

Presenilins are required for the formation of comma- and S-shaped bodies during nephrogenesis

Pei Wang^{1,2}, Fred A. Pereira^{1,2,3}, Duane Beasley¹ and Hui Zheng^{1,2,4,*}

¹Huffington Center on Aging, Baylor College of Medicine, Houston, TX 77030, USA

²Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

³Department of Otolaryngology, Baylor College of Medicine, Houston, TX 77030, USA

⁴Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

*Author for correspondence (e-mail: huiz@bcm.tmc.edu)

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Summary

Mammalian presenilins consist of two highly homologous proteins, PSEN1 and PSEN2, which share redundant activities in Notch processing and signaling. To bypass the early lethality of the *Psen1*- and *Psen2*-double (PSEN) null embryos, we used a human *PSENI* transgene to rescue the somite patterning defects in PSEN-null animals and to allow a determination of the function of presenilins in late embryogenesis. We report here that expression of the human *PSENI* transgene supported the survival of PSEN-null embryos to the perinatal stage. However, presenilin deficiency in the kidney led to severe nephrogenesis defects and virtually no comma- or S-shaped bodies, or mature glomeruli were formed. We document that the mesenchyme was induced which could further progress to renal vesicles in the PSEN-null kidney, indicating that the presenilins are not essential for the inductive interactions and mesenchyme to epithelium transition. However, renal vesicles failed

to pattern to form proximal tubules and glomerular epithelium. A presenilin-dependent, signaling-competent form of Notch1 was detected in mesenchymal derivatives but not in the ureteric buds of wild-type mice. Consistent with an obligatory role of presenilins in Notch processing and activation, the active form of Notch1 and its downstream target *Hesr1* were absent in the PSEN-null kidney. Importantly, sustained Notch1 signaling was required for the maintenance of Notch ligand *Jag1* expression. These results identify presenilins as one determinant of renal vesicle patterning in the developing mouse kidney, and we hypothesize that they act through the Notch signaling pathway.

Key words: Presenilin, Notch, Kidney, Nephrogenesis, Patterning, Proximal tubule

Introduction

Presenilins (PSEN1 and PSEN2) are polytopic transmembrane proteins that are essential to process the β -amyloid precursor protein (APP) at the γ -secretase site to generate A β 40 and A β 42, peptides that constitute major components of the β -amyloid plaques characteristic of Alzheimer's disease (AD) (De Strooper et al., 1998; Selkoe, 1998). Autosomal dominant inheritance of mutations in *PSEN1* and *PSEN2* leads to familial Alzheimer's disease and these mutations are known to affect the γ -secretase activity and to foster the β -amyloid plaque pathology (Selkoe, 1998). Through similar mechanisms, presenilins are required for the proteolytic cleavage of Notch (De Strooper et al., 1999), molecules that are crucial in various cell-fate specification processes, and this activity is highly conserved (Levitan et al., 1996; Levitan and Greenwald, 1995; Struhl and Greenwald, 1999; Ye et al., 1999). In mammalian systems, the two presenilins play compensatory roles in the Notch signaling pathway, as revealed by gene knockout studies. Specifically, *Psen1*-null mice die pre- or perinatally, exhibiting a Notch1-associated somite segmentation defect (Shen et al., 1997; Wong et al., 1997). Although the *Psen2* knockout does not have overt impairment, *Psen1*- and *Psen2*-double (PSEN) null embryos die at embryonic day 9.5 (E9.5)

with complete absence of somite patterning, which resembles a complete *Notch1* loss-of-function, or more precisely, *Notch1*- and *Notch4*-double knockout phenotypes (Conlon et al., 1995; Donoviel et al., 1999; Herreman et al., 1999; Krebs et al., 2000). The redundant activities of PSEN1 and PSEN2 may be the explanation for the lack of overt organogenesis defects in each of the single knockouts. The early lethal phenotype of the PSEN-null embryos prevents the evaluation of potential physiological roles of presenilins in mid-to-late embryogenesis.

Organogenesis is a highly regulated developmental program involving cell-fate decisions, pattern formation and differentiation. In mammals, organogenesis initiates after the body axes are established. Kidney organogenesis in mice begins at E11 and involves reciprocal inductive interactions between the ureteric bud epithelium and metanephric mesenchyme. The tips of the ureteric bud induce nephrogenic mesenchyme to form condensates followed by pretubular aggregates, which then undergo mesenchyme to epithelium transition (MET) to progress into polarized epithelia of renal vesicles and comma- and S-shaped bodies (Saxen, 1987). Morphogenesis and patterning of the epithelial structures then leads to the formation of distal and proximal tubules and the

glomerulus; the latter consists of podocytes, mesangial cells and endothelial cells (Saxen, 1987).

A number of ductal- and mesenchymal-derived molecules have been identified and loss-of-function studies have established their crucial roles in nephrogenesis (<http://golgi.ana.ed.ac.uk/kidhome.html>). Relevant to this study, *Ret* is expressed at the tips of the newly formed branches of the ureteric bud and is crucial for the inductive interactions through binding to its ligand GDNF, which resides in the condensing mesenchyme (Moore et al., 1996; Pachnis et al., 1993; Schuchardt et al., 1994). The paired box genes *Pax2* and *Pax8* are expressed in various mesenchymal derivatives and ureteric bud epithelium (Dressler et al., 1993; Plachov et al., 1990; Rothenpieler and Dressler, 1993), and together they regulate and define the nephric lineage (Bouchard et al., 2002). *Wnt4* has been documented as an essential signaling molecule from the metanephric mesenchyme for the mesenchymal to epithelial cell conversion (Kispert et al., 1998; Stark et al., 1994). *WT1* is expressed in both uninduced mesenchyme and podocyte precursor cells of S-shaped bodies and mature glomeruli. It is required in the earliest phase of kidney formation (Armstrong et al., 1993; Kreidberg et al., 1993). The winged helix transcription factor *BF2* is specifically expressed in the interstitial stromal mesenchyme and has been shown to modulate the transition of condensed mesenchyme into tubular epithelium (Hatini et al., 1996).

The Notch pathway has been implicated in kidney development. Specifically, *Notch1* and *Notch2* and their ligands *Delta-like1* (*Dll1*) and *Jag1* are expressed in the maturing nephron and glomerulus (Beckers et al., 1999; McCright et al., 2001; Weinmaster et al., 1991; Weinmaster et al., 1992). In the *Xenopus* pronephros, Notch signaling has been proposed to be involved in both the early determination of duct versus tubule fate as well as in controlling tubule patterning (McLaughlin et al., 2000). Furthermore, mice with a hypomorphic expression of *Notch2* exhibit a kidney glomerulogenesis defect (McCright et al., 2001).

We reported earlier that restricted expression of a human *PSEN1* transgene in the developing brain and vertebral/spinal column driven by the human *Thy-1* promoter could rescue the *Psen1*-null patterning defects and lethal phenotype (Qian et al., 1998). To bypass the early lethality of the PSEN null embryos, we bred the transgene onto the *Psen1*- and *Psen2*-double knockout background. We showed that expression of the human *PSEN1* transgene supported the survival of the PSEN-null embryos to the perinatal stage. However, loss of presenilins resulted in profound nephrogenesis defects prior to development of comma- and S-shaped bodies. We provide evidence that presenilins play an important role, probably through the Notch signaling pathway, in the patterning of renal epithelial structures.

