γ-Secretase activity is dispensable for mesenchyme-to-epithelium transition but required for podocyte and proximal tubule formation in developing mouse kidney

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Summary

Notch signaling is involved in pronephros development in *Xenopus* and in glomerulogenesis in mice. However, owing to early lethality in mice deficient for some Notch pathway genes and functional redundancy for others, a role for Notch signaling during early stages of metanephric development has not been defined. Using an antibody specific to the N-terminal end of γ-secretase-cleaved Notch1, we found evidence for Notch1 activation in the comma and S-shaped bodies of the mouse metanephros. We therefore cultured mouse metanephroi in the presence of a γ-secretase inhibitor, *N*-*S*-phenyl-glycine-*t*-butyl ester (DAPT), to block Notch signaling. We observed slightly reduced ureteric bud branching but normal mesenchymal condensation and expression of markers indicating that mesenchyme induction had occurred. However, fewer renal epithelial structures were observed, with a severe deficiency in proximal tubules and glomerular podocytes, which are derived from cells in which activated Notch1 is normally present. Distal tubules were present but in reduced numbers, and this was accompanied by an increase in intervening, non-epithelial cells. After a transient 3-day exposure to DAPT, proximal tubules expanded, but podocyte differentiation failed to recover after removal of DAPT. These observations suggest that γ-secretase activity, probably through activation of Notch, is required for maintaining a competent progenitor pool as well as for determining the proximal tubule and podocyte fates.

Supplemental data available online

Key words: Metanephric culture, Notch, gamma-secretase, DAPT, Wt1, Mouse

Introduction

Notch proteins are conserved receptors mediating short-range intercellular communications that allow proper allocation of cell fates during development (Lewis, 1998) and in the adult (Brittan and Wright, 2002; Gridley, 1997; Ohishi et al., 2002; Radtke et al., 2002). In most instances, Notch acts in a core pathway as a membrane-bound transcription factor that is released by two proteases in response to ligand binding (Fortini, 2002; Kopan, 2002; Mumm and Kopan, 2000). The freed Notch intracellular domain (NICD) enters the nucleus, where it switches a DNA-bound protein (CSL) associated with a co-repressor complex into an activating complex, leading to expression of target genes. In mammals, four Notch genes (*Notch1* to *Notch4*) and five ligands [Jagged 1 (Jag1), Jag2, Delta-like (Dll) 1, Dll3 and Dll4] have been identified.

Notch signaling plays a role in mammalian kidney development, but the precise cellular steps that require Notch signaling are still obscure. The permanent functional kidney, the metanephros, begins to form in the mouse at ~11 days post coitum (dpc) when a collection of mesenchymal cells (the metanephric blastema) induces an outgrowth from the Wolffian duct called the ureteric bud, which invades the blastema (Saxen and Sariola, 1987). The ureteric epithelium then induces adjacent mesenchymal cells to condense and to epithelialize to form the renal vesicle (Schedl and Hastie, 2000). Concomitantly, the mesenchymal derivatives induce branching and growth of the ureteric bud. This process of reciprocal induction continues at the periphery to form new nephrons and new ureteric bud branches in the mouse until 1-2 weeks after birth, resulting in ~15,000 nephrons per kidney.

The renal vesicle differentiates and alters its morphology to form comma- and then S-shaped bodies, from which arise the nephrons that consist of glomerulus, proximal tubule and distal tubule. The ends of the distal tubules fuse with the inducing

branches of the ureteric bud, which give rise to the collecting ducts. Each nephron segment expresses different proteins that serve as both cell fate and functional markers. The glomerular podocytes, together with endothelial cells, establish the glomerular filter between the blood stream and the urinary space.

McCright and colleagues (McCright et al., 2001) have investigated the role of Notch2 in mammalian kidney development. *Notch2* mRNA was detected in comma-shaped bodies and in tubules, including podocyte precursors. In addition, ureteric bud branching and the mesenchyme-to-epithelial transition appeared normal in mice homozygous for a hypomorphic *Notch2* mutant allele. However, these mice developed hypoplastic kidneys with glomerular defects. As the initial formation of nephron precursors was normal in the mutant kidneys, Notch2 may only be essential for glomerulogenesis. Alternatively, redundancy with other Notch molecules may compensate for the deficiency in earlier Notch2 functions. For example, *Notch1* mRNA is detected in the renal epithelial component of the mouse metanephros (Weinmaster et al., 1991; Weinmaster et al., 1992), and Notch3 protein is present in the developing mouse kidney (data not shown). The ligands Dll1 and Jag1 were detected in the comma- and S-shaped bodies, and Jag1 expression persisted in the center of the developing glomerulus (Beckers et al., 1999; McCright et al., 2001). Therefore, it is likely that the Notch signaling pathway plays a broad role in mammalian kidney development.

Ligand binding to Notch triggers shedding of its extracellular domain by a metalloprotease (Brou et al., 2000; Lieber et al., 2002; Mumm et al., 2000). Subsequently, γ-secretase-dependent proteolysis within the transmembrane domain releases NICD (De Strooper et al., 1999; Huppert et al., 2000; Schroeter et al., 1998). All four Notch molecules undergo intramembrane proteolysis (Mizutani et al., 2001; Saxena et al., 2001). To investigate the role of Notch signaling in kidney development without the problems associated with early embryonic lethality (Gridley, 1997) and functional redundancy, we combined a pharmacological approach with metanephric organ culture (Rogers et al., 1991; Saxen and Lehtonen, 1987). By culturing metanephroi in the presence of the γ-secretase inhibitor DAPT (Dovey et al., 2001), we have been able to block all γ-secretase activity – and therefore all Notch signaling – and record the consequences for kidney development. Similar pharmacological studies with fetal thymus organ cultures (Doerfler et al., 2001; Hadland et al., 2001) or whole animals (Geling et al., 2002; Micchelli et al., 2003) were extremely informative and demonstrated specificity for Notch signaling by inhibition of γ-secretase with no general toxicity. Moreover, by transient application of DAPT at different time points, this method has allowed us to define precise temporal dependence on γ-secretase. These studies have revealed an absolute requirement for γ-secretase in the transition from primitive epithelia to proximal nephron; missing are proximal tubules and podocytes. Thus, Notch signaling appears to be involved in patterning the proximal-distal axis of the nephron during metanephrogenesis.

Materials and methods

Metanephric organ culture

Mouse metanephric organ cultures were performed as described by Rogers et al. (Rogers et al., 1991). Briefly, kidneys were removed from 11.5, 12.5 or 14.5 days post-coitum (dpc) CD1 mouse embryos and cultured on transwell filters (Falcon, pore size 1 μm) at an air-fluid interface in a serum-free medium consisting of equal volumes of Dulbecco's modified Eagle medium and Ham's F12 medium containing 25 mM HEPES, sodium bicarbonate (1.1 mg/ml), 10 nM $Na_2SeO_3.5H_2O$, 10^{-11} M prostaglandin E1, and iron-saturated transferrin (5 μg/ml). From each embryo, one kidney was cultured with DMSO (1 μl/ml), and the other with DAPT in DMSO (at a final concentration of 0.5 or 1 μM in the medium). Both concentrations were equal to 100 times the IC_{50}, which was either 5 nM or 10 nM, depending on two different DAPT batches. In the recovery experiments, both kidneys were initially cultured with DMSO plus DAPT. After a specified interval (2, 3 or 3.5 days), one of the kidneys was cultured in media containing DMSO only. The other was continuously fed with DAPT-containing media. In all cases, the medium was refreshed twice a day to maintain the inhibition by DAPT. We followed and photographed the daily growth of each individual cultured metanephros under a Nikon TMS inverted microscope equipped with a digital camera.

Ret activation assay

Ret-expressing Neuro2a cells stably transfected with GFRα1-FLAG were pre-treated (1 hour) with DMSO (1 μl/ml) or 1 μM DAPT in serum-free media followed by stimulation with 40 ng/ml Gdnf in the presence of DMSO or DAPT for the times indicated. Proteins were extracted for SDS-PAGE and blotted as described (Tansey et al., 2000). Membranes were probed with rabbit polyclonal antibody specific for phospho-p42/p44 (Thr202/Tyr204) from Cell Signaling Technology (a division of New England Biolabs). Phospho-Ret PY905, PY1062 antibodies were kindly provided by Drs Brian Tsui-Pierchala and Eugene Johnson (Tsui-Pierchala et al., 2002).

