Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation

Brent McCright1, Xiang Gao1,2,*, Liya Shen2,4, Julie Lozier1, Yu Lan1,§, Maureen Maguire2,¶, Doris Herzlinger3, Gerry Weinmaster4, Rulang Jiang1,§ and Thomas Gridley1,2,**

1The Jackson Laboratory, Bar Harbor, ME 04609, USA
2Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110, USA
3Department of Physiology and Biophysics and Department of Urology, Cornell University Medical College, New York, NY 10021, USA
4Department of Biological Chemistry, UCLA School of Medicine, Los Angeles, CA 90024, USA
*Present address: Institute of Molecular Medicine, Nanjing University, Nanjing 210093, China
†Present address: National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA
§Present address: Center for Oral Biology and Department of Biology, University of Rochester, Rochester, NY 14642, USA
¶Present address: Schering-Plough Research Institute, Kenilworth, NJ 07033, USA
**Author for correspondence (e-mail: gridley@jax.org)

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SUMMARY

The Notch gene family encodes large transmembrane receptors that are components of an evolutionarily conserved intercellular signaling mechanism. To assess the in vivo role of the Notch2 gene, we constructed a targeted mutation, Notch2 del1. Unexpectedly, we found that alternative splicing of the Notch2 del1 mutant allele leads to the production of two different in-frame transcripts that delete either one or two EGF repeats of the Notch2 protein, suggesting that this allele is a hypomorphic Notch2 mutation. Mice homozygous for the Notch2 del1 mutation died perinatally from defects in glomerular development in the kidney. Notch2 del1/Notch2 del1 mutant kidneys were hypoplastic and mutant glomeruli lacked a normal capillary tuft. The Notch ligand encoded by the Jag1 gene was expressed in developing glomeruli in cells adjacent to Notch2-expressing cells. We show that mice heterozygous for both the Notch2 del1 and Jag1 dDSL mutations exhibit a glomerular defect similar to, but less severe than, that of Notch2 del1/Notch2 del1 homozygotes. The co-localization and genetic interaction of Jag1 and Notch2 imply that this ligand and receptor physically interact, forming part of the signal transduction pathway required for glomerular differentiation and patterning. Notch2 del1/Notch2 del1 homozygotes also display myocardial hypoplasia, edema and hyperplasia of cells associated with the hyaloid vasculature of the eye. These data identify novel developmental roles for Notch2 in kidney, heart and eye development.

Key words: Notch2, Glomerulogenesis, Kidney, Hypomorphic mutation, Persistent hyperplastic primary vitreous, Mouse

INTRODUCTION

The Notch signaling pathway is an evolutionarily conserved intercellular signaling mechanism, and mutations in its components disrupt cell-fate specification and embryonic development in organisms as diverse as insects, sea urchins, nematodes and mammals. Genes of the Notch family encode large transmembrane receptors that interact with membrane-bound ligands encoded by Delta, Serrate and Jagged family genes. The signal induced by ligand binding is transmitted intracellularly by a process involving proteolysis of the receptor and nuclear translocation of the intracellular domain of the Notch protein (reviewed by Artavanis-Tsakonas et al., 1999; Weinmaster, 2000).

Proteins of the Notch family share several repeated peptide motifs. The extracellular domain of the protein contains a large number (>30) of tandemly repeated copies of an epidermal growth factor (EGF)-like motif. The extracellular domain of these proteins also contains three repeats of another motif, unique to this protein family, termed the Notch/lin-12 repeat. The intracellular domain of Notch family proteins contains six copies of another conserved motif termed the cdc10/ankyrin repeat, as well as nuclear localization signals and domains required for interaction with a number of cytoplasmic and nuclear proteins.