Materials and methods

Breeding and genotyping of PSEN rescue mice

The generation and genotyping of *Psen1*- and *Psen2*-null and human *PSEN1* transgenic mice have previously been described (Donoviel et al., 1999; Qian et al., 1998; Wong et al., 1997). The breeding strategy is shown in Fig. 1A. The mice analyzed in this study were on a mixed genetic background of three strains (129, C57 and SJL). The day when the vaginal plug was observed was considered to be embryonic day

(E) 0.5. Embryos with at least one wild-type allele of *Psen1* gene were used as littermate controls.

Embryonic histology

Embryos were dissected and fixed in 10% neutral buffered formalin (Sigma) for 24–48 hours and dehydrated through graded alcohols and stored in 70% ethanol at 4°C. Embryos were vacuum-embedded in paraffin wax, sectioned at 5 µm and stained with Hematoxylin and Eosin.

In situ hybridization

In situ hybridization was carried out using digoxigenin-labeled or [³⁵S]UTP-labeled antisense riboprobes. Samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections were cut at 5 µm for radioactive probes and at 9 µm for nonradioactive probes. The protocol used for radioactive in situ was essentially as published (Qiu et al., 1994). For non-radioactive in situ, a modified protocol of Wilkinson was employed (Wilkinson, 1992). In brief, sections were dewaxed and dehydrated, then fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature. After washing with PBS, sections were treated with proteinase K (20 µg/ml) for 10 minutes, refixed with PFA and washed with PBS. Samples were acetylated for 20 minutes, washed and hybridized (in 50% formamide, 20 mM Tris-HCl (pH 8.0), 500 µg/ml tRNA, 1×Denhardt's solution, 10% dextran sulfate, 300 mM NaCl, 10 mM NaPO₄ (pH 8.0), 5 mM EDTA) with 200 ng probe/slide overnight at 65°C in a humidified chamber. Sections were washed for 40 minutes with 0.5×SSC/20% formamide at 60°C followed by treatment with RNase for 30 minutes at 37°C, and washed again for 20 minutes with 0.5×SSC/20% formamide at 60°C. Sections were blocked with blocking solution (100 mM maleic acid, 150 mM NaCl, 1% Boehringer blocking agent) for 1 hour at room temperature and incubated with alkaline phosphatase-conjugated sheep polyclonal antidigoxigenin antibody (Roche) diluted 1:2000 in TBS buffer overnight at 4°C. After washing twice with TBS, alkaline phosphatase activity was detected in the presence of NBT/BCIP (Roche).

Immunohistochemical staining

PSEN-null and the littermate control kidneys were dissected at various developmental stages as specified in the figure legends. Immunohistochemical staining was performed in frozen (laminin α1, gift from D. Abrahamson) or paraffin-fixed sections (all others). Sections were blocked with 5% goat serum, incubated with primary antibodies at 4°C overnight, washed in PBS, incubated with 1/1000 Alexa Fluor-488 or Alexa Fluor-594 conjugated secondary antibody (Molecular Probe) for 1 hour at room temperature, washed in PBS, mounted in glycerol/PBS. Digital images were obtained with a Zeiss microscope (Axioskop 2). The primary antibodies used were Laminin (Sigma, L9393), E-cadherin (Signal Transduction, C20820), WT1 (Santa Cruz, sc192), cleaved Notch1 (val1744) (Cell Signaling, 2421), cleaved caspase 3 (Asp175) (Cell Signaling, 9661), NCAM monoclonal antibody, developed by T. Jessell, and TROMA1 monoclonal antibody, developed by P. Brulet, were obtained from the Developmental Studies Hybridoma Bank.

Embryonic proliferation and apoptosis

Antibody to phosphorylated histone H3 (Upstate Biochemicals) was used to detect mitotic cells (Wei et al., 1999). TUNEL assay was performed as described (Gavrieli et al., 1992). All sections were stained with methyl green to identify nucleated cells. Four-hundred to 600 cells per section were counted and the proportion of mitotic or apoptotic cells was determined as a fraction of the total number of nucleated cells, four kidneys/stage/genotype, five sections/kidney were counted.

Kidney organ culture and immunohistochemistry

Embryonic day 12.5 kidneys were isolated and cultured in MEM/F12 containing 10% fetal bovine serum on Millicell culture plate insert

(Millipore) at the medium/gas interface for 3 days. Kidneys were fixed in 2% paraformaldehyde for 10 minutes, then in 95% methanol for 15 minutes and washed in PBST (phosphate-buffered saline 0.1 M, pH 7.4, 0.1% Tween 20). They were stained whole-mount with anti-pancytokeratin (Sigma, C2562) and WT1 (Santa Cruz, sc192) antibodies at 4°C overnight, washed in PBS, incubated with 1/1000 Alexa Fluor-488 or Alexa Fluor-594 conjugated secondary antibody (Molecular Probe) for 2 hours at room temperature, washed in PBS, mounted in glycerol/PBS and viewed under a Zeiss confocal microscope (LSM510).

Results

Morphological and histological characterization of presenilin rescue mice

To generate mice carrying the human *PSEN1* transgene on the endogenous mouse *Psen1*- and *Psen2*-double knockout (*Psen1*^{-/-}*Psen2*^{-/-}*PSEN1*, or presenilin rescue) background, we bred mice that were heterozygous for mouse *Psen1* and positive for *PSEN1* (*Psen1*^{+/-}*PSEN1*, line 16-4) (Qian et al., 1998) with *Psen2*-null (*Psen2*^{-/-}) (Donoviel et al., 1999) to produce offspring that were compound heterozygous for endogenous presenilins and positive for *PSEN1* (*Psen1*^{+/-}*Psen2*^{-/-}*PSEN1*). These mice were then crossed twice with animals heterozygous for *Psen1* and homozygous for *Psen2* (*Psen1*^{+/-}*Psen2*^{-/-}) to yield the desired mutant genotype (*Psen1*^{-/-}*Psen2*^{-/-}*PSEN1*) (Fig. 1A).

We reported earlier that expression of the *PSEN1* transgene was able to rescue the somite patterning defect of the *Psen1*-null mice (Qian et al., 1998). Similarly, such transgene expression restored the somite structures that were completely absent in *PSEN1*-null embryos (Fig. 1B) (Donoviel et al., 1999). Presenilin rescue mutants were indistinguishable when compared with their littermate controls prior to E12.5. At E13.5, the mutants were overtly similar to their littermates, although they were slightly smaller in size, and this difference became more dramatic over time (Fig. 1B). The mutants could be unambiguously identified because their eyes lacked pigmentation, a phenotype that is the subject of a separate study.

The presenilin rescue mutants died within 2 hours of birth. Examination of the organs revealed that, at P0, the kidneys were dramatically smaller than that of the littermate controls (Fig. 1C), suggesting a defect in kidney development. Western blot analysis showed that the *PSEN1* protein could be readily detected in control kidneys (Fig. 1D, lane 1,2). However, as it was not detectable in *Psen1*^{-/-}*Psen2*^{-/-}*PSEN1* rescue mutant (Fig. 1D, lane 3), it suggests that the defect is due to the loss of presenilins and the mutant kidney is herein referred as *PSEN1*-null.