Immunohistochemistry

The metanephroi or the embryos were fixed in Bouin's fixative or in 4% paraformaldehyde (for DBA, LTL, or laminin α1 staining), embedded in paraffin wax and sectioned at 7 μm. The sections were boiled in Trilogy (Cell Marque) for antigen retrieval. The antibodies, the lectins and their dilutions were as follows: WT1 (1:100, Santa Cruz), Pax2 (1:300; Covance), Ncam (1:300, Sigma), E-cadherin (1:150; Transduction Labs), cytokeratin 8 (TROMA1; 1:10; Developmental Studies Hybridoma Bank), rat anti-mouse laminin α1 (1:1000; clone 8B3, kindly provided by Dale Abrahamson, Kansas City, KS) (Abrahamson et al., 1989), rabbit anti-mouse cadherin 6 (1:300; kindly provided by Dr Dressler (Cho et al., 1998), Jag1 (1:150; Santa Cruz), and FITC-conjugated DBA and LTL (1:100; Vector Laboratories). Hoechst (0.5 μg/ml, Sigma) was used for nuclear staining. Fluorescein- and Cy3-conjugated anti-IgG corresponding to the species of the primary antibodies were used to visualize the antigen in Fig. 5K,L and Fig. 8B, biotinylated anti-mouse IgG and avidin-AMCA (vector) were used to detect E-cadherin.

Owing to the low abundance of activated Notch1 in metanephroi, a modified method was used to detect the epitope. After incubation of the primary antibody Val1744 (1:500, Cell Signaling Technology, a division of New England Biolabs), the sections were treated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1000; Jackson ImmunoResearch), tyramide-conjugated FITC (NEN, PerkinElmer; 1:1000), and then HRP-conjugated anti-fluorescein antibody. The antigen was then visualized with tyramide-conjugated Cy3 (Cy3 Plus, NEN; 1:100). The localization of the activated Notch1 displayed in Fig. 1 was performed on sections of an E14.5 embryo incubated with Val1744 (1:200, NEB), followed by a biotinylated anti-rabbit IgG (1:4000). After treatment with the avidin/biotinylated HRP complex (Vector) followed by the amplification reagent biotinyl-tyramide (1:50; NEN), the sections were incubated with streptavidin-HRP (1:100; NEN) and developed using the substrate DAB (Pierce).

For whole-mount antibody staining, metanephroi were fixed in 4%

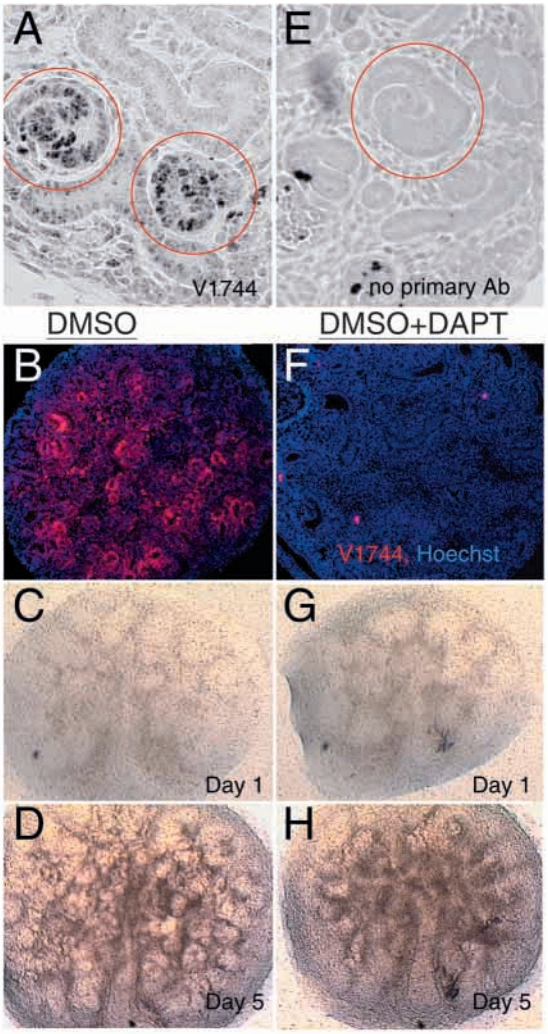

Fig. 1. Inhibition of γ-secretase in cultured metanephroi eliminates Notch activation and reduces tissue complexity. (A,E) Activated Notch1, detected with an antibody against the antigen generated by γ-secretase (VLLS) (Schroeter et al., 1998), is present in the nucleus of S- and comma-shaped bodies (red circles) in 14.5 dpc embryonic kidney. (E) Control with no primary antibody. (B-D) Metanephroi cultured in DMSO-containing media or (F-H) 1 μM DAPT-containing media. NICD is present in control (B) but is undetectable after 5 days in DAPT (F). After the first day of culture, phase contrast images of control (C) and DAPT-treated metanephroi (G) are indistinguishable. After 5 days, the control metanephros is filled with high contrast structures (D), while the DAPT-treated contralateral metanephros has branches but less tissue complexity (H).

paraformaldehyde, washed in PBS, and incubated with PBST (PBS plus 0.1% Tween-20) for 1 hour at room temperature. For DBA staining, the specimen was directly incubated with FITC-DBA (1:200) for 1 hour, and washed in PBST. For WT1 detection, the specimen was boiled in CT (10 mM sodium citrate, pH 6.0, 0.1% Tween-20) for 10 minutes, and then incubated in the blocking solution (MABT:100 mM maleic acid pH 7.5, 150 mM NaCl, 0.1% Tween-20; plus 2% Blocking Reagent; Boehringer Mannheim) for 1 hour before adding WT1 antibody (1:100) for overnight incubation at room temperature. After extensive wash in MABT, the specimen was incubated with the Cy3-conjugated anti-rabbit IgG (1:1000). After this step, FITC-DBA could be added for double staining.

Quantification of branching morphogenesis

Focused, full-framed day 5 DBA whole-mount stained images were imported into Adobe PhotoShop 5 for layout. To obtain the number of the branch tips, the outermost branched tips pointing toward the margin of the specimen were counted. Using the software's measure tool, the diameter of each marginal branch tip at its widest was determined. The result was presented as mean±s.e.m. for DMSO (n=61) and DAPT groups (n=29). Tracing back from the tip to the main collecting duct, each end-branch could be assigned as the result of a bifurcation number (one specimen in DMSO group, n=29; two specimens in DAPT group, n=29). The result was presented as the frequency distribution of the bifurcation number for both groups. Student's t-test was used to compare the difference, and $P<0.01$ was considered to be statistically significant.

Results

Activated Notch1 is detectable in comma- and S-shaped bodies

γ-Secretase cleavage of Notch1 generates a novel NICD N-terminal epitope, n-VLLS. The presence of this epitope serves as a unique marker for Notch1 activation. We therefore used a commercial antibody against this epitope (Val1744) to identify cells that have experienced Notch activation during embryonic development. In 14.5 dpc mouse embryos, the strongest NICD signal outside the CNS was detected in the embryonic kidney. NICD was detected in the comma and S-shaped bodies (Fig. 1A), where *Notch2* mRNA is also present (McCright et al., 2001). This indicates that Notch signaling probably contributes to the early stages of nephrogenesis.

Inhibition of γ-secretase with DAPT prevents Notch activation

To study the role of Notch signaling in early mammalian kidney development, we treated cultured mouse metanephroi with DAPT in order to inhibit γ-secretase activity and therefore block all Notch signaling. As controls, we cultured the contralateral kidney in the presence of the solvent used, dimethyl sulfoxide (DMSO). NICD immunoreactivity (Fig. 1B) disappeared when DAPT was present (Fig. 1F). This demonstrates that DAPT penetration of cultured metanephroi was sufficient to completely block Notch proteolysis.

