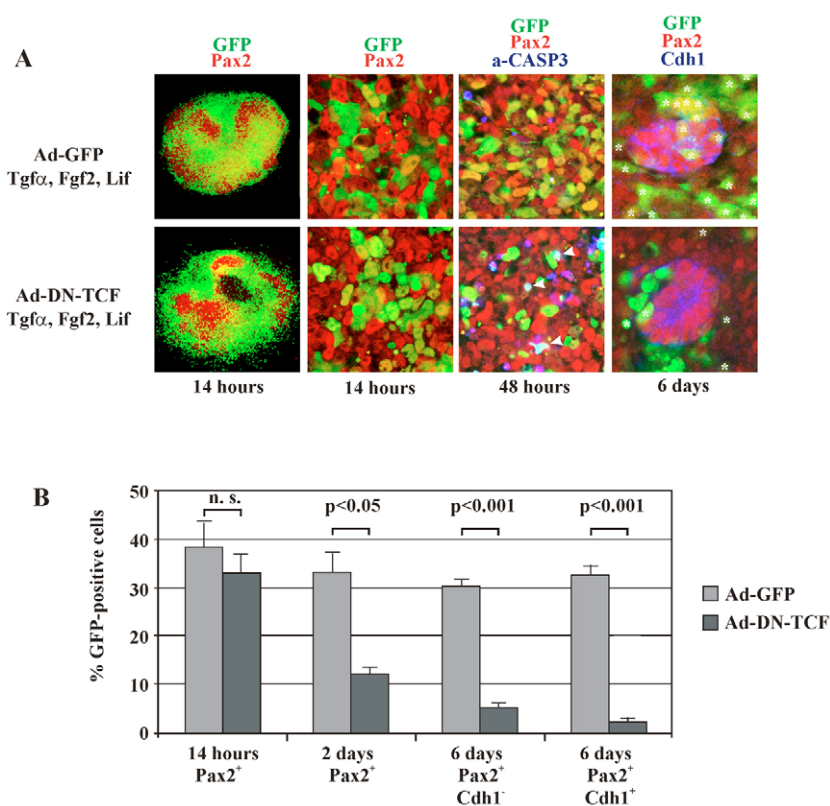


Fig. 7. β -catenin signaling triggers survival and proliferation of epithelial progenitors, but not tubulogenesis. (A) Introduction of stabilized β -catenin (Ad-CTNNB_{537A}) into epithelial progenitors marked by Pax2 (red) prevents apoptosis determined by immunostaining for activated caspase 3 (a-CASP3, blue) observed after 3 days of culture under control conditions (Ad-GFP only). This anti-apoptotic response is blocked by dominant-negative TCF (Ad-DN-TCF). (B) Proliferative activity measured by phospho-histone H3 staining (blue) is observed in Pax2-expressing (red) progenitors following infection with Ad-CTNNB_{537A}, but not Ad-GFP (arrows). Note the presence of a small number of phospho-histone-positive cells in the Pax2-negative cell compartment in both conditions (arrowheads). (C) Only scattered Pax2-positive progenitors (red) are initially infected by Ad-CTNNB_{537A}, as detected after 16 hours of culture following infection and staining for the HA epitope on the adenovirally coded protein (green). Infected cells display a strongly positive membrane pool of HA-CTNNB_{537A} and a nuclear pool of variable intensity, which colocalizes with Pax2 (yellow areas). After 3 days of culture, HA-negative progenitors have largely disappeared suggesting positive selection of cells infected by Ad-CTNNB_{537A}, which indicates that the effect of the adenovirus is cell-autonomous. (D) Metanephric mesenchymes infected with Ad-CTNNB_{537A} develop into cell aggregates, but do not display evidence of tubulogenesis or segmentation. Conversely, tubular and glomerular-like structures are abundantly observed after treatment with a low dose of the Gsk3 β inhibitor BIO. (E) Aggregates induced by Ad-CTNNB_{537A} stain negative for Cdh1 (green), a marker of polarized epithelial cells. By contrast, metanephric mesenchymes induced with the Gsk3 β inhibitors lithium chloride or BIO for the same time period display Cdh1-positive tubules. Note that treatment with BIO also induces glomerular-like structures marked by podocalyxin expression (Podxl, red). (F) As detected by real-time RT-PCR, markers of polarized epithelial cells are strongly induced after epithelial induction with BIO for 4 days (d4 BIO) or Lif for 7 days (d7 Lif) (time points of robust epithelial differentiation), but remain negative 4 days (d4) and 7 days (d7) after infection with Ad-CTNNB_{537A} (S37A). Values are compared with freshly isolated metanephric mesenchymes (d0), expression levels of which were set to 1. *, $P < 0.05$ versus metanephric mesenchyme. Gl, glomerular-like structure; Tb, tubule.