Histological analysis at various stages of kidney development revealed that, at E12.5, the ureteric buds were readily identified and were surrounded by condensed mesenchyme in both the control and the *PSEN1*-null kidneys (Fig. 2A,B, asterisks). This result suggests that the inductive interactions between the ureteric bud epithelium and the metanephric mesenchyme proceeded normally in the absence of presenilins. However, at E13.5, while the kidney development was further advanced in the control, as evidenced by the appearance of pretubular aggregates/renal vesicles (distinguished upon further analysis, see Fig. 4) and comma- and S-shaped bodies (Fig. 2C, thin arrow and arrowheads

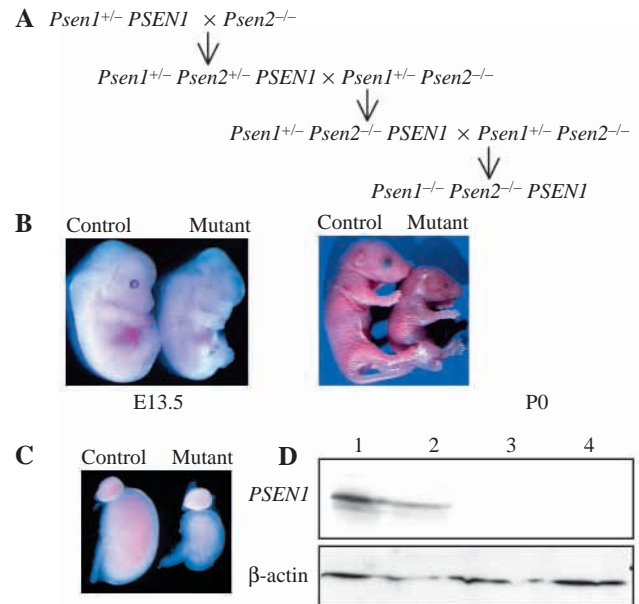


Fig. 1. Generation, morphological and biochemical characterization of presenilin rescue mice. (A) Mice carrying a single copy of the human *PSEN1* transgene onto endogenous mouse *Psen1*- and *Psen2*-double knockout (*Psen1*^{-/-}*Psen2*^{-/-}*PSEN1*, or presenilin rescue) background were produced by three generations of breeding as outlined. (B) Whole-mount photographs of presenilin rescue embryos (mutant) along with their littermates (control) at E13.5 (left) and P0 (right). (C) Dramatically reduced kidney size in presenilin rescue (mutant) compared with that of a littermate control (control). (D) Western blot analysis of *PSEN1* expression of P0 kidneys from control (lanes 1 and 2), presenilin rescue mutant (lane 3) and *PSEN1*-null (lane 4), documenting the absence of *PSEN1* expression in the presenilin rescue kidney. Lower panel is hybridization with an anti- β -actin antibody as a loading control.

respectively), only pretubular aggregates/renal vesicles (Fig. 2D, thin arrow) could be found in the *PSEN1*-null kidney but no comma- and S-shaped bodies were identified. This result indicates that presenilins play a critical role in the progression of pretubular aggregates/renal vesicles towards comma- and S-shaped bodies during nephrogenesis. Analysis of kidneys at later stages (E15.5 and P0) supports this view as mature glomeruli, which could be readily detected in the controls (Fig. 2E,G, thick arrows), were completely absent in the *PSEN1*-null kidneys (Fig. 2F,H). The same kidney defect is present in another *PSEN1* rescue line (17-3) (data not shown), and we therefore conclude that the phenotype is the result of the loss of presenilin expression rather than transgene integration.

Analysis of inductive interactions

As kidney organogenesis involves reciprocal inductive interactions between the ureteric bud epithelium and the metanephric mesenchyme, we performed in situ hybridization of various ductal and mesenchymal markers to delineate the origin of the defect. Analysis of *Gdnf* and *Bmp7* showed that both molecules were expressed in *PSEN1*-null kidneys similar to their littermate controls (data not shown), suggesting that the mesenchymal stem cells were preserved. To investigate the inductive processes, we analyzed the expression of *Pax2*, *Pax8* and *Wnt4*. At E12.5, comparable levels of *Pax2* in

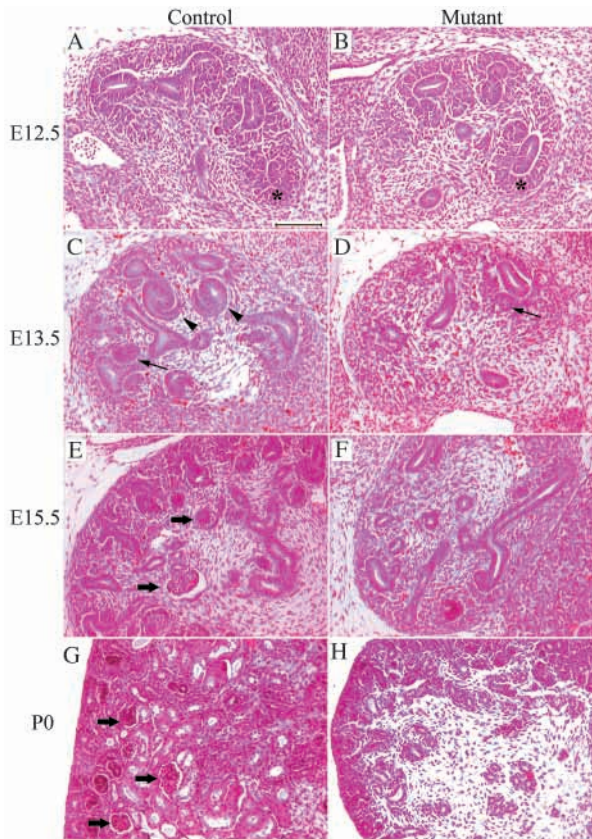


Fig. 2. Hematoxylin and Eosin analysis of kidney development. Right column, presenilin-null (Mutant); left column: littermate controls (control). At E12.5 (A,B), condensed mesenchyme (asterisks) surrounding ureteric bud could be identified in both PSEN-null mutant (B) and the control (A). At E13.5 (C,D), while pretubular aggregates (arrows) could be seen in both the control (C) and the mutant (D), further advanced structures, such as comma- and S-shaped bodies (arrowheads), could be detected only in the control but not in PSEN-null kidney. At E15.5 (E,F) and P0 (G,H), numerous glomeruli were formed in the control (E,G, arrows). These structures were absent in the mutant (F,H). Scale bar: 100 μ M.

mesenchymal condensates were observed in PSEN-null kidney and the control (Fig. 3A, compare a with b), but expressions of *Pax8* and *Wnt4* were undetectable regardless of the genotype at this stage (not shown). At E13.5, levels of *Pax2* (Fig. 3A, part f), *Wnt4* (Fig. 3B, part b) and *Pax8* (Fig. 3C, part b) were clearly detectable in mesenchymal derivatives, including aggregates (arrows), in PSEN-null kidney. However, there was reduced expression of these genes which is probably attributed to the lack of differentiation to comma- and S-shaped bodies (Dressler et al., 1990; Plachov et al., 1990; Stark et al., 1994). Examination of these molecules at E15.5 of development yielded similar results (data not shown).