DAPT attenuates branching morphogenesis without affecting the Gdnf-Ret pathway

After 3 days in DAPT, fewer epithelial structures were present compared to the untreated contralateral side, and this became more obvious after 5 days (compare Fig. 1C,D with 1G-H). The missing epithelial structures could result from defects in ureteric bud branching, mesenchyme-to-epithelium transition, epithelial growth and differentiation, or a combination of these. To analyze the effects of γ-secretase inhibition on branching morphogenesis, we used fluorescein-conjugated *Dolichos biflorus* agglutinin (DBA), which labels the ureteric buds and their derivatives, collecting ducts (Laitinen et al., 1987). After 2 days of culture, there was no discernible difference in branching. However, after 3 days, the treated metanephroi had reduced arborization and more dilated end-branches (Fig. 2). We used three parameters to quantify the changes in several 5-day-old specimens: the number of bifurcations, the number of branch tips at the periphery and the average diameter of branch tips (see Materials and methods). The distribution of

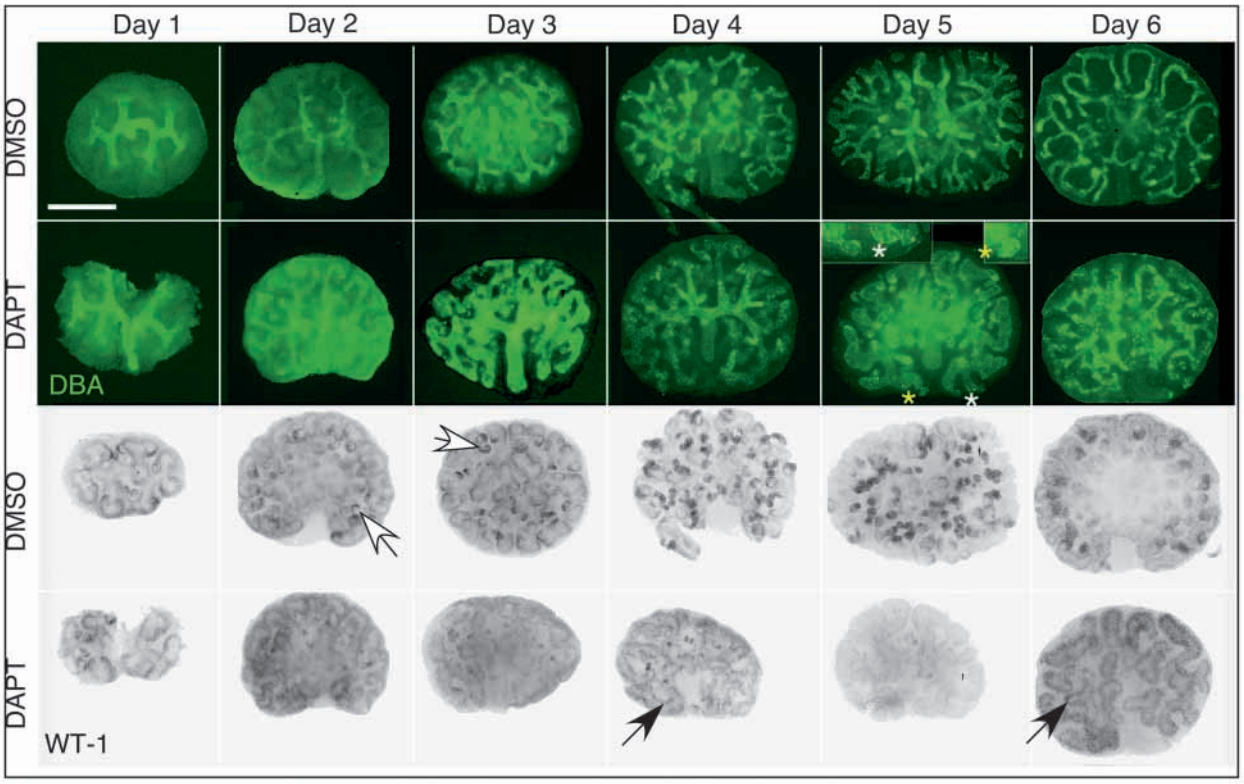

Fig. 2. Whole-mount images of cultured 12.5 dpc metanephroi. Top two rows, DBA stain (ureteric bud/collecting ducts). Bottom two rows, Wt1 stain. In both cases representative metanephroi are shown. The treatment and time in culture are indicated in the figure. With DAPT, less extensive branching is evident after 3 days in culture. Ducts appear dilated, and the epithelium at the tip of a few end branches formed bubble-like structures (day 5, asterisk and insets). Wt1 staining reveals fewer $Wt1^{HIGH}$ structures (white arrows) and more $Wt1^{LOW}$ cells (black arrows) in DAPT-treated metanephroi from day 2 onwards. Scale bar: 0.5 mm.

branch bifurcations between the two growth conditions was significantly different (P<0.01): control metanephroi had ~10 on average, and no branches had fewer than six branch points (9.8±0.3, n=29); DAPT-treated ones had at most eight branch points (7.1±0.3, n=29; Fig. 3A). As a consequence, controls contained twice as many branch tips at the periphery (32 or 29 in DMSO versus 14 or 15 in DAPT). Control branch tips appeared finer (46.3±1.6 μm, n=61, versus 80.0±3.3 μm, n=29; P<0.01) with a constant diameter. By contrast, the end-branches of DAPT-treated metanephroi were larger and irregular in diameter and had longer segments lacking ramification compared to untreated metanephroi. Thus, DAPT retarded branching. In addition, small cone-shaped protrusions emerged out of branch shafts (not shown), and the epithelium at the tip of a few end branches pinched to form bubble-like structures (Fig. 2, inset). These may represent incomplete or aborted branching or ectopic branch points.

Next, we wanted to examine if Gdnf/Ret signaling, essential for branching (Ehrenfels et al., 1999), was impacted nonspecifically by the dose of DAPT used in our study. As shown in Fig. 3B, neither Gdnf-induced Ret tyrosine phosphorylation (at the autocatalytic tyrosine Tyr905 and at

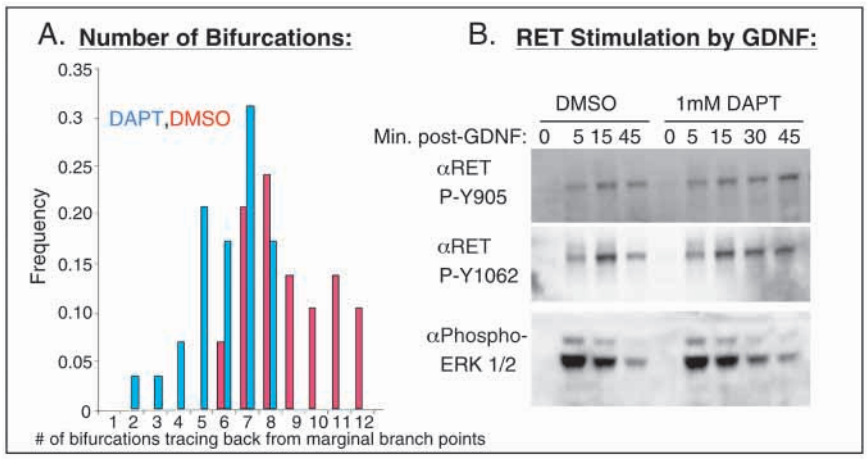

Fig. 3. (A) Distribution of bifurcations in 5 day DMSO- and DAPT-treated metanephroi. DBA-stained metanephroi were examined as described in the Materials and methods, and the frequency of branches with the given number of bifurcations was determined. Note the shift to fewer bifurcations in DAPT. This is not due to loss of Ret signaling, because even in the presence of DAPT, Ret was activated in response to 40 ng/ml Gdnf, as judged by Ret tyrosine phosphorylation (at the autocatalytic tyrosine Tyr905 and at Tyr1062) and subsequent activation of the downstream MAPK pathway (B).

Tyr1062) (De Vita et al., 2000; Grimm et al., 2001; Hayashi et al., 2000; Hayashi et al., 2001) nor activation of the downstream MAPK pathway was inhibited by 1 μM DAPT in cultured cells.