Four Notch genes have been identified in mammals (Notch1, Notch2, Notch3 and Notch4). Analysis of both inherited human diseases and mutant mice has shown that some of these genes are essential for embryonic development and adult homeostasis (Swiatek et al., 1994; Conlon et al., 1995; Joutel et al., 1996; Hamada et al., 1999; Krebs et al., 2000). Similarly, genes encoding ligands for the Notch family of receptors are also
essential in both humans and mice (Hrabé De Angelis et al., 1997; Li et al., 1997; Oda et al., 1997; Sidow et al., 1997; Jiang et al., 1998; Kusumi et al., 1998; Xue et al., 1999; Bulman et al., 2000). The Notch2 gene was the second of the mammalian Notch family receptors cloned (Weinmaster et al., 1992). Hamada et al. have constructed and analyzed a Notch2 mutant allele in which the Escherichia coli β-galactosidase-coding region replaces all but one of the ankyrin repeats in the intracellular domain of the mouse Notch2 protein (Hamada et al., 1999). Embryos homozygous for this Notch2 mutant allele die before 11.5 days of gestation.

In this study, we have constructed and analyzed another targeted mutation of the Notch2 gene. Unexpectedly, alternative splicing of our Notch2 mutant allele leads to the production of two different in-frame transcripts that delete either one or two EGF repeats of the Notch2 protein. Mice homozygous for this Notch2 mutant allele die as neonates, owing to defects in kidney development, and also exhibit defects in development of the heart and eye vasculature. This probable Notch2 hypomorphic allele has therefore permitted us to identify and analyze additional developmental decisions that are dependent on Notch2 function.

Our work indicates that Notch2 function is required for development of the kidney. The vertebrate kidney is formed by a series of inductive interactions between epithelial and mesenchymal tissues (Saxén, 1987). In mice, formation of the adult kidney, or metanephros, begins at embryonic day (E) 11 and is driven by reciprocal induction between the ureteric bud epithelium and the metanephric mesenchyme (for recent reviews, see Davies and Bard, 1998; Clark and Bertram, 1999; Kuure et al., 2000; Schedl and Hastie, 2000). The ureteric bud branches from the Wolffian duct and grows into the metanephric mesenchyme. Branching and growth of the ureteric bud is induced by signals from these mesenchymal cells. At the tips of the ureteric bud branches, the mesenchyme is induced by the epithelial cells of the ureteric bud to form aggregates. These aggregates then undergo a mesenchymal to epithelial transition to form an epithelial vesicle. The vesicles go through a series of well-characterized morphological stages, which have been termed the comma-stage, the S-shape stage, the capillary loop (or cup-shape) stage and the maturing glomerulus stage. At the S-shape stage, the region of the S-shape body destined to form the distal tubule fuses with the terminal tips of the ureteric bud derivatives, which will form the collecting ducts and ureters of the kidney; vascularization of the glomerulus also commences with the migration of endothelial cells into the glomerular cleft. These endothelial cells form a capillary loop, which branches to produce the complex capillary tuft of the mature glomerulus. Mesangial cells also populate the inner part of the glomerulus and maintain the looped configuration of the capillaries of the glomerular capillary tuft. Formation of the capillary tuft is accompanied by differentiation of glomerular epithelial cells into podocytes.

We report here that Notch2 function is required for glomerulogenesis in the kidney, as well as for development of heart and eye vasculature. We also demonstrate by gene expression analyses and genetic interaction studies that the Notch ligand encoded by the Jag1 gene is required for Notch2 signaling in the developing glomerulus.

MATERIALS AND METHODS

Targeting vector construction

A Notch2 genomic clone was isolated from a 129/Sv mouse genomic phage library. The genomic organization of a portion of the mouse Notch2 locus was determined by restriction enzyme mapping, blot hybridization and nucleotide sequencing. To construct the targeting vector, a 2.8 kb Scal-Xbal fragment of Notch2 was subcloned upstream of a PGK-neo expression cassette (Soriano et al., 1991), and a 3.5 kb HindIII-Xbal Notch2 fragment was subcloned downstream of the PGK-neo cassette. This resulted in the deletion of a 0.4 kb genomic fragment, deleting codons for 22 amino acids in EGF repeat 14 (amino acids 538-560 of the Notch2 protein), the splice donor site, and 0.3 kb of intron sequence. An HSV-tk cassette (Mansour et al., 1988) was introduced for negative selection. We refer to this allele as Notch2del1.