To test whether an activation of β -catenin signaling in Pax2-positive epithelial progenitors promoted proliferation in addition to this anti-apoptotic effect, we assayed the number of proliferating cells in the progenitor compartment. Co-staining for Pax2 and phospho-histone H3, a marker of cells in metaphase, revealed that approximately 10% of epithelial progenitors were undergoing cell division 24 hours after infection with Ad-CTNNB_{537A} ($n=4$, Fig. 7B). Conversely, under control conditions (Ad-GFP), Pax2-positive epithelial progenitors showed no evidence of proliferation, although a few phospho-histone H3-positive cells were observed

in the Pax2-negative cell population (Fig. 7B). This result indicated that β -catenin signaling induced proliferation in epithelial progenitor cells.

We reasoned that if β -catenin signaling exerted a cell-autonomous anti-apoptotic and proliferative effect in Pax2-positive epithelial progenitors, this should lead to a positive selection of the subset of cells infected by Ad-CTNNB_{537A} during the course of the experiment. To test this, we stained the developing cellular clusters for the HA epitope present on the adenovirally introduced stabilized β -catenin after 3 days in culture. This revealed that at this point, $95.9 \pm 2.3\%$ of



Pax2-positive cells in the developing clusters also stained positive for the HA tag as compared with only $30.2 \pm 1.2\%$ of initially infected cells detected after 16 hours ($n=3$, Fig. 7C). This finding indicated that Ad-CTNNB_{S37A} promoted positive selection of the infected subset of cells – consistent with cell-autonomous anti-apoptotic and proliferative effects. Notably, in addition to a prominent membranous pool of stabilized β -catenin, we consistently observed a small nuclear pool (Fig. 7C), suggesting an interaction with chromatin.

We tested whether the biological effects of stabilized β -catenin were mediated through TCF/Lef-type transcription factors. For this purpose, we co-infected Ad-DN-TCF in metanephric mesenchymes treated with Ad-CTNNB_{S37A}. Consistent with our hypothesis, Ad-DN-TCF, but not Ad-GFP, blocked the survival effect induced by Ad-CTNNB_{S37A} ($n=6$, Fig. 7A). These data indicated that TCF/Lef-type transcription factors mediated the anti-apoptotic effects of β -catenin signaling in epithelial progenitor cells.

Finally, we tested whether β -catenin signaling alone was sufficient to induce epithelial differentiation of the metanephric mesenchyme. For this purpose, we examined the histomorphology of the Pax2-positive cell aggregates induced by stabilized β -catenin. However, even with extended incubation in vitro for up to 7 days (a time period after which tubulogenesis is robustly observed with all known epithelial inducers), none of these aggregates displayed evidence of tubulogenesis. In contrast to Gsk3 β inhibitors, which induced either unsegmented (10 mM lithium chloride) or segmented (4 μ M BIO) renal epithelia (Fig. 7D,E), Ad-CTNNB_{S37A} induced the formation of virtually unorganized cellular clusters ($n=5$, Fig. 7D) and Cdh1, a marker of polarized epithelial cells in the kidney, remained absent in these aggregates ($n=10$, Fig. 7E). In addition, by real-time RT-PCR, several markers of mature renal epithelial cells, including *Cdh1*, *Lama1* and *Cdh16*, failed to be upregulated, even after 7 days in culture (Fig. 7F). To exclude the possibility that this response was related to an excess level of β -catenin signaling in the presence of the

Fig. 8. Progenitors with compromised TCF/Lef signaling are depleted from the epithelial lineage. (A) Metanephric mesenchymes are infected with Ad-GFP or GFP-tagged dominant-negative TCF (Ad-DN-TCF) followed by culture in the presence of inductive media containing Tgfa, Fgf2 and Lif and virus withdrawal after 14 hours. Efficiency of infection of Pax2-positive epithelial progenitors (red) with Ad-GFP (green, cytoplasmic staining) or Ad-DN-TCF (green, nuclear staining) is similar as determined after 14 hours of culture. Conversely, after 48 hours, cells expressing DN-TCF are observed at a decreased frequency in the Pax2-positive cell population as compared with cells expressing GFP only. Occasionally, staining for activated caspase 3 (blue) is observed in Pax2-positive cells infected by Ad-DN-TCF (arrowheads), indicating ongoing apoptosis in this population (note occasional apoptosis also in the Pax2-negative cell population with both viruses). After 6 days of culture, DN-TCF-expressing cells (green) are rarely observed in Pax2-positive Cdh1-negative (Pax2⁺ Cdh1⁻) epithelial progenitors or arising Pax2⁺ Cdh1⁺ epithelial clusters, whereas cells infected with Ad-GFP (green) readily contribute to either of these pools (asterisks). (B) Quantitative evaluation of percentage of green-fluorescent cells in either Pax2⁺ progenitors or Pax2⁺ Cdh1⁺ epithelia demonstrates progressive depletion from the differentiating epithelial lineage of cells expressing GFP-tagged DN-TCF, but not GFP only.