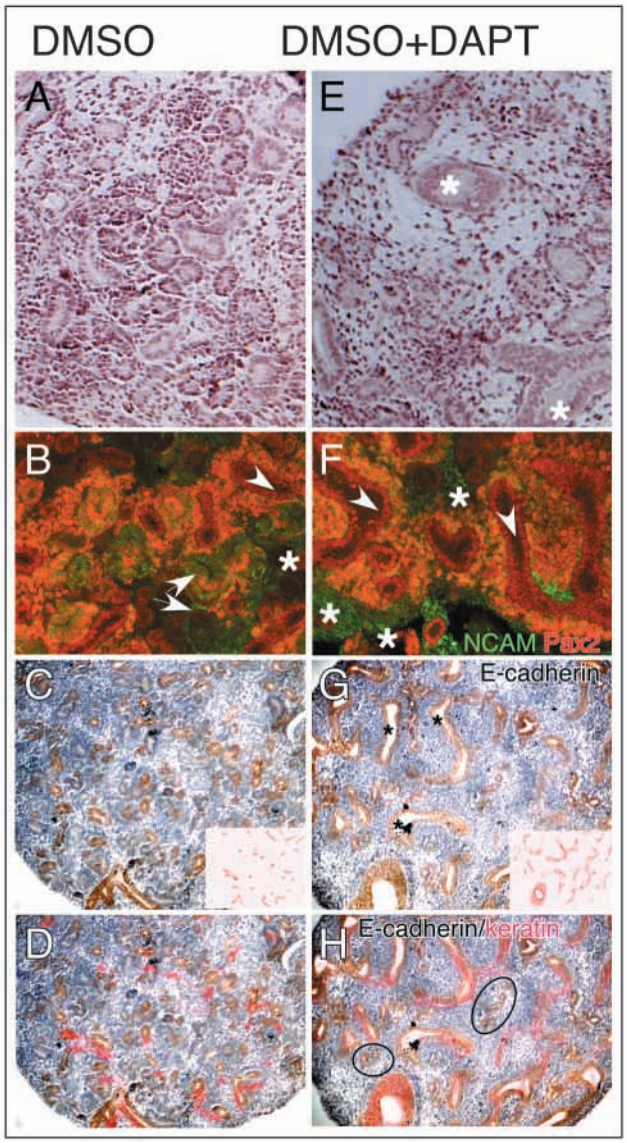

Fig. 4. Fewer epithelial structures in DAPT-treated metanephroi. Hematoxylin and Eosin-stained sections of metanephroi cultured for 5 days in DMSO (A) or DAPT (E). Fewer renal tubular epithelia and more interstitial cells are seen in the DAPT-treated metanephroi. Note dilated collecting ducts in the DAPT-treated metanephroi (asterisks in E and G). (B-D,F-H) Expression of several epithelial cell markers. (B,F) Ncam (green) and Pax2 (red). Pax2 only: ductal cells (arrowheads). Pax2 + Ncam: condensing mesenchyme (orange). Ncam only: epithelial cells (B, arrows) or uninduced mesenchyme (asterisks). (C,D,G,H) Expression of E-cadherin (brown), an epithelial marker present in ducts and enriched in distal tubules, on Hematoxylin counterstained (purple) slides. Insets in C and G: detection of cytokeratin 8 (red), a ductal marker, in sections adjacent to C and G. These images were overlaid using Canvas (Deneba software) on C and G to create images D and H. E-cadherin was highly expressed in both collecting duct and nephron epithelia. Fewer non-ductal epithelia (i.e. cytokeratin 8 negative) developed in DAPT-treated metanephroi (circled, H).

DAPT diminishes, but does not completely abolish, the mesenchymal-epithelial transition

Hematoxylin and Eosin stained sections of metanephroi cultured for 5 days revealed fewer renal tubular epithelia and more interstitial cells in the DAPT-treated metanephroi (Fig. 4), consistent with the overall morphology (Fig. 1). Few, if any, comma-shaped and S-shaped bodies or glomeruli were visible in DAPT-treated samples. A slight increase in activated caspase 3 staining was observed at the periphery of DAPT-treated metanephroi, consistent with the possibility that cell death was increased (data not shown). As seen in the DBA whole-mount stains, dilated collecting ducts were found in the DAPT-treated metanephroi (asterisks in Fig. 4E,G). To identify epithelial cells accurately, we analyzed the expression of several epithelial cell markers. Neuronal cell adhesion molecule (Ncam, green) is expressed in uninduced and condensed mesenchyme and in comma and S-shaped bodies, and then it is lost (Klein et al., 1988; Nouwen et al., 1993). The same sections were stained for Pax2 (red in Fig. 4B,F), a paired domain-containing transcription factor expressed in the condensing mesenchyme, the newly formed renal epithelia and the collecting ducts, but not in the uninduced mesenchyme or in the podocytes (Dressler et al., 1990; Patterson and Dressler, 1994). Pax2 staining was conducted on adjacent section of most panels (not shown). Ducts (Pax2 positive, Ncam negative; Fig. 4B,F, arrowheads) in both control and treated metanephroi were surrounded by condensing mesenchymal cells positive for both Ncam and Pax2. Strikingly, fewer organized epithelial structures lacking Pax2 but expressing Ncam (Fig. 4B, arrow, inset) were detected in DAPT-treated metanephroi compared with control. Instead, increased abundance of Pax2-negative, Ncam-positive cells were present in DAPT-treated metanephroi (Fig. 4F, asterisks). Ncam-positive cells closer to the tips of the ureteric bud were likely to have received inductive signals, and therefore proceeded to express the marker Pax2, identifying them as condensing mesenchyme. However, many of these cells failed to epithelialize (Fig. 4E,F). This indicates that γ-secretase inhibition did not block mesenchymal condensation, but attenuated the transition to epithelium.

To investigate further the degree of epithelialization, the expression of E-cadherin, an epithelial marker present in collecting ducts and enriched in distal tubules (Cho et al., 1998), was examined. To differentiate between ducts and distal tubules, we stained the adjacent sections with the cytokeratin 8 antibody, a marker of collecting ducts (J.H.M., unpublished; Fig. 4) (Hemmi and Mori, 1991). E-cadherin was highly expressed in the control metanephroi in both collecting ducts and nephron epithelia (Fig. 4C,D, brown). In DAPT-treated metanephroi, E-cadherin expression was found mainly in collecting ducts (Fig. 4G-H). The staining pattern indicated that some non-ductal epithelia did develop in DAPT-treated metanephroi (circled, Fig. 4H). These data again demonstrate the paucity of non-ductal epithelia in the absence of Notch signaling.

γ-Secretase activity is required for the differentiation of the proximal epithelial derivatives

Next, we sought to determine to what extent γ-secretase inhibition prevented differentiation of primitive renal epithelia into mature nephron segments. To identify additional differentiated epithelial cells, we first examined the expression