Electroporation, selection and screening of ES cells and mouse genotyping

C57 embryonic stem (ES) cells were electroporated with 25 μg of linearized targeting vector, placed under positive-negative selection in G418 and FIAU, and screened for homologous recombination by Southern blot hybridization as previously described (Swiatek and Gridley, 1993). For Southern blot analysis, 10 μg of DNA was digested with HindIII, fractionated on 0.8% agarose gels, transferred to Zeta-Probe GT membranes (BioRad) and hybridized with a 1.0 kb ClaI-XbaI fragment. ES cells containing the expected recombination event were injected into blastocysts from C57BL/6J mice. Male chimeras were bred with C57BL/6J females, and germine transmission was obtained for three independently targeted ES cell clones. Animals were genotyped by Southern blot analysis or by PCR. PCR primers for the wild-type Notch2 allele were sp3 (5′-CCAG-TGTGCCCAGGTAAGTG-3′), located in the deleted fragment in the Notch2del1 mutant allele, and sp4 (5′-TCTCCATATTGAGGCAAGCTC-3′), located in the genomic region 3′ from the deletion in the mutant allele; the primers for the Notch2del1 allele were sp4 and sp6 (5′-TTCTTGACTAGGGGAGGAGTAG-3′), located in the neo cassette.

RT-PCR analysis

Total RNA was isolated from whole embryos (E10.5). A Notch2-specific primer, N2-3 (bases 2220-2199 of Notch2 cDNA, GenBank Accession Number D32210) and AMV reverse transcriptase (Promega) were used for the reverse transcription reaction. Primers N2-10 (bases 1337-1357) and N2-2 (bases 2156-2136) were used to generate the PCR product. The PCR products from the wild-type and mutant RNAs were then sequenced to determine the nucleotides present in the alternatively spliced Notch2del1 mRNA. Genomic DNA was sequenced in the deletion region to identify splice donor and acceptor sites.

Northern blot

Poly(A+) RNA was isolated from E14.5 embryos, and 2.5 μg of RNA was separated on a 1% formaldehyde gel and transferred to Zeta-Probe GT membrane (BioRad). An 800 bp fragment from the 3′ end of Notch2 cDNA was used for generation of a 32P-dCTP labeled probe. Hybridization was at 42°C in a buffer containing 30% formamide, 2× SSC, 5× Denhardt’s, 100 μg/ml denatured salmon sperm DNA and 1% SDS.

Histological analysis

Embryos and organs for histological analysis were fixed in Bouin's solution. Fixed embryos were dehydrated through graded alcohols, embedded in paraffin wax or methacrylate, sectioned at 7 μm (paraffin wax) or 2 μm (methacrylate), and stained with Hematoxylin and Eosin or Toluidine Blue.
Non-radioactive in situ hybridization

Embryonic kidneys were dissected and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, washed with PBS, then equilibrated in PBS containing 30% glycerol. Samples were embedded in OCT, sectioned on a cryostat at 10-14 μm and mounted on Superfrost Plus (Fisher) slides. Tissue sections were permeabilized with 0.5% Triton X-100 and endogenous peroxidase activity was quenched in 3% H2O2/10% methanol. Blocking was allowed to develop 6-18 hours. Probes used for RNA in situ were Jag1 (Dudley et al., 1995); Vgf, nucleotides 517-864 of IMAGE clone M95200; Wt1, nucleotides 2449-3042 of IMAGE clone M55512; Pdgfrb (Lindahl et al., 1998); Wnt4 (Stark et al., 1994); Pax2 (Dressler et al., 1990).

Immunohistochemistry

Kidneys from embryos at E18.5 were dissected, fixed, and cryosectioned as for in situ hybridization. The sections were then permeabilized with 0.5% Triton X-100 and endogenous peroxidase activity was quenched in 3% H2O2/10% methanol. Blocking was carried out with 10% goat serum/TTBS. Antibody to mouse platelet endothelial cell-adhesion molecule 1 (PECAM, Pharmingen) was used in 2% goat serum/TTBS at a dilution of 1:100. A peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Jackson ImmunoResearch), at a 1:100 dilution in TTBS, was used to detect PECAM and to develop the DiaminoBenzidine (DAB, Sigma) substrate with 0.2% NiCl added. Polyclonal antibody to desmin (Sigma) was used at 1:100 dilution in 2% goat serum/TTBS at a dilution of 1:100 dilution in 2% goat serum/TTBS and was detected using a peroxidase-conjugated donkey anti-rabbit IgG secondary antibody, at a 1:100 dilution in TTBS (Jackson ImmunoResearch).