Ret marks the tips of newly formed ureteric bud branches and its signaling is essential for inductive interactions (Pachnis et al., 1993). At E13.5, *Ret* expression was comparable in the control and the mutant (Fig. 3D, compare a with b), indicating that the primary branching was not affected by the loss of presenilins at this stage. As impaired nephrogenesis was unambiguously documented at this stage, this result suggests that the ureteric bud signaling is not the primary cause for the

defect. The reduced number of *Ret*-positive tips in the mutant at E15.5 (Fig. 3D, compare c with d) would thus probably be the result of impaired secondary or tertiary branching morphogenesis at this and later stages.

To exclude a possible contribution of stromal mesenchyme to the defects, we further evaluated stromal mesenchyme marker *Bf2* expression and its levels were not significantly different in the mutants and controls (data not shown). Therefore, the combined data suggest that loss of presenilins does not disrupt the early inductive interactions and that the nephrogenic phenotype in PSEN-null kidney is probably due to a defect intrinsic to the mesenchyme.

Analysis of mesenchymal to epithelial cell conversion

The presence of mesenchymal condensates and aggregates but absence of comma- and S-shaped bodies in PSEN-null kidney prompted us to examine the mesenchyme to epithelium transition (MET). The neuronal cell adhesion molecule (NCAM) is highly expressed only in mesenchymal derivatives, but not in the ureteric buds. NCAM-positive structures (green) were readily detected in both the control and PSEN-null kidney at E14.5 (Fig. 4A, parts a,b) and E16.5 (Fig. 4A, parts c,d). The majority of NCAM-positive structures were also positive for pan-laminin (Fig. 4A, red), which is found in the basement membranes of both ductal and renal epithelia (Cho et al., 1998). These data suggest that the mesenchymal derivatives were competent for epithelization.

The proper expression of laminin A chain has been shown to be crucial for epithelial polarization (Ekblom et al., 1990; Klein et al., 1988). Polarized, laminin α 1-positive renal vesicles were readily identifiable in PSEN-null kidney (Fig. 4Bb, arrow). The epithelial nature of these mesenchymal derivatives was further confirmed by their positive staining for E-cadherin (present in both ductal and renal epithelia, green) and negative for cytokeratin 8 (ductal epithelium only, red) (Fig. 4C). These results establish the notion that presenilins are not required for MET.

Analysis of nephron patterning

Detailed examination of NCAM-positive structures revealed that multiple types were identified, including laminin-low irregular aggregates (Fig. 4A, asterisks), laminin-positive organized renal vesicles with lumen (Fig. 4A, thin arrows), and elongated laminin-expressing tubules (Fig. 4A, part d, thick arrow), suggesting that the pretubular aggregates progressed into renal vesicles and tubules in PSEN-null mutant kidney. Consistent with this assessment, the E-cadherin-positive renal derivatives in PSEN-null mutant, similar to the littermate control, have formed immature tubular structures which were either in close proximity (Fig. 4C, part b, arrowhead) or appear to have connected with the duct (thick arrows in d and arrowhead in f, Fig. 4C). These characteristics suggest the formation of distal tubules. However, in contrast to the control in which advanced tubules with adjoining glomerular clefts could be recognized (Fig. 4C, part e), the mutant structures failed to undergo further differentiation and patterning.

To investigate a possible defect in proximal tubule development in the PSEN-null kidney, we performed staining with Lotus Tetragonolobus lectin (LTL), which is a specific marker for proximal tubules (Cho et al., 1998). Remarkably,