mutant stabilized β -catenin construct, we also tested the effect of Ad-CTNNB_{WT}, a virus expressing wild-type non-stabilized β -catenin. Although this treatment was sufficient to induce survival of Pax2-positive cell aggregates, they were substantially smaller in size than those induced by stabilized β -catenin, but also did not initiate tubulogenesis or express the above markers of differentiated epithelial cells (data not shown). These results indicated that β -catenin signaling alone, as opposed to Gsk3 β inhibition, was insufficient to induce the complete program of epithelial differentiation in the metanephric mesenchyme.

Metanephric progenitors with compromised TCF/Lef signaling are progressively depleted from the epithelial lineage

Although the above results indicated sufficiency of β -catenin/TCF/Lef signaling to maintain viability and control cellularity of the metanephric mesenchymal progenitor pool in the absence of exogenous growth factors, it was unclear whether this pathway was also required for these processes in the experimental setting of epithelial differentiation. To test this, we examined the effect of compromised TCF/Lef signaling in epithelial progenitors in the organ culture system. We infected freshly isolated metanephric mesenchymes with either Ad-DN-TCF or Ad-GFP and cultured them in the presence of Tgfa, Fgf2 and Lif to induce epithelial conversion. Viruses were removed after 14 hours and culture was continued in virus-free inductive media to prevent continuous infection of differentiating cells during the course of the experiment. The GFP tags on both constructs enabled us to follow the fate of infected progenitor cells during the process of epithelial differentiation. Initial infection rates of Pax2-positive epithelial progenitors by Ad-GFP and Ad-DN-TCF (determined 14 hours after infection) were similar ($n=5$, Fig. 8A,B). However, during the course of the experiment, the fraction of Pax2-positive

progenitors expressing DN-TCF was progressively reduced, whereas the fraction of GFP-expressing Pax2-positive progenitors remained similar (Fig. 8A,B). Following epithelial differentiation after 6 days of culture, DN-TCF-expressing cells were depleted from both the Pax2-positive and Cdh1-negative epithelial progenitor pool and from Pax2-positive Cdh1-positive epithelial progeny ($n=22$, Fig. 8A,B). Co-staining for activated caspase 3 after 48 hours of culture revealed frequent apoptosis in cells expressing dominant-negative TCF, but not in control cells expressing GFP, suggesting that compromised TCF/Lef signaling in these cells promoted apoptosis ($n=3$, Fig. 8A). Cells expressing dominant-negative TCF were occasionally observed in Cdh1-positive epithelia (Fig. 8B), suggesting that they were still competent to undergo epithelial conversion, but we cannot exclude the possibility that they were infected subsequent to epithelial conversion by small amounts of residual virus in the culture media. In summary, these data unequivocally demonstrated that TCF/Lef signaling is crucially involved in the regulation of cellularity in the epithelial lineage and suggested a requirement of this pathway for cell survival and stage progression during epithelial differentiation.

DISCUSSION

We have developed an approach to follow transcriptional events during the course of renal epithelial differentiation in an organ culture model closely resembling the differentiation of epithelial progenitors in the developing kidney in vivo. Our data indicate that a β -catenin/TCF/Lef-dependent transcriptional program (1) is activated during the process of epithelial differentiation of renal progenitors; (2) is sufficient to maintain viability of mesenchymal progenitor cells and induce their proliferation; and (3) is necessary for the regulation of cellularity in the epithelial progenitor/progeny compartment in the experimental setting of epithelial differentiation. Although WNT ligands have been implicated in epithelial differentiation of mesenchymal progenitors (Carroll et al., 2005; Herzlinger et al., 1994; Kispert et al., 1998; Stark et al., 1994), these data provide the first evidence that TCF/Lef signaling constitutes a central downstream signal.