Terminal transferase-mediated dUTP nick end labeling (TUNEL) assay

Kidneys from E15.5 embryos were dissected, fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Cell death was detected using the In Situ Cell Death Detection Kit (Boehringer Mannheim), according to the manufacturer’s instructions. The average number of TUNEL-positive cells was determined by making 25 measurements on sections of both wild-type and homozgyous mutant kidneys. The field size used for counting the TUNEL-positive cells was 0.04 mm2, and measurements were taken for both medullary and cortical regions of the kidneys. Four individual wild-type and four Notch2del1/Notch2 del1 mutant kidneys at E15.5 were used for this analysis.
kidneys were fixed in 4% paraformaldehyde and processed into paraffin blocks as for histological analysis. 7 mm sections were then rehydrated and peroxidase activity was quenched in 3% H2O2/10% methanol. Tissue was permeabilized with 20 μg/ml proteinase K and the DNA was denatured using 2N HCl. An anti-BrdU monoclonal antibody (Pharmingen) was used at 1:100 dilution in 2% goat serum/TTBS. It was detected with a peroxidase-conjugated donkey anti-mouse IgG secondary antibody at a 1:100 dilution in TTBS (Jackson ImmunoResearch). Diaminobenzidine (DAB, Sigma) substrate with 0.2% NiCl added was used for detection. Quantitation was performed as described above for detection of TUNEL-positive cells.

Generation and analysis of double heterozygous mice
Official nomenclature and references for the mutant alleles used in these studies are Jag1 dDSL (Xue et al., 1999): Jag1 tm1Grid; Notch2 del1 (this report): Notch2 del1/Notch2 del1 and Dll1 tm1Go (Hrabé de Angelis et al., 1997). Heterozygous Notch2 del1 mice were mated with either Jag1 dDSL or Dll1 tm1Go heterozygous mice to generate double heterozygotes. Kidneys were dissected at postnatal day 6 and were processed for histological analysis as described above. Double heterozygous, single heterozygous and wild-type kidneys were analyzed.

RESULTS
Targeted disruption of the Notch2 gene
To analyze the in vivo role of the Notch2 gene, a targeting vector was constructed that deleted 0.4 kb of genomic sequence of the Notch2 gene (Fig. 1A). The deleted fragment interrupts the exon encoding EGF repeat 14 of the protein, deleting codons for 22 amino acids in EGF14, the splice donor site, and 0.3 kb of intron. We refer to this allele as Notch2 del1. Hamada et al. recently described construction of a targeted mutation of the Notch2 gene (Hamada et al., 1999). They observed completely penetrant embryonic lethality by E11.5 in

Kidneys were dissected at postnatal day 6 and were processed for histological analysis as described above. Double heterozygous, single heterozygous and wild-type kidneys were analyzed.
Analysis of a hypomorphic Notch2 mutation

mouse embryos homozygous for their targeted allele. However, when we set up timed matings of Notch2del1 heterozygous mice, we observed survival of Notch2del1/Notch2del1 homozygous mutant embryos to gestational ages later than E11.5 (see below). We therefore examined whether any potentially functional Notch2 transcripts could be transcribed from the Notch2del1 mutant allele. Northern blot hybridization using a probe from the 3' end of the Notch2 cDNA identified similar transcripts present in homozygous Notch2del1/Notch2del1 embryos and in wild-type embryos (Fig. 1C). In addition, in situ hybridization analyses of homozygous Notch2del1/Notch2del1 embryos using the same 3' Notch2 probe demonstrated the presence of Notch2 transcripts in the mutant embryos (data not shown). Since transcription termination and polyadenylation signals are present in the neo selection cassette contained in the Notch2del1-targeted allele, the presence of Notch2 transcripts containing sequences 3' of the targeted region suggested that there may be splicing around the neo cassette in the Notch2del1 allele. Therefore, we performed a series of RT-PCR analyses to determine the nature of the Notch2del1 transcript (Fig. 1D). RT-PCR analysis of Notch2del1 RNA and subsequent DNA sequencing of the RT-PCR products identified two alternative transcripts that are the result of in-frame splicing around both the deleted region of the Notch2del1 allele and the neo cassette (Fig. 1D,E). The alternatively spliced transcripts should result in the deletion of either one or two EGF repeats from the Notch2 protein. The shorter protein isoform joins the first half of EGF12 to the second half of EGF14, while the longer isoform joins the first half of EGF13 to the second half of EGF14 (Fig. 1E). The levels of these alternative transcripts are similar to the amount of wild-type transcript. These data demonstrate that the Notch2del1 mutant allele is not a null allele, and most likely is a hypomorphic allele of the Notch2 gene.