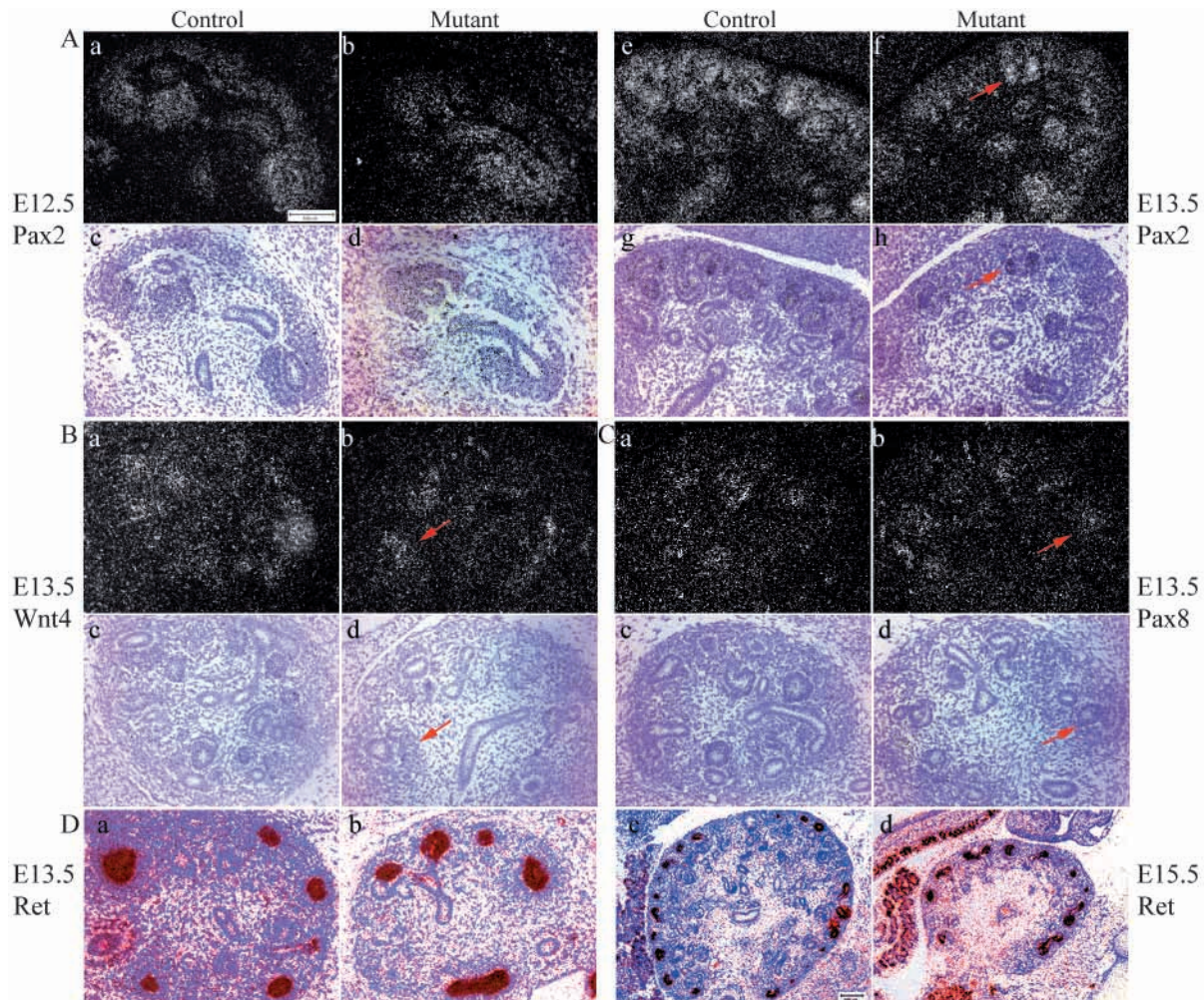


Fig. 3. In situ hybridization analysis of *Pax2* (A), *Wnt4* (B), *Pax8* (C) and *Ret* (D) expression. In A-C, the top panels are dark field and lower panels are corresponding bright field. (A) At E12.5, the levels of *Pax2* were comparable between the control (a,c) and PSEN-null (mutant) (b,d). At E13.5, reduced *Pax2* expression was observed in the mutant (f,h) compared with the control (e,g). This was also the case for *Wnt4* (B; compare b with a) and *Pax8* (C; compare b with a). Arrows indicate aggregate structures that are positive for all the markers. (D) At E13.5, *Ret*-positive structures were similar in the control (a) and the PSEN-null mutant (b). At E15.5, the number of *Ret*-positive tips was reduced in the mutant (d) when compared with the control (c). Scale bars: in A part a, 100 μ m for A-C and D, parts a,b; in D, part c, 100 μ m for D parts c,d.

although the control kidney showed strong staining with LTL (Fig. 5A, parts a,c), no positive staining could be detected with LTL in the mutant (Fig. 5A, part b,d). Immunostaining for WT1, which is highly expressed in the podocyte precursor cells of S-shaped bodies and mature glomeruli (Ryan et al., 1995), revealed a complete absence of WT1 expression in PSEN-null kidney as well (Fig. 5B). Thus, presenilins are absolutely required for the formation of proximal structures including proximal tubules and glomeruli.

To strengthen our *in vivo* findings, we next studied the kidney development in organ cultures. Control and PSEN-null kidneys were dissected from E12.5 embryos and cultured for 3 days. Whole-mount immunostaining with an anti-pan-cytokeratin antibody documented significant branching in both the control and PSEN-null kidneys after three days of culture (Fig. 5C), consistent with the notion that the presenilins do not exert an effect on primary branching morphogenesis. In agreement with our *in vivo* results, PSEN-null kidneys were devoid of WT1 (Fig. 5C,

part b). Close up examination of the ureteric bud tips showed that, in PSEN-null culture, weak pan-cytokeratin positive renal tubules were formed and at least some appeared to have fused with the ureteric bud, which were strongly positive for pan-cytokeratin (Fig. 5C, part d). However, compared with the control (Fig. 5C, part c), the PSEN-null renal derivatives were truncated and were negative for WT1 expression (Fig. 5C, part d). Similar results were also obtained when the control kidney cultures were treated with a presenilin γ -secretase inhibitor (data not shown). These *in vitro* organ culture experiments thus lend support for an essential role of presenilins in the patterning and differentiation of renal epithelial derivatives.

Effect of proliferation and apoptosis during nephrogenesis

Analysis of cells undergoing active division with an anti-phosphorylated histone H3 antibody showed that the cell proliferation profile was similar between the mutant kidney and

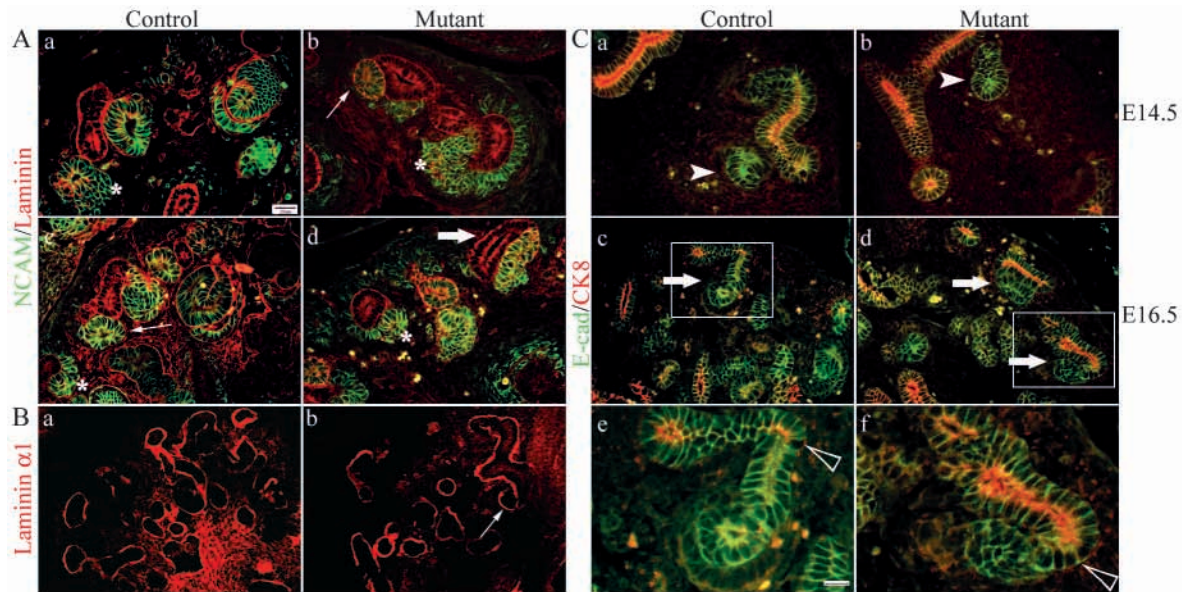


Fig. 4. Immunohistochemical characterization of renal derivatives. (A) Double staining with anti-NCAM (green) and anti-pan-laminin (red) antibodies at E14.5 (a,b) and E16.5 (c,d) of development. NCAM-positive, laminin-low (or negative) aggregates (asterisks) and NCAM- and laminin-positive renal vesicles (thin arrows) and tubules (thick arrow) were present in both the controls and PSEN-null (mutant). (B) E16.5 sections labeled with an anti-laminin $\alpha 1$ antibody, which identified polarized renal epithelium in PSEN-null mutant (b, thin arrow). (C) Double staining with anti-cytokeratin 8 (CK8, red, ductal only) and anti-E-cadherin (renal and ductal, green) antibodies. (a,b) E14.5. (c,d) E16.5. (e,f) Magnified view of highlighted structures in c,d, respectively. E-cadherin-positive, cytochrome 8-negative renal epithelial derivatives could be identified that were either in close proximity (white arrowheads) or appear to have connected with the ureteric bud terminals (thick arrows). Open arrowheads in e,f indicate site of fusion. Scale bars: in A, part a: 20 μm for A,B and C, parts a-d; in C, part e, 8 μm for C, parts e,f.

the control at both E13.5 (data not shown) and E15.5 (Fig. 6A, parts a,b). Double staining with the anti-NCAM (Fig. 6A, parts c,d) and cytochrome 8 antibodies (not shown) indicated that the proliferation rate of renal and ductal derivatives, respectively, were also similar. However, we cannot rule out the possibility that minor differences in cell proliferation in renal structures affected the tubule growth and patterning in presenilin mutant. This finding is not unexpected because much of the mesenchymal derivatives that required active cell division have already formed in the PSEN-null kidney.