Although part of our data rely on experiments on cultured explants of rat metanephric mesenchyme, several facts point to the relevance of our observations in vivo and in other species, e.g. mouse. First, the transcriptional program induced in the course of epithelial differentiation in our organ culture system is associated with an activation of a transcriptional signature that closely recapitulates gene expression in the developing kidney in vivo. Second, the β -catenin/TCF/Lef target genes identified in the in vitro system are activated in close temporal and spatial correlation in vivo in pretubular aggregates and in the emerging early epithelia and their sites correlate with that of a TCF/Lef reporter in vivo. Third, TCF/Lef target gene expression in vivo is identical in rat and mouse kidneys pointing towards a conserved process. Fourth, the promoter region of β -catenin/TCF/Lef target genes identified in vitro is occupied by β -catenin-containing transcriptional complexes in vivo, implying that β -catenin and its downstream targets might be directly involved in their regulation. Furthermore, Wnt1, which triggers β -catenin-dependent signaling in many cell types (although this remains to be confirmed in the case of the metanephric mesenchyme), when misexpressed from the ureteric bud of Wnt9b-deficient mice, rescues a transcriptional program downstream of Wnt9b (Carroll et al., 2005). This program includes *Pax8*, a gene that we identify here to be a TCF/Lef-dependent target of β -catenin signaling in the

metanephric mesenchyme. Together, these observations suggest that β -catenin signaling controls a differentiation-associated transcriptional subprogram in differentiating cells of the renal epithelial lineage.

Previous studies have suggested a role for β -catenin signaling during differentiation of the developing renal epithelial lineage in vitro. First, in the isolated metanephric mesenchyme from rats or mice, co-cultivation of fibroblasts expressing Wnt1 triggers survival and differentiation of tubular and glomerular-like epithelia (Herzlinger et al., 1994; Kispert et al., 1998). Secondly, lithium chloride and BIO, which increase cellular β -catenin levels by inhibiting Gsk3 β (Klein and Melton, 1996; Sato et al., 2004), induce epithelial structures in isolated metanephric mesenchymes from rats or mice (Davies and Garrod, 1995; Kuure et al., 2007). Third, in the model of Lf-induced differentiation of the rat metanephric mesenchyme, epithelial differentiation is accompanied by an occupation of TCF/Lef sites in mobility shift assays and is blocked by exogenous application of Sfrp1, a secreted WNT antagonist (Plisov et al., 2001). Now, our study provides the first functional link between β -catenin/TCF/Lef signaling and anti-apoptotic and proliferative effects in the epithelial lineage and defines a set of target genes in epithelial progenitors. However, β -catenin signaling in our model phenocopies only part of the effects of Gsk3 β inhibitors or WNT-expressing cell lines in that we detect neither epithelial cells nor polarized tubules, hallmarks induced by Gsk3 β inhibitors and co-expressed WNTs. In a recent study, lotus lectin- and peanut agglutinin-positive structures were detected following homozygous deletion of exon 3 of β -catenin in metanephric mesenchyme suggesting that stabilization of β -catenin might be sufficient to trigger expression of surface markers of proximal tubules and glomeruli (Kuure et al., 2007). Their data and ours strongly support the concept that β -catenin signaling induces stage progression in the epithelial lineage. However, additional pathways downstream of WNT signaling and Gsk3 β inhibitors appear to cooperate with β -catenin/TCF/Lef signaling to induce polarized and segmented renal epithelia.

An important remaining question is the nature of the extracellular signal that triggers β -catenin/TCF/Lef signaling in the organ culture system and in vivo. Wnt4 seems an obvious candidate based on an upregulation we observe in differentiating metanephric explants and co-expression of Wnt4 and TCF/Lef target genes in vivo. Furthermore, in the present study, inhibition of β -catenin/TCF/Lef signaling in epithelial progenitors decreases but incompletely abolishes their participation in epithelial differentiation, which is reminiscent of the reduction, but not complete absence, of epithelial progeny in Wnt4-deficient kidneys (Kobayashi et al., 2005). Wnt9b and Wnt7b, both of which are formed by the ureteric bud, may participate in controlling β -catenin signaling, but the fact that we observe activation of the TCF/Lef-responsive transcriptional program in the absence of the ureteric bud and surrogate WNT molecules argues for a mesenchymal-derived autocrine signal that activates β -catenin/TCF/Lef signaling. In this regard, it is also notable that *Frzb*, a class B gene expressed in pretubular aggregates, has been shown to act as an antagonist of WNT/ β -catenin signaling (Lin et al., 1997). This secreted molecule might act to sharpen the gradient of TCF/Lef transcriptional activity in the vicinity of the pretubular aggregate.

In summary, our data suggest that β -catenin/TCF/Lef drives a transcriptional program in differentiating renal epithelia that participates in maintenance, proliferation and stage progression of the renal epithelial lineage. Additional transcriptional programs downstream or in parallel to this pathway might participate in

epithelial differentiation, and the global transcriptional profile of epithelial conversion identified herein will provide a valuable tool to elucidate their molecular nature.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/17/3177/DC1>

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