Defects in glomerulogenesis in Notch2del1/Notch2del1 mice

To examine whether mice homozygous for the Notch2del1 mutation were viable, heterozygous F1 animals were intercrossed. We observed that some of the progeny of intercrosses of Notch2del1 heterozygous mice died within the first 24 hours of birth. Genotypic analyses revealed that these dying neonatal animals were homozygous for the Notch2del1 mutant allele. Gross anatomical analysis of the Notch2del1/Notch2del1 neonates revealed that their kidneys were
hypoplastic and exhibited vascular lesions near the cortical surface (Fig. 2A).

Histological analysis revealed defects in glomerulogenesis in the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> kidneys. At E13.5, mutant and control kidneys were similar in size, and both exhibited ureteric bud growth and branching (Fig. 2B,C). In both mutant and control kidneys, at the tips of the bud branches the metanephric mesenchyme condensed and formed epithelial vesicles. However, by E16.5, kidneys from the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> embryos were smaller than those of wild-type and heterozygous littermate controls, and while ureteric bud branches and embryos were smaller than those of wild-type and heterozygous

However, by E16.5, kidneys from the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> embryos could be observed in the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> homozygous mutant kidneys (Fig. 2D-K).

Two types of abnormal glomeruli were observed in the mutant kidneys. Most commonly, differentiation of the mutant glomeruli appeared to arrest just before the capillary loop stage. In these mutant glomeruli, the capillary tuft of the normal mature glomerulus was absent and the glomerulus appeared to be a disorganized clump of cells (Fig. 2G,H). In the second type of abnormal mutant glomeruli, the glomerular capillary tuft was replaced by a capillary aneurysm-like structure that filled the region of Bowman’s capsule with red blood cells (Fig. 2L,K). Similar structures have been observed in mice mutant for the platelet-derived growth factor gene (Pdgfb; Levén et al., 1994; Lindahl et al., 1998) or the PDGFRβ receptor gene (Pdgfrb; Soriano, 1994). It has been demonstrated for the Pdgfb and Pdgfrb mutants that these glomerular capillary aneurysms arise due to defects in mesangial cell differentiation.

Expression of Notch2 and of genes encoding Notch ligands during kidney development

The Notch2 gene is expressed widely in mouse embryos and adults (Weinmaster et al., 1992; Lardelli and Lendahl, 1993; Swiatek et al., 1994; Williams et al., 1995; Lindsell et al., 1996). To study the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> kidney phenotype in more detail, the expression patterns of Notch2 and the genes encoding its potential ligands were assayed by RNA in situ hybridization. Since metanephric kidney development does not proceed synchronously, examination of kidneys during late embryogenesis permits examination of multiple developmental stages (Saxén, 1987). Notch2 is expressed in both the ureteric bud-derived collecting duct system, and in condensations and vesicular structures (such as metanephric vesicles and comma- and S-shape bodies) derived from the metanephric mesenchyme (Fig. 3A). In maturing glomeruli Notch2 is expressed primarily at the periphery, where glomerular epithelial cells are differentiating into podocytes (Fig. 3A,B,H).