Measurement of apoptosis using the TUNEL assay revealed that, at E13.5, the number of apoptotic cells was low in both the control and PSEN-null kidney, and no appreciable differences could be identified (data not shown). However, at E15.5, the number of apoptotic cells was significantly higher in the PSEN-null kidney when compared with the control (Fig. 6B, compare a with b). Quantitative analysis showed that only 0.93% of the cells was apoptotic in the control, while the percentage of apoptotic cells reached 6.34% in PSEN-null. The apoptotic cells were widespread with the majority localized to the peripheral mesenchyme (Fig. 6B, part b, arrowheads). The enhanced general apoptosis would contribute to the reduced kidney size in PSEN-null mutant.

Using co-immunostaining to detect the activated form of caspase 3 and NCAM (Fig. 6B, parts c,d), we were able to evaluate the apoptotic profile of the renal structures. Similar to the control, pretubular aggregates and renal vesicles in PSEN-null exhibited no caspase 3 activity. However, high caspase 3 staining was observed in NCAM-positive tubular structures in the PSEN-null kidney (Fig. 6Bd, arrow). Similar to the TUNEL staining, cortical mesenchyme also showed higher caspase 3

activation (Fig. 6B, part d, arrowheads). Overall, our results suggest that defective patterning of immature distal tubules in the absence of presenilins leads to the attenuation of nephrogenesis and enhanced apoptosis.

Analysis of Notch pathway molecules in kidney development

Because an impaired Notch signal transduction is considered the leading mechanism for the somite patterning defect of PSEN-null mice, we assessed the expression of Notch pathway molecules in E14.5 PSEN-null kidneys. First, we examined cell types exhibiting the presenilin γ -secretase activity by immunostaining with an antibody that recognizes a PSEN-cleaved and activated form of Notch1, the Notch intracellular domain (NICD) (Fig. 7A). Co-staining with an anti-cytokeratin 8 antibody (CK8) marked the ductal derivatives. NICD was not expressed above background in ureteric bud epithelium in both the control and PSEN-null samples (Fig. 7A). In the control kidney, NICD immunoreactivity could be detected in pretubular aggregates/renal vesicles (Fig. 7Aa), and comma- and S-shaped bodies (data not shown). PSEN-null kidney was devoid of NICD although pretubular aggregates were present (Fig. 7A, part b). Notch activation was correlated with expression of its downstream target *Hes1* (*Hey1* – Mouse Genome Informatics) (Kokubo et al., 1999) in the control (Fig. 7B, part a). By contrast, *Hes1* expression cannot be detected in PSEN-null mutant (Fig. 7Bb), consistent with an obligatory role of presenilins in Notch processing and signaling. The residual signal probably results from staining of blood vessels that also express *Hes1*. These results support the idea that a presenilin-mediated nephrogenic signal is derived from the

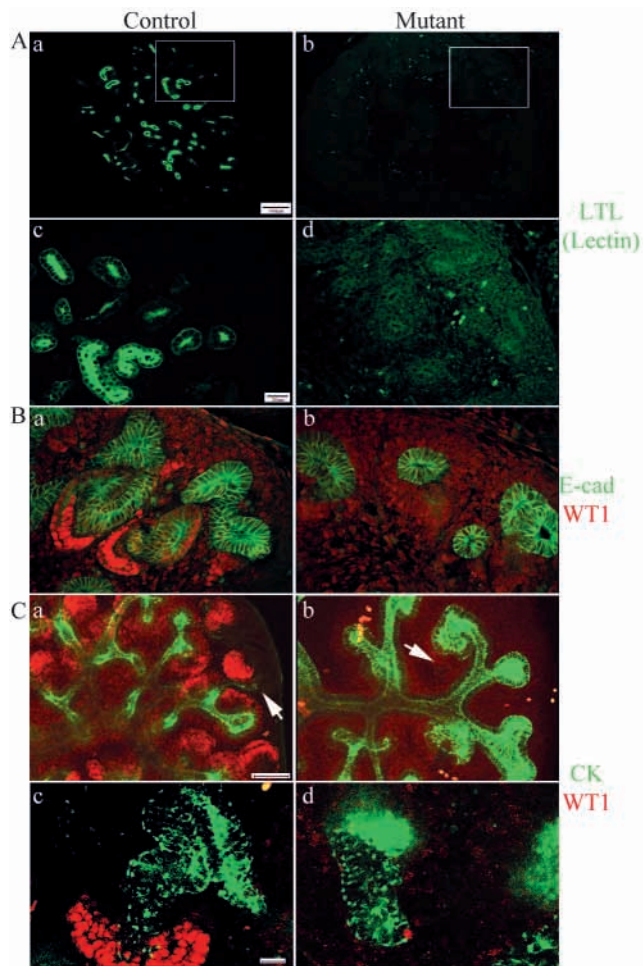


Fig. 5. Analysis of proximal derivatives. (A) Lotus Tetragonolobus lectin (LTL) staining revealed complete absence of mature proximal tubules in PSEN-null (mutant) (b,d). (c,d) Magnified view of highlighted structures in a,b, respectively. Residual positives in d probably result from background staining of blood vessels. (B) Double staining with anti-E-cadherin (green) and anti-WT1 (red) antibodies documented the lack of WT1-expressing podocyte precursors in PSEN-null kidney (b). (C) Whole-mount immunostaining of kidney organ cultures with anti-pan-cytokeratin (green) and anti-WT1 (red) antibodies. (c,d) Magnified view of highlighted structures in a,b, respectively, revealing the connection of weak-cytokeratin positive, truncated renal tubules with strong cytokeatin positive ureteric bud terminal in PSEN-null kidney culture (d). Scale bars: in A, parts a,b, 100 μ m; in A, parts c,d and B, 20 μ m; in C, parts a,b, 50 μ m; in C, parts c,d, 10 μ m.

mesenchyme, and that presenilin-dependent Notch activation and signaling is the mechanism in operation.

We next investigated Notch ligands *Jag1* and *Dll1* expression by in situ hybridization. At E14.5, *Jag1* was not expressed in condensed mesenchyme (Fig. 7C, part a,b, asterisks), but its expression was turned on in pretubular aggregates in both the PSEN-null kidney and the control (Fig. 7C, part a,b, arrows) and in comma-shaped bodies of the control kidney (Fig. 7C, part a, arrowhead). Interestingly, at E16.5, although *Jag1* was strongly expressed in the control kidney (Fig. 7C, part c, arrowhead), its expression was almost undetectable in the mutant (Fig. 7C, part d). The loss of *Jag1*

