**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 (Ccd1) 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 (Ccd1), 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 epithelium. 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 (TGFβ), 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 Gsk3β and casein kinase 1 (CK1; 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 (Barash et al., 1999). Gsk3β inhibitors were used as lithium chloride (Sigma-Aldrich, St Louis, MO) and 6-bromoindirubin-3'-oxime (BIO; EMD Biosciences, San Diego, CA).

Isolation of non-heparin-binding fraction (NHBF)

Approximately 200 I f serum-free media, conditioned by monolayers of ureteric bud cells (Barash et al., 1996), were concentrated, desalted and applied to heparin-Sepharose (GE Healthcare, Piscataway, NJ) in 10 mM NaPO4 (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 μg of total protein was subjected to immunoblotting for Lif (polyclonal anti-mouse Lif; R&D) and NGAL (100 μg total protein per ml). Labeled RNA 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).

Microarray analysis

Metanephric mesenchymes were cultured in basal media supplemented with Fgf2 (50 ng/ml; R&D Systems) and Tgfα (20 ng/ml; R&D Systems) and one of the following inducers: Lif (50 ng/ml; R&D Systems), siderophore-loaded NGAL (100 μg/ml) (Yang et al., 2002b) or NHBF (100 μg total protein per ml). Labeled RNA 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 (Novak 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(TA)(AT)CAAG-3'] on both strands. Only sites conserved among 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 χ2 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=fTcf/fcon, where fTcf is the percentage of 10 kb promoters from class B and C genes that contain at least one occurrence of the nucleotide matrix and fcon 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-CTNNB1WT and Ad-CTNNB1S37A were described previously (Masckauchan et al., 2005). Ad-DN-TF, was prepared from a pEGFP-DN-Tcf14 construct (a kind gift of P. Petzeltbauer 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 1.5×107 plaque forming units of adenovirus for 18 h, with or without metallothionein-induced BMP induction (Brembeck et al., 2006).
forming units (PFU) of the appropriate adenovirus in 150 μl 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 assessed 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, mouse anti-Cdh1 (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 Tgfα 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.,...
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).

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, $Fzrb$, 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
the thick ascending limbs of Henle [Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (Nkcc2; also known as Slc12a1)] and distal tubules (Hnf1\(\beta\), AP-2\(\beta\) 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, 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 (Ccnd1) 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 (χ² 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-CTNNB157A) or stabilized (Ad-CTNNBS37A) 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-CTNNBS37A is effectively blocked by co-infection of Ad-DN-TCF. (B) As determined by real-time RT-PCR, Ad-CTNNBS37A 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-CTNNBS37A and Ad-DN-TCF (n=3, values are represented as mean±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 (Frb2, 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-CTNNBS37A+Ad-GFP (P<0.05).
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 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.

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.

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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 has 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
detected ‘target sequences’ by PCR using primers that encompassed one of the putative TCF/Lef binding sites in the promoters of Pax8, Ccnd1 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 within the same genes, and we did not observe such enrichment (Fig. 6C). 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 Gsk3β 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 mesenchymes 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-CTNNB37A 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.
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-CTNNB37A, 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). Only scattered Pax2-positive progenitors (red) are initially infected by Ad-CTNNB37A, 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-CTNNB37A 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-CTNNB37A, which indicates that the effect of the adenovirus is cell-autonomous.

(D) Metanephric mesenchymes infected with Ad-CTNNB37A 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 Gsk3β inhibitor BIO. (E) Aggregates induced by Ad-CTNNB37A stain negative for Cdh1 (green), a marker of polarized epithelial cells. By contrast, metanephric mesenchymes induced with the Gsk3β 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-CTNNB37A (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.
Pax2-positive cells in the developing clusters also stained positive for the HA tag as compared with only 30.2±1.2% of initially infected cells detected after 16 hours (n=3, Fig. 7C). This finding indicated that Ad-CTNNB137A 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-CTNNB137A. Consistent with our hypothesis, Ad-DN-TCF, but not Ad-GFP, blocked the survival effect induced by Ad-CTNNB137A (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 Gsk3β inhibitors, which induced either unsegmented (10 mM lithium chloride) or segmented (4 μM BIO) renal epithelia (Fig. 7D,E), Ad-CTNNB137A 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 mutant stabilized β-catenin construct, we also tested the effect of Ad-CTNNBWT, 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 Gsk3β 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 Tgfβ1, Fgf2 and Lif to induce continuous infection of differentiating cells expressing GFP-tagged DN-TCF, but not GFP only.

![Fig. 8. Progenitors with compromised TCF/Lef signaling are depleted from the epithelial lineage.](image)

(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 Tgfβ1, 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.
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 Cdhl-negative epithelial progenitor pool and from Pax2-positive Cdhl-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 Cdhl-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 Gsk3β (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 Lif-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 Gsk3β inhibitors or WNT-expressing cell lines in that we detect neither epithelial cells nor polarized tubules, hallmarks induced by Gsk3β inhibitors and co-expressed WNTs. In a recent study, lots 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 Gsk3β 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
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References


sequence weighting, position-specific gap penalties and weight matrix choice.