Several genes encoding Notch ligands were also expressed in the developing kidney. TheDll1 gene was expressed in tubules and in comma- and S-shape bodies at E16.5 (Fig. 3C,D; see also Beckers et al., 1999). The Jag1 gene was expressed in the mesenchymal condensations and vesicular structures of the developing glomeruli (Fig. 3E-G). Jag1 expression was primarily observed in the inner region of the developing glomerulus, in a position consistent with expression in differentiating mesangial and/or endothelial cells (Fig. 3G). The Jag1 and Notch2 genes appeared to be expressed in adjacent cells in the developing glomerulus (Fig. 3G,H). Jag1 expression was also detected in the collecting ducts. The Dll4 gene, which encodes a recently identified Notch ligand, is expressed in endothelial cells of the glomerulus (Shutter et al., 2000). The Jag2 gene was expressed at low levels (much lower than the expression levels of Dll1 and Jag1) throughout the kidney at E16.5, and we did not detect expression of the Dll3 gene in the kidney (data not shown).

Notch2 is not required for mesenchymal condensation and epithelialization, but is essential for glomerular differentiation and patterning

To define the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> kidney phenotype in further detail, and to determine at which step in development the mutants deviate from the normal pathway, we analyzed expression of a panel of molecular markers. Both the Pax2 (Dressler et al., 1990) and Wnt4 (Stark et al., 1994) genes are expressed in the condensing mesenchyme and pretubular aggregates of the developing metanephric kidney. By in situ analysis, both Pax2 (Fig. 4A,B) and Wnt4 (Fig. 4C,D) were expressed normally in these structures in Notch2<sup>del1</sup>/Notch2<sup>del1</sup> kidneys. The Bmp7 gene is expressed early during nephrogenesis in the ureteric bud, and expression is subsequently observed in the metanephric mesenchyme and in the pretubular aggregates (Dudley et al., 1995; Luo et al., 1995). In Notch2<sup>del1</sup>/Notch2<sup>del1</sup> kidneys, Bmp7 expression in the metanephric mesenchyme and the condensing aggregates was normal (Fig. 4E,F). Thus, the early stages of metanephric kidney development, including ureteric bud growth and branching and mesenchymal condensation and vesicle formation, occurred relatively normally in the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> mutant kidneys. However, there appeared to be a quantitative difference in these processes in the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> mutants, in that far fewer condensations and vesicles formed in the mutants than in wild-type and heterozygous littermate controls.

We assessed differentiation and patterning of the glomerulus using molecular markers for the three primary cell types of the mature glomerulus: endothelial cells, mesangial cells and podocytes. Endothelial cell differentiation was assayed using a monoclonal antibody to PECAM1 (platelet-endothelial cell adhesion molecule 1) (Baldwin et al., 1994). This analysis revealed that few endothelial cells were present in the abnormal mutant glomeruli (Fig. 5A,B). However, some endothelial cells could be observed migrating into the cleft of the comma- and S-shape bodies in the mutants (Fig. 5B). The migration of endothelial cells into the cleft of the comma- and S-shape bodies is consistent with the development of capillary aneurysm-like structures in a subset of glomerular mutants (Fig. 2L,K).

Mesangial cell differentiation was assayed using an antibody to desmin (Lindahl et al., 1998; Miner and Li, 2000) and an in situ hybridization probe for Pdgfrb (Seifert et al., 1998; Lindahl et al., 1998). This analysis showed there were no glomeruli that contained desmin-positive cells in the mutant kidneys (Fig. 5C,D). Pdgfrb expression was also not observed in the mutant glomeruli (Fig. 5E). In the wild-type and heterozygous control kidneys, Pdgfrb expression was present in the center of cup-staged glomeruli, consistent with its expression in mesangial cells (Fig. 5E). These data reveal the absence of mesangial cells in the abnormal glomeruli of the Notch2<sup>del1</sup>/Notch2<sup>del1</sup> mutants.

Podocyte differentiation was assayed using in situ hybridization probes for the vascular endothelial growth factor

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A (Vegf) gene (Breier et al., 1992; Kitamoto et al., 1997; Tufró, 2000) and the Wilms’ Tumour (Wt1) gene (Kreidberg et al., 1993). In wild-type and heterozygous littermate control kidneys, both genes were expressed in the cup-shaped semicircle of glomerular epithelial cells that are differentiating into podocytes (Fig. 5G,I). In Notch2del1/Notch2del1 homozygous mutant kidneys, Vegf- and Wt1-positive cells were present in the abnormal mutant glomeruli, but the cells expressing these markers were clumped together in the center of the glomerulus, and did not form the cup-shaped epithelial layer observed in the controls (Fig. 5H,J).