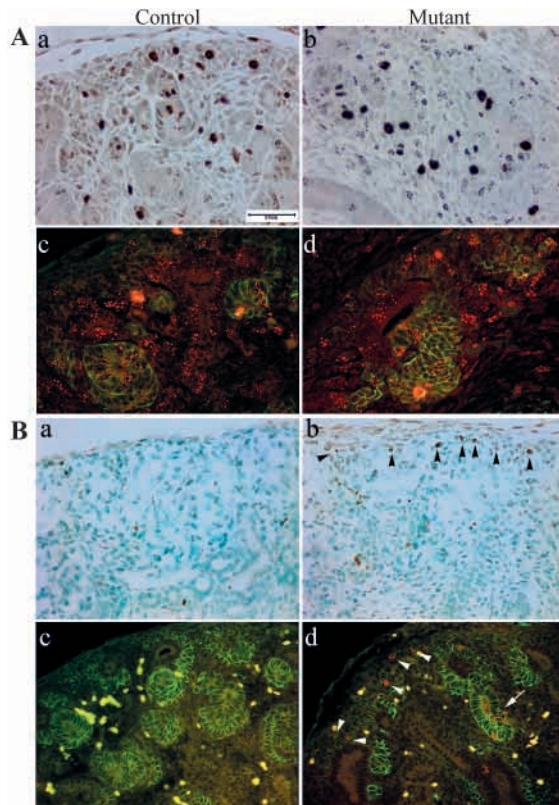


Fig. 6. Cell proliferation and apoptosis analysis. Left column, littermate controls; right column, presenilin-null. (A) Anti-phosphorylated histone H3 was used to stain for mitotic cells (a,b). Double staining with anti-phosphorylated histone H3 (red) and anti-NCAM (green) antibodies labeled the renal derivatives undergoing active division (c,d). (B) TUNEL assay revealed that the percentage of apoptotic cells (arrowheads) was increased dramatically in the mutant (b) when compared with the control (a). (c,d) Double staining with anti-cleaved caspase 3 (red) and anti-NCAM antibodies (green) showed increased apoptosis in NCAM-positive renal derivatives (arrow) as well as in cortical mesenchyme (arrowheads). The yellow dots are auto-fluorescence of the blood. Scale bar: 50 μ m.

is not caused by the absence of mesenchymal derivatives as NCAM-positive pretubular aggregates and renal vesicles could be readily identified in PSEN-null at this stage (Fig. 4A, part d). Therefore, this result suggests that Notch signaling is required for the maintenance of *Jag1* expression during kidney organogenesis.

In situ hybridization analysis of another Notch ligand, *Dll1*, showed that, in contrast to *Jag1*, *Dll1* was not expressed in condensed mesenchyme or pretubular aggregates. *Dll1*-positive staining could be seen in comma- and S-shaped bodies in the control kidney (Fig. 7D, part a, arrowheads), but no *Dll1* expression could be detected in the PSEN-null at any stages of nephrogenesis (Fig. 7D, part b). This negative staining could be attributed by the lack of *Dll* induction caused by defective Notch signaling or by the absence of comma- and S-shaped bodies in PSEN-null mutant kidney. It is interesting to note that *Dll1* expression seems to be restricted to a specific region of comma- and S-shaped bodies (Fig. 7D, part a, arrowheads), although the fate of these cells is not clear. Similar to NICD and Hesn1, both Notch ligands were not expressed in the ureteric

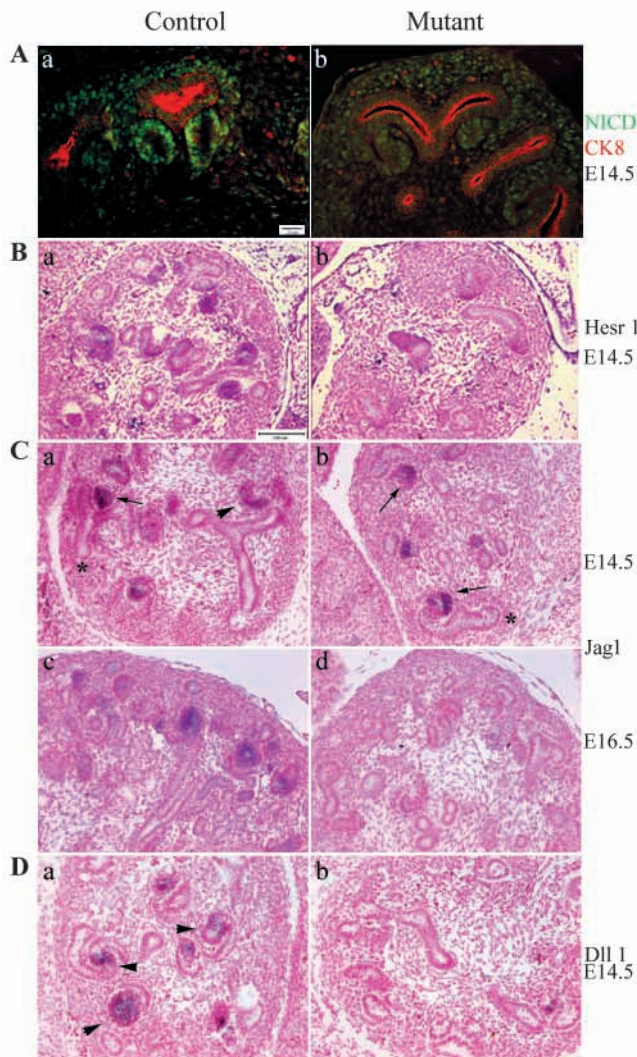


Fig. 7. Notch pathway analysis in the control (left) and PSEN-null (right) kidneys. (A) Immunohistochemical staining of presenilin-dependent NICD (green) expression. Ureteric bud was marked by cytokeratin 8 (CK8, red). (B-D) In situ hybridization analysis of Notch downstream target *Hesr1* (B), Notch ligands *Jag1* (C) and *Dll1* (D). All are at E14.5 except C, parts c,d, which are at E16.5. Thin arrows indicate pretubular aggregates/renal vesicles; arrowheads indicate comma- and S-shaped bodies. Scale bars: in A, 20 μm for A; B, 100 μm for B-D.

bud, again supporting a cell autonomous mechanism of presenilin-dependent Notch signaling within the mesenchyme.

Discussion

Role of presenilins in nephrogenesis

Histological examination at various stages of kidney development showed that presenilin deficiency does not affect the reciprocal inductive interactions between the ureteric bud epithelium and the metanephric mesenchyme. At E12.5, the mutant kidney was indistinguishable from that of littermate controls, as evidenced by the formation of condensed mesenchyme surrounding the ureteric buds. However, the difference became apparent at E13.5. While the control kidney

undergoes further development to form pretubular aggregates, renal vesicles and comma- and S-shaped bodies, only pretubular aggregates and renal vesicles can be found in the PSEN-null kidney. In situ hybridization analyses of kidney development markers revealed that *Gdnf* and *Bmp7* were normally expressed in PSEN-null kidney (data not shown), demonstrating that the uninduced mesenchyme was not affected by the loss of presenilins. The positive staining of *Pax2*, *Pax8* and *Wnt4* in the mutant established that the metanephric mesenchyme was competent in receiving signals from the ureteric bud to form condensates and aggregates. Last, the fact that *Ret*, which is a marker for the newly formed ureteric bud tips, was expressed similarly in the control and mutant at E13.5 suggests that presenilins do not exert a direct effect on primary branching morphogenesis. The reduced levels of mesenchymal markers *Pax2*, *Pax8* and *Wnt4* in the mutant at E13.5 and E15.5 are likely caused by the absence of comma- and S-shaped bodies. The combined effect led to impaired secondary and tertiary branching, as evidenced by the decreased number of *Ret*-positive tips in the mutant at E15.5. This notion is supported by kidney organ culture experiments in which loss of presenilins only affected branching in a minor fashion while formation of glomeruli were completely blocked. Limited secondary and tertiary branching and increased apoptotic cell death at later stages may in turn be the result of the failed progression through nephrogenesis in the PSEN-null kidney. These results, combined with our finding that presenilin γ -secretase activity is restricted to the mesenchymal derivatives, support a role for presenilins in the differentiation from pretubular aggregates and renal vesicles towards comma- and S-shaped bodies within the mesenchyme.