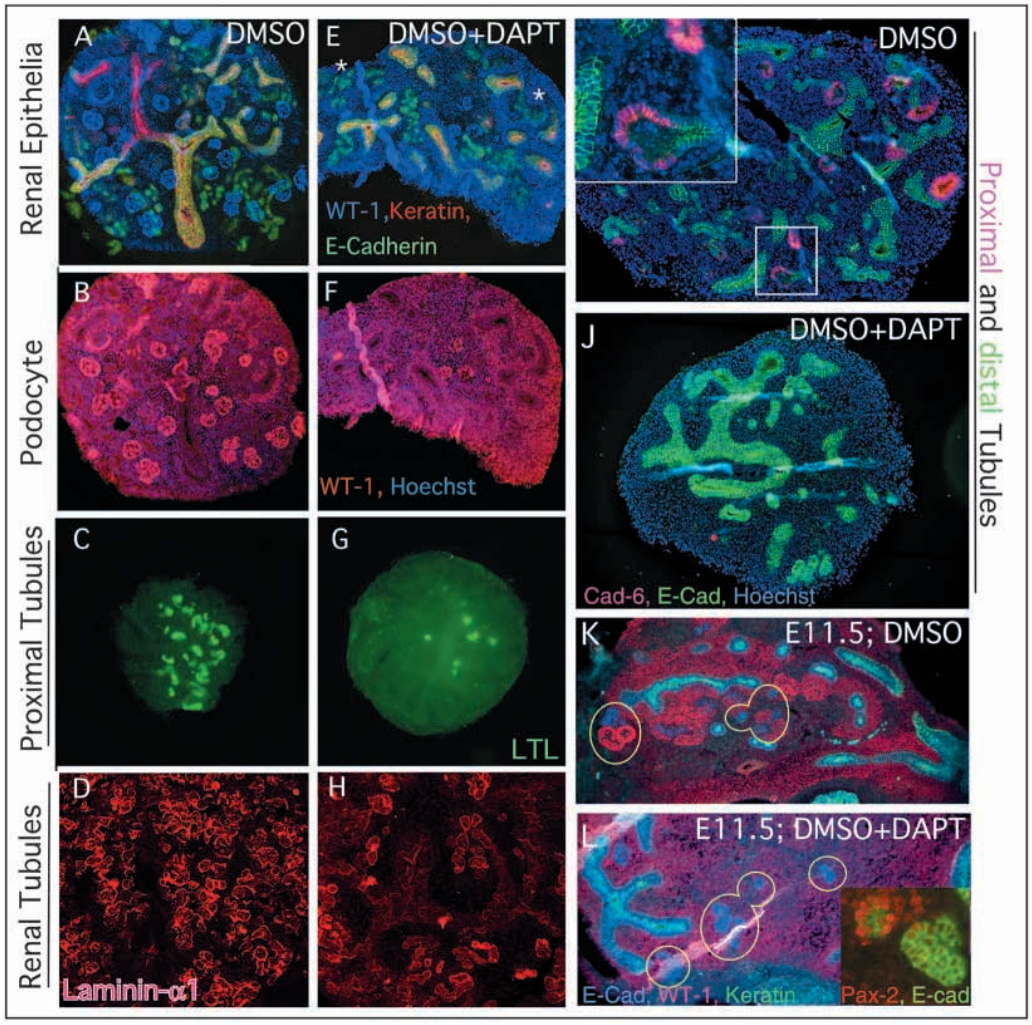

Fig. 5. Proximal epithelial structures are missing in DAPT-treated metanephroi. (A,E) E-cadherin (green), cytokeratin 8 (red) and Wt1 (blue, overlay of A on B and E on F with Canvas). Distal tubules (green only) formed in DAPT-treated metanephroi throughout the section (E, asterisk). (B,F): $Wt1^{HIGH}$ (glomerular podocytes) and $Wt1^{LOW}$ (induced/condensed mesenchyme and primitive epithelia) are detected in both DMSO- and DAPT-treated metanephroi. Only a few podocytes and well-defined glomeruli are found in the center of DAPT-treated metanephroi, and an increase in $Wt1^{LOW}$ cells is also evident (F). (C,G) Detection of proximal tubules with *Lotus tetragonolobus* Lectin (LTL, green). Note the overall reduction in the number and the central location of proximal epithelia in DAPT-treated metanephroi. (D,H) Laminin α1 (red) was detectable in the basal laminae of tubules in both control and treated metanephroi; fewer renal tubules were detected in DAPT-treated cultures. (I,J) Proximal tubules are abundant in untreated metanephroi (cadherin 6, pink, inset in I) but are missing in DAPT-treated metanephroi (J). Distal tubules (E-cadherin, green) are present in both. (K,L) De novo formation of epithelia occurs in DAPT. Distal tubules (circled in K and L) but no podocytes ($Wt1^{HIGH}$, K) are present in 11.5 day metanephroi cultured for 6 days in DAPT. (Inset in L) Pax2 (red), expressed in induced mesenchyme, persists in newly formed epithelia at the periphery of DAPT treated cultures (stained with E-cadherin; green). A more central epithelial cluster loses staining of Pax2.

of the Wilms' tumor protein Wt1, a zinc-finger transcription factor that is expressed at a low level in uninduced and induced metanephric mesenchyme, in condensing mesenchyme, and in early epithelia, but is strongly upregulated in glomerular podocytes (Schedl and Hastie, 2000). Metanephroi were first examined for global up-regulation of Wt1 expression by whole-mount antibody staining (Fig. 2). After 1 day in culture, the expression of Wt1 was mainly detected in the region surrounding the bud tips (Fig. 2). At that time point, DAPT-treated metanephroi showed a similar expression domain at comparable levels. After 3 days in culture, elevated Wt1 expression appeared in podocyte clusters within developing glomeruli (arrows, Fig. 2). As with branching, differences in podocyte/glomerulus formation became obvious with every successive day in culture: in the control samples, numerous intensely positive Wt1 ($Wt1^{HIGH}$) glomeruli could be seen (Fig. 2, Fig. 7A), but few such structures were found in the treated samples. Their numbers did not increase after day 3, and they were always located near the center (Fig. 2, Fig. 7B). In both the treated and untreated samples, weak Wt1 expression ($Wt1^{LOW}$) could be detected in the region surrounding the collecting ducts each day of culture (Fig. 2). We further examined Wt1 expression in cross-sections of cultured metanephroi. In the control, the $Wt1^{HIGH}$ podocytes formed aggregates throughout the tissue, including the periphery (Fig. 5A-B, Fig. 7E). In DAPT-treated metanephroi, only few podocytes and well-defined glomeruli formed, primarily in the center (Fig. 5E-F, Fig. 7F). This is in contrast to E-cadherin-positive, cytokeratin 8-negative (mesenchyme-derived) epithelia that formed in DAPT-treated metanephroi throughout the section, including the periphery. This result implies that new renal epithelia are still forming at the periphery even in the presence of DAPT (Fig. 5B,F, asterisk, podocyte overlay in blue, see below), but they are unable to give rise to podocytes.

Next, we analyzed the expression of several proximal tubular markers: *Lotus tetragonolobus* lectin (LTL, exclusive for mature proximal tubules) (Laitinen et al., 1987), cadherin 6, another marker expressed exclusively in proximal tubule precursors (Cho et al., 1998) and laminin α1 (enriched in mature proximal tubules but also deposited in other epithelial basal laminae) (Abrahamson et al., 1989; Sorokin et al., 1997).

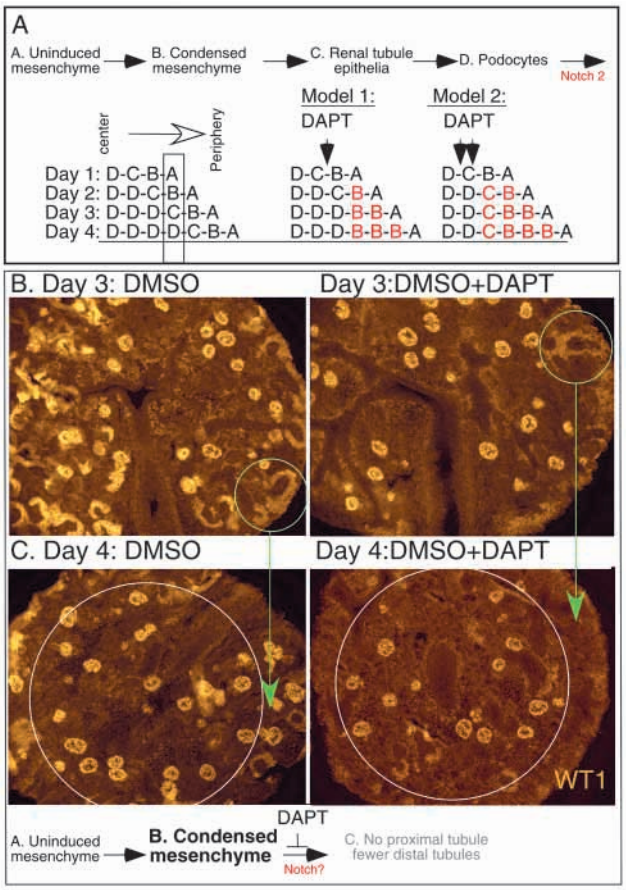

Fig. 6. (A) Hypothetical responses of metanephroi to DAPT. At 14.5 dpc, the metanephros contains mature structures in the center (D, podocytes) and immature ones at the periphery (A, uninduced mesenchyme). The peripheral tissue progressively differentiates (vertical box), flanked with mesenchymal stem cells at more peripheral positions. If DAPT only blocks the induction but not differentiation of proximal epithelia (model 1), induced epithelia can still progress to podocytes. If DAPT also blocks podocyte differentiation (model 2), the deficiency in podocytes ($Wt1^{HIGH}$ cells) will manifest earlier, at day 3 (D-D-D-B-B-A in model 1; D-D-B-B-B-A in model 2). After 3 days in culture, there is no appreciable difference in formation of podocyte/glomeruli near the periphery between the control and DAPT-treated cultures (green circles in B), compatible with model 1. (C) Twenty-four hours later, peripheral regions (green arrows in C) have produced mature $Wt1^{HIGH}$ cells in DMSO but not in DAPT. We therefore assign the DAPT-sensitive step at the formation of proximal renal epithelia, with a possible secondary effect reducing the number of distal epithelia. The affected cell population is the one containing NICD.

Very few LTL-positive cells were seen in whole-mounts after 5 days of DAPT treatment. As with $Wt1^{HIGH}$ cells, the LTL-positive epithelia were only detected near the center, indicating an inhibition of the formation of new mature proximal tubules in the absence of Notch signaling (Fig. 5G,H). Laminin α1 was used to detect renal tubules; staining was detectable in the basal laminae of renal tubules in both control and treated metanephroi (Fig. 5D,H), demonstrating an overall reduction in the number of renal epithelia. Cadherin 6 was abundantly expressed in the control metanephros [Fig. 5I; a junction between the proximal (cadherin-6-positive) and distal (E-cadherin-positive) epithelium is shown in the inset]. Compatible with the above findings, there was no detectable cadherin 6 in the DAPT-treated kidney (Fig. 5J). DAPT-treated metanephroi thus contained most of their proximal tubules and glomeruli in the middle of the organ.

Finally, we analyzed the E-cadherin-positive, cytokeratin 8-negative (Figs 4, 5) epithelial structures we classified as distal tubules. We postulated these cells were derived from a population of $Wt1^{LOW}$/Pax2-positive cells at the periphery of DAPT-treated metanephroi (Pax2 staining not shown). However, the reduced numbers of these structures could indicate that a general block in epithelialization is imposed during DAPT treatment, and that the observed distal structures formed from renal epithelial cells pre-existing in the E12.5 metanephroi. If epithelia were able to form from mesenchyme in the presence of DAPT, we reasoned that they should express both E-cadherin and Pax2 shortly after epithelialization, while Pax2 would be downregulated as the epithelium matured (Klein et al., 1988; Patterson and Dressler, 1994). Indeed, we detected Pax2 in the nuclei of E-cadherin-positive, cytokeratin 8-negative epithelia (inset in Fig. 5L, periphery on the left), suggesting that they were newly formed from conversion of mesenchyme. A more definitive approach to clarify the origin of epithelia in the presence of DAPT was to harvest the metanephroi from E11.5 mouse embryos, before any renal epithelia are formed. The first T-shaped bifurcation of the ureteric bud formed after 1 day in culture (data not shown), indicating that renal epithelial cells could not have existed prior to tissue collection. After 6 days of culture with DAPT, E-cadherin-positive, cytokeratin 8-negative epithelia formed, demonstrating that mesenchymal-epithelial transition indeed occurs in the absence of γ-secretase activity (Fig. 5L). Similar to the experiment with E12.5 metanephroi, $Wt1^{HIGH}$ podocytes appeared only in the DMSO-treated control (Fig. 5K).

γ-Secretase activity is not essential for podocyte differentiation

In the section above we established that distal tubules form but proximal structures (podocytes, proximal tubules) are completely missing when DAPT is present during the induction process. However, in addition to this early requirement for γ-secretase activity, the defects in mice expressing a hypomorphic allele of Notch2 suggest that later differentiation events may also require this enzyme (McCright et al., 2001). To determine whether Notch signaling is directly involved in podocyte differentiation, we inhibited γ-secretase in older metanephroi, isolated at 14.5 dpc. At this age, the center of the tissue contains glomeruli, and less mature cells are present peripherally [Fig. 6A, from the center outwards: D, podocytes ($Wt1^{HIGH}$); C, comma and S-shaped bodies (Pax2, Ncam, E-cadherin, NICD, Jag1); B, condensed mesenchyme (Pax2, Ncam, $Wt1^{LOW}$); A, mesenchymal stem cells (Ncam, $Wt1^{LOW}$]. If only induction of proximal structures requires γ-secretase, the many S-shaped bodies that are present at 14.5 dpc should be able to form new glomeruli at peripheral positions in both treated and untreated metanephroi after 3 days in culture (the time it takes for $Wt1^{HIGH}$ cells to appear in 12.5 dpc metanephroi; Fig. 2). Only after 4 days in culture, a period of time sufficient to generate glomeruli from mesenchyme, should there be a difference (model 1, Fig. 6A). However, if Notch signaling is required not only for the induction of

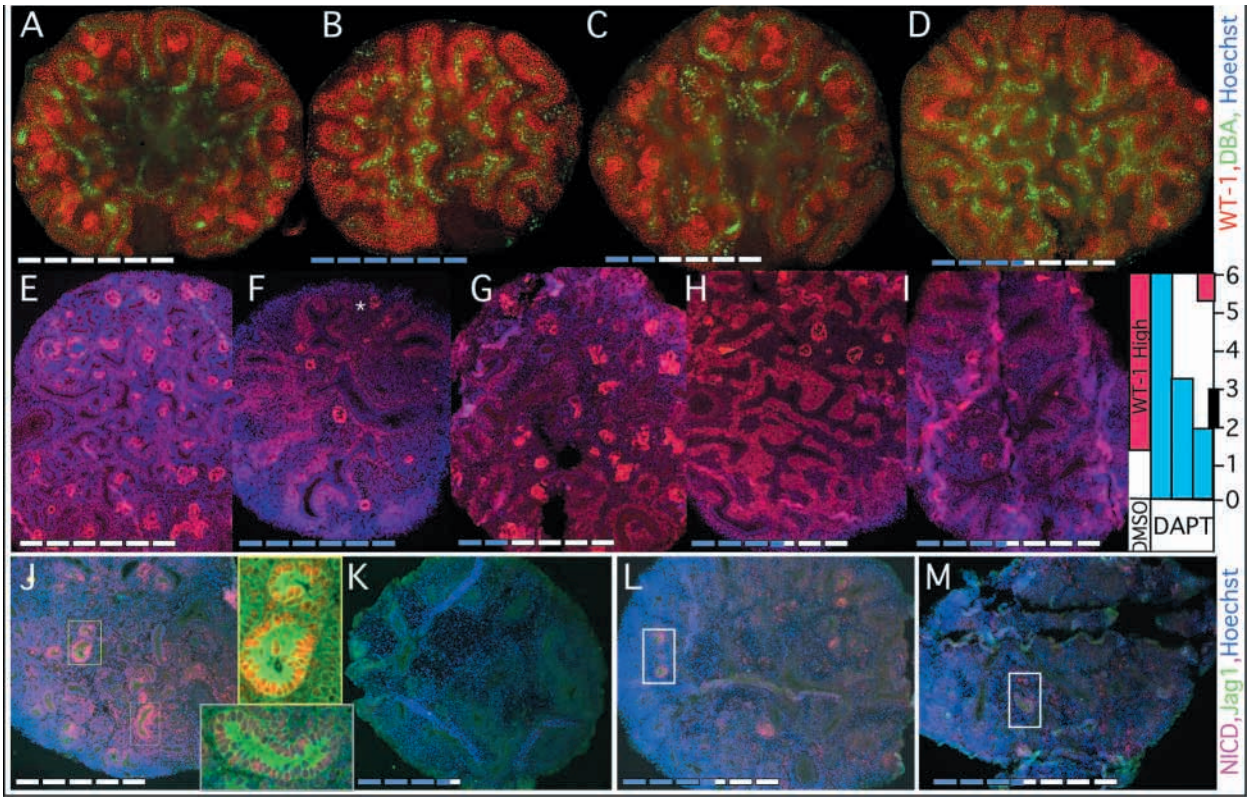

Fig. 7. Reversibility of DAPT treatment. (A-D) Whole-mount double staining of metanephroi with anti-Wt1 and DBA; (E-M) sections through metanephroi cultured under conditions similar to the wholemounts. The antigens detected are indicated on the right in the appropriate colors. In all sections, bars at the bottom reflect days in DMSO (white bars) and/or in DAPT (blue bars). Note that NICD and Jag1(J) reside in the same cells (insets and frames in J). Expression of both is inhibited by DAPT (K) but recovers after DAPT removal (frames in L,M). After a 2-day inhibition, $Wt1^{HIGH}$ cells are again detected throughout the metanephroi (C,G). After 3.5 days, despite recovery of NICD and Jag1 (K,L), no $Wt1^{HIGH}$ cells appear at the periphery, and only $Wt1^{LOW}$; Pax2-positive (not shown) cells increase in abundance, and branching resumes (D,H,I). We conclude that a crucial period exists between day 2 and 3 in DAPT, after which restoration of Notch signaling fails to rescue podocyte differentiation. This is summarized in I.