Progression from pretubular aggregates to comma- and S-shaped bodies requires the conversion and patterning of mesenchymal cells into highly organized and polarized epithelia. Analysis of cell adhesion molecules and extracellular matrix proteins, NCAM and E-cadherin, revealed that renal vesicles and tubular derivatives were partially formed in the PSEN-null kidney. Importantly, laminin stained these renal structures suggesting that they developed polarized cells with basement membranes. Thus, presenilins are not essential in the mesenchyme to epithelium transition. However, despite the formation and apparent fusion of some E-cadherin positive distal renal tubule structures to the bud epithelium, mature proximal tubules and glomeruli were completely absent in PSEN-null kidney. This conclusion was supported by the negative binding to LTL and lack of expression of WT1, respectively. From these data, we conclude that presenilins play an indispensable role in the patterning and differentiation of renal vesicles leading to proximal tubules and glomeruli.

In contrast to inductive interactions and MET, our current understanding about nephron patterning is limited. There are candidate regulatory mechanisms in tubule patterning by molecules such as the cadherins, which are differentially regulated in the various renal epithelial compartments (Cho et al., 1998). However, gene knockout studies have not supported a clear function of these molecules in nephron patterning (Dahl et al., 2002; Dressler, 2002; Mah et al., 2000). The presenilins represent one of the first examples of molecules that play an essential role in patterning and differentiation of renal vesicle derivatives.

Role of presenilins in Notch signaling during nephrogenesis

Presenilins are indispensable for the processing and signaling of Notch. This activity is highly conserved and likely accounts for the role of presenilins during somite patterning (Donoviel et al., 1999; Takahashi et al., 2000). Our analysis showed that the presenilin γ -secretase activated form of Notch1 (NICD), Notch downstream target *Hesr1* and ligands *Dll1* and *Jag1* were expressed in mesenchymal derivatives and that their expressions were critically impaired in PSEN-null kidneys. Thus, the Notch pathway, particularly signaling through Notch1, may be the underlying mediator for presenilin activity in kidney development. It is noteworthy that mice expressing a hypomorphic allele of *Notch2* exhibit kidney glomerulogenesis defects (McCright et al., 2001). As all four mammalian Notch proteins are presenilin substrates in vitro (Saxena et al., 2001), a defective Notch2-mediated pathway may also account for the PSEN-null kidney phenotype. However, the presence of glomeruli in Notch2 mutant kidneys, albeit structurally abnormal, indicates that Notch2 functions downstream of tubule patterning (McCright et al., 2001).

Although an essential role for the presenilins in Notch signaling has been well established, how this regulation affects Notch ligand expression has been the subject of controversy. Some support a lateral inhibition model in which loss of Notch signaling resulting from presenilin deficiency leads to ectopic overexpression of the ligand (Donoviel et al., 1999; Handler et al., 2000); Others suggest that presenilin-mediated Notch signaling is necessary for proper induction of its ligand (Takahashi et al., 2000). The differences may be caused in part by variations of the systems of study; as ligand expression seems to be subjected to strict temporal-, spatial- and cell type-specific regulation.

In kidney development, detailed examination of expression patterns of *Jag1* and *Dll1* yielded some interesting findings.

(1) *Jag1* is not expressed in condensed mesenchyme but its expression can be detected in pretubular aggregates and renal vesicles. This distinct *Jag1* staining pattern was similar in the control and the PSEN-null kidneys at E14.5, suggesting that transition from condensed mesenchyme to pretubular aggregates does not depend on presenilins. Interestingly, although pretubular aggregates and renal vesicles still exist in PSEN-null mutant at E16.5, *Jag1* expression is lost. These data suggest that sustained Notch activation is necessary for the maintenance of *Jag1* expression during nephrogenesis.

(2) In contrast to *Jag1*, *Dll1* message cannot be detected in pretubular aggregates. In the control kidney, *Dll1* expression seems to be restricted to a specific region of the comma- and S-shaped bodies at E14.5. Although the origin of these cells is not known, this spatiotemporally restricted expression pattern may imply functional significance. Indeed, during somite patterning, caudally restricted expression of *Dll1* has been shown to be important for prefiguring somite identity, and proper *Dll1* induction requires presenilin-dependent Notch signaling (Takahashi et al., 2000). Equally possible, however, the absence of *Dll1* expression could also be due to the physical lack of comma- and S-shaped bodies in the PSEN-null kidney.

Besides Notch and APP, numerous other proteins have been reported as substrates of presenilin-dependent proteolysis (Lammich et al., 2002; Marambaud et al., 2002; Ni et al., 2001).

Although the physiological significance is not clear, impaired processing of these molecules cannot be excluded as a potential mechanism for the kidney defects. Through processing-independent mechanisms, PSEN1 has been shown to interact with β -catenin and to facilitate its turnover (Kang et al., 2002; Xia et al., 2002). Therefore, it is possible that deregulation of the β -catenin pathway in the PSEN-null may contribute to the kidney phenotype. The Wnt pathway has been implicated in the inductive phase of kidney development (Dressler, 2002), but is essentially unaffected in the PSEN-null. In addition, we have created mice with a deletion of exon 10 of the endogenous *Psen1* essential for β -catenin interaction. Homozygous exon 10-deleted mice, when crossed onto the *Psen2*-null background, are viable and do not exhibit kidney defects (H.Z., unpublished). These observations thus argue against a potential β -catenin involvement in the PSEN-null kidney.

In addition to the kidney patterning defect, *Psen1*- and *Psen2*-double null embryos display other abnormalities, most noticeably defects in heart looping and vascular remodeling (Donoviel et al., 1999; Herreman et al., 1999). These defects were apparently corrected by the human *PSEN1* transgene presumably because of its early expression in mesoderm and in relevant cell derivatives. Indeed, the human Thy-1 promoter has been shown to be active in endothelial cells (Gordon et al., 1987), and expression of *Hesr1*, although absent in developing nephrons of the PSEN-null kidney, can be detected in blood vessels. The analysis of mechanisms leading to these phenotypes is currently ongoing.

In summary, using our novel presenilin 'rescue' system, we have identified a novel function of the presenilins in nephrogenesis. Specifically, presenilins are indispensable for the patterning of renal epithelial structures to form mature proximal tubules and glomeruli. Loss of presenilins is associated with failed progression from renal vesicles to comma- and S-shaped bodies. On the molecular level, we reveal an obligatory role of presenilins in the activation of Notch signaling and maintenance of the Notch ligand *Jag1* expression in the mesenchymal derivatives during kidney development.

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