proximal structures but also for differentiation of proximal tubule cells and podocytes, new glomeruli will not be generated from pre-existing S-shaped bodies in DAPT-treated cultures. Hence, the $Wt1^{HIGH}$ glomeruli are expected to mainly exist in the center of the developing kidney at the 3-day point (model 2 in Fig. 6A). Consistent with Model 1, 3-day culture of 14.5 dpc metanephroi results in similar distributions of $Wt1^{HIGH}$ glomeruli throughout the organs in both control and DAPT-treated tissues (Fig. 6B). At day 4, the number of glomeruli at the periphery was markedly decreased in DAPT-treated metanephroi compared with the controls, and the glomeruli were now exclusively found in the center of the sections (Fig. 6C). These findings suggest that γ-secretase inhibition blocks the formation of proximal structures but not their differentiation once S-shaped epithelia form.

The differentiation potential of renal epithelial cells is irreversibly lost after prolonged inhibition of γ-secretase activity

We next wanted to determine whether renal epithelial cells retained the potential to differentiate after transient γ-secretase inhibition. A transient block in γ-secretase activity could result in accumulation of progenitors fully capable of further differentiation. Alternatively, γ-secretase activity (and therefore, Notch signaling) may be essential to retain competence for proximal fates. In its absence, nascent epithelia may lose their ability to respond to Notch signaling, or may even acquire alternative developmental potentials. To distinguish between these two possibilities, we designed a series of recovery experiments. First, 12.5 dpc metanephroi were kept for 2 days in DAPT, followed by regular culture media for 4 days (Fig. 7, blue bars indicate days in DAPT. Fig. 7C,G), while the contralateral metanephroi were maintained under continuous DAPT treatment (Fig. 7B,F). Four days after DAPT withdrawal, glomerulogenesis had resumed throughout the metanephroi (Fig. 7C,G), and the distribution of glomeruli was indistinguishable from that of untreated controls (Fig. 7A,E). By contrast, when metanephroi were kept for 3.5 days in DAPT and then allowed to recover for 3 or 4 days, we did not detect any increase in the numbers of $Wt1^{HIGH}$ glomeruli. However, with recovery there was a massive expansion of $Wt1^{LOW}$ cells (Fig. 7H,I) and extensive ureteric bud branching, as judged by DBA staining of wholemounts (compare Fig. 7B-D) and cross-sections (Fig. 7E-H).

To confirm that γ-secretase activity was restored, we assayed for activated Notch1, as above. Twelve hours and 36 hours after DAPT removal, no NICD was detected (Fig. 7K and data not shown); however, NICD immunoreactivity returned by 60

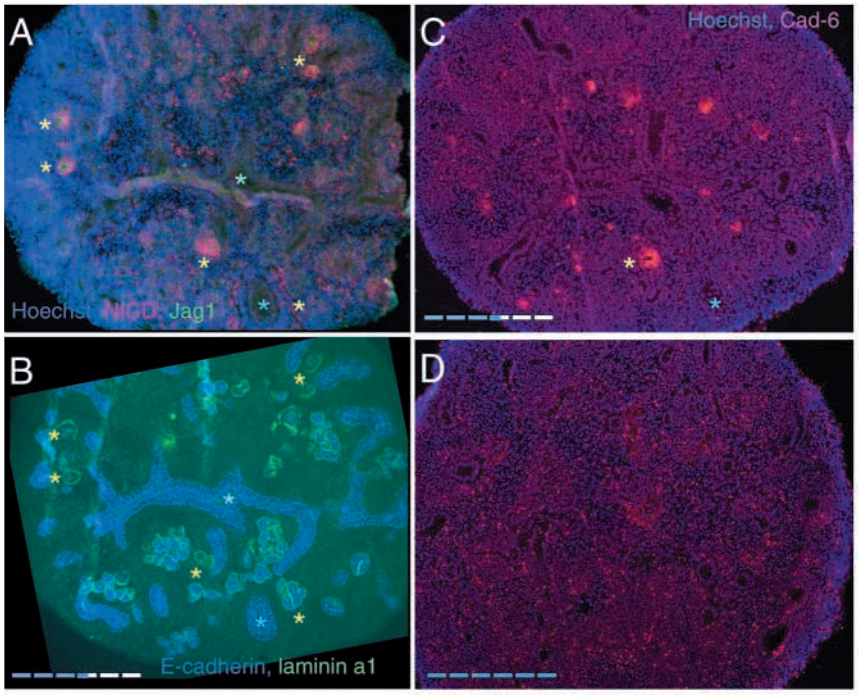

Fig. 8. (A,B) Proximal epithelia can form in the absence of podocytes after DAPT removal. The section shown in Fig. 7L is shown here (A) next to an adjacent section stained with laminin α1 (green) and E-cadherin (blue; B). The sections are aligned to the same orientation (ducts are marked with blue asterisks). NICD-positive (pink) cells are surrounded by laminin α1 and are E-cadherin^{LOW} (yellow asterisks). In addition to these proximal epithelial, laminin α1 and E-cadherin^{HIGH} distal epithelia are also detected. Ducts did not stain with laminin α1 in this experiment. (C) To confirm the proximal tubule identity, another section from the same metanephros was stained for cadherin 6. (D) Contralateral metanephros cultured continuously in DAPT contained no cadherin 6-positive cells. Bars indicate days with DAPT (blue) or DMSO (white).

hours (Fig. 7L). We could also detect a few comma- or S-shaped bodies that formed within 60 hours, and persisted after 84 hours following DAPT removal (NICD/Jag1-positive, Fig. 7J,L,M). This observation prompted us to investigate whether any proximal epithelial differentiation occurred. We therefore examined the expression of laminin α1 and E-cadherin. There were many laminin α1 and E-cadherin^{Low} double-stained epithelial structures after 3 or 4 days recovery following 3.5 days of DAPT treatment (Fig. 8A,B). These NICD/Jag1 structures could be newly formed proximal renal epithelia as they started to synthesize basement membrane, and showed a low level of E-cadherin. Indeed, cadherin 6-positive proximal tubules were detected in the same metanephros after 2.5 days without DAPT (Fig. 8C). We confirmed that NICD-expressing epithelia were also cadherin 6-positive (data not shown). Thus, after 2 days of DAPT treatment, cells retained the ability to choose all proximal fates, but after 3.5 days of DAPT treatment, cells lost the ability to form podocytes while retaining the ability to form proximal tubules.

Discussion

Several factors hamper a traditional genetic approach aimed at deciphering the role of Notch signaling either in vivo or in cultured mouse metanephroi. *Notch4* and *Notch3* mutant mice are viable, but mice homozygous for null *Notch1* or *Notch2* alleles (Conlon et al., 1995; Hamada et al., 1999; Swiatek et al., 1994) die between 9.5 and 11.5 dpc. *Notch1/Notch4* double mutants have a more severe phenotype than *Notch1* mutants alone (Krebs et al., 2000). Mice lacking RBP-Jk (Oka et al., 1995) or presenilins, the catalytic center of γ-secretase, die even earlier (Donoviel et al., 1999; Herreman et al., 1999). Conditional loss of single Notch proteins may not reveal an interesting phenotype because of redundancy. Genetic removal of presenilin 1 leads to increase in β-catenin caused by an axin-like secondary role as a priming scaffold (Kang et al., 1999; Xia et al., 2001); β-catenin is essential for kidney development (Kispert et al., 1998; Kispert et al., 1996; Vainio and Uusitalo, 2000). Both genetic and pharmacological block of γ-secretase activity could impact other γ-secretase substrates besides Notch and, in addition, pharmacological inhibition of γ-secretase may inhibit related enzymes such as signal peptide protease (SPP) (Lemberg and Martoglio, 2002; Weihofen et al., 2002). However, such inhibition does not affect β-catenin stability and thus provides a means to evaluate the global contribution of the Notch signaling pathway. Despite all these caveats, we can conclude that γ-secretase is clearly crucial for renal epithelial differentiation.

Pharmacological inhibition of Notch activation in cultured mouse metanephroi attenuates branching and reveals a crucial role in formation of proximal epithelial structures

We find that ureteric bud branching proceeded quite extensively even in the absence of γ-secretase activity. The difference in branching between the control and the treated kidneys was statistically significant but not dramatic. As γ-secretase also cleaves other Type I transmembrane proteins including Erbb4, a receptor tyrosine kinase (Lee et al., 2001; Ni et al., 2001), we examined the possibility that DAPT might directly affect the Gdnf-Ret pathway, a major regulator of the ureteric branching mediated through the receptor tyrosine kinase. Our data did not support such an effect. Collectively, our results indicate that although branching persists at a reduced rate, the inhibition of γ-secretase interrupts the tightly regulated association between growth and ramification (Davies and Davey, 1999), leading to an increase in shaft diameter without bifurcation. One possibility is that the branch-inducing cells are diminished in the treated metanephroi, or that their inductive potential is altered. Another possibility is that unknown γ-secretase substrate(s) in the ductal cells are directly affected.

Mature nephrons form in two steps: first, mesenchymal-epithelial transition (MET) is induced by the ureteric bud; second, segmental differentiation of the renal epithelium occurs to form the podocyte, the proximal tubule and the distal tubule, in that order (Saxen, 1987). Given that a general reduction in the number of renal epithelial cells (fewer E-cadherin-positive structures) was observed in all DAPT treated metanephroi, we considered the following four possibilities: (1) γ-secretase inhibition blocks MET; (2) cellular differentiation still occurs, but proper epithelialization fails; (3) inhibition of formation of the earlier cell type (podocytes) precludes differentiation of later cell types (proximal and distal epithelia); or (4) γ-secretase inhibition interrupts only the differentiation of a particular epithelial cell type. Our data using 11.5 dpc metanephroi, which did exhibit MET in the presence of DAPT (Fig. 5), argue against the first scenario. However, MET was significantly attenuated, and this may be due to the presence of unknown MET-promoting γ-secretase substrates in mesenchymal cells the activities of which are blocked by DAPT. In the second scenario, the few epithelial structures (or non-epithelial cells surrounding the ducts) should express differentiation markers of all cell types, but they do not. In the third scenario, all segment-specific markers will be lost, but they are not, as E-cadherin^{HIGH} distal tubules are present. In the last scenario, only some cell types would be lost, and that is what we found.

Interestingly, once formed, podocyte precursors were able to differentiate to WT1^{HIGH} cells even in the presence of DAPT, and this may seem inconsistent with the phenotype of mice homozygous for a hypomorphic *Notch2* allele (McCright et al., 2001). The Notch2-sensitive step discovered by McCright et al. during glomerulogenesis may require the vasculature or is reached at a stage not recapitulated in organ cultures. Notch3 is expressed in the podocytes, but *Notch3*-null mice are apparently normal, suggesting no or a minimal role in glomerular formation and function. A model for the role of Notch signaling in early metanephros development is presented in Fig. 6.

Prolonged inhibition of the γ-secretase activity may eliminate the differentiation potential of the renal epithelial cells

From our recovery experiments, we conclude that all epithelial cell types, including podocytes, can form after 2 days without γ-secretase activity (inset in Fig. 7I). If devoid of γ-secretase activity for more than 3 days, renal epithelial cells irreversibly lose their ability to form podocytes. Instead NICD-containing epithelial structures express low levels of E-cadherin, laminin α1 and cadherin 6, indicating that proximal tubules formed in the absence of podocytes (Fig. 8 and data not shown). Furthermore, the correlation between the resumption of branching and Notch activation suggests that NICD-positive epithelial cells contribute indirectly to branching.

Our finding that all cell types can form after 2 days inhibition, proximal and distal tubules form after 3 days inhibition, and only distal tubules form under continuous inhibition is intriguing. Based on the experiment in which mesenchyme was induced by exogenous spinal cord signals, Saxen (Saxen, 1987) suggested that the differentiation of a nephron is segmental and sequential (podocytes→proximal tubule→distal tubules). This suggested sequence was deduced from the observation that short induction time (<24 hours) produced predominantly glomeruli and proximal tubule but no detectable distal tubules. Our results demonstrate the inverse order of sensitivity to γ-secretase inhibition; γ-secretase activity, probably through Notch1, is required specifically for the proper induction of the proximal epithelial structures (but is not needed for the differentiation of podocytes once they have been induced, Fig. 6). We therefore can conclude that each segment is able to differentiate without signals from the cells proximal to it. Although differentiation of more proximal cells is not a prerequisite for the subsequent differentiation of the distal structures, it may still be required for maximal success in epithelialization in our experiments.

A related finding was reported earlier regarding the role of Notch in cell fate determination in vertebrate pronephros development (McLaughlin et al., 2000). In *Xenopus*, *Notch1* mRNA was first detectable in the developing pronephrotic anlage during early tail bud stages. During late tail bud stages, *Notch1* expression became restricted in the epithelial pronephric tubules. When Notch signaling was blocked by injecting the embryos with RNA encoding a dominant-negative CSL, the tubular cell fate was converted to the ductal fate. Notch is thus essential for selecting the type of renal epithelial cell both in the *Xenopus* pronephros and in the mouse metanephros.

Is Notch1 participating in lateral interaction within the metanephros? Overlap of Notch expression with its ligands has been noted in the kidney (Leimeister et al., 1999). The precise cellular colocalization of activated Notch1 with its ligand, Jag1 (Fig. 7J, inset, enlarged image in supplemental Fig. S1 at http://dev.biologists.org/supplemental/), and their uniform intensity is inconsistent with the salt-and-pepper pattern of NICD accumulation anticipated from a simple lateral inhibitory role for Notch signaling within the renal epithelium (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Bray, 1998). In addition, Jag1 expression may depend on γ-secretase and therefore on Notch; it reappears with NICD when DAPT is removed (Fig. 7K-M; H. Zheng and P. Wang have noted this as well, personal communication). This, too, is contrary to the predicted behavior of Notch ligand in laterally interacting cells (Wilkinson et al., 1994). Determination of the precise role of Notch1 in kidney development, whether the Wt1^{LOW}/Pax2-positive cells acquired novel properties or are simply expanding nephron progenitors, and how Jag1 expression is regulated are beyond the scope of this report.

Finally, our results have significant implications for treatment of Alzheimer's disease. As the presenilin-dependent γ-secretase processes the amyloid precursor peptide to generate fragments that are causally related to Alzheimer disease, γ-secretase is currently an important therapeutic target (Dovey et al., 2001). Although individuals with Alzheimer's disease need not worry about any drug effects on fetal development, the kidney is a major collateral target organ of disease processes and drug toxicity in the elderly. Preservation of its limited regenerative capacity is a very important clinical concern. Imgrund et al. (Imgrund et al., 1999) showed that *Pax2*, which is not normally expressed in adult tubules, was expressed in the regenerating proximal tubular epithelium following tubular necrosis induced by folic acid, implying that the kidney may recapitulate its developmental process to regenerate. If the role of γ-secretase (and Notch) during kidney regeneration is

similar to the early developmental process, γ-secretase inhibitors may prevent an injured kidney from regenerating properly.

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