Analysis of cell death and proliferation in Notch2del1/Notch2del1 kidneys

Since by the later stages of embryogenesis the kidneys of Notch2del1/Notch2del1 mutant embryos were significantly smaller than kidneys of control littermate embryos, we analyzed cell proliferation and cell death in developing kidneys. Cell proliferation was analyzed by BrdU labeling of kidneys isolated at E15.5. Cell proliferation was approximately equal in both wild-type and mutant kidneys in the highly proliferative cortex of the kidney (Fig. 6A,B). There were 51.1±5.8 (mean±s.d.) BrdU-positive cells/0.04 mm² in wild-type sections compared with 47.2±8.1 BrdU-positive cells/0.04 mm² in mutant sections in this region. However, while proliferating cells were observed inside the morphologically normal glomeruli of wild-type and heterozygous littermate controls, no proliferating cells were observed inside the abnormal glomeruli that were present in the Notch2del1/Notch2del1 mutant kidneys.

Apoptotic cell death was assessed by TUNEL assay. We detected an increased amount of cell death in Notch2del1/Notch2del1 mutant kidneys (Fig. 6C,D). There were 9.1±3.4 TUNEL-positive cells/0.04 mm² in wild-type sections compared with 17.0±4.8 TUNEL-positive cells/0.04 mm² in mutant sections. Higher numbers of TUNEL-positive cells were detected at the periphery of the mutant kidneys, immediately beneath the renal capsule. These results suggest that increased cell death may contribute to the growth retardation observed in the Notch2del1/Notch2del1 mutant kidneys.

Notch2del1/Jag1dDSL double heterozygous mice exhibit glomerular defects

Phenotypes sensitive to gene dose are a common feature of Notch pathway mutants in both vertebrates and invertebrates (Artavanis-Tsakonas et al., 1991; Li et al., 1997; Oda et al., 1999; Krebs et al., 2000). Since the Notch2 and Jag1 genes were expressed in adjacent regions of the developing glomerulus, we examined kidneys from mice heterozygous for both the Notch2del1 and Jag1dDSL mutant alleles. These double heterozygous mice exhibited kidney defects (Fig. 7). The kidneys of the double heterozygotes (n=6) were about half the size of kidneys from the littermate controls (which consisted of wild-type mice, and Notch2del1 and Jag1dDSL single heterozygotes) (Fig. 7A). The number of glomeruli was greatly reduced in the double heterozygotes, and about a quarter of the glomeruli present lacked glomerular capillary tufts and exhibited the capillary aneurysms similar to those observed in Notch2del1/Notch2del1 homozygous mutant kidneys (compare Fig. 7C with Fig. 2L,K). These results demonstrate that Notch2del1/Jag1dDSL/+ double heterozygotes have a glomerular defect similar to, but less severe than, those of the Notch2del1/Notch2del1 homozygous mutants.

Since theDll1 gene is expressed in the developing glomerulus, we also generated mice heterozygous for both the Notch2del1 and Dll1im/Gi (Hrabé de Angelis et al., 1997) mutant alleles. However, we observed no kidney defects in Notch2del1/Dll1im/Gi double heterozygous mice (n=3).

Mice homozygous for the Notch2del1 mutation exhibit defects in development of the hyaloid vasculature of the eye

In addition to hypoplastic kidneys, other defects were observed in Notch2del1/Notch2del1 mutant embryos and neonates. Gross anatomical analysis of Notch2del1/Notch2del1 neonates revealed that all mutants displayed bilateral microphthalmia (Fig. 8A). Histological analysis of the eyes of Notch2del1/Notch2del1 neonates typically revealed an aberrant bulbous structure at the terminus of the hyaloid artery (Fig. 8B). Numerous small capillaries were observed emanating from this structure (Fig. 8C). At earlier embryonic stages, a pronounced retrolenticular hyperplasia was evident in sections of eyes from the Notch2del1/Notch2del1 embryos (Fig. 8D,E). Eyes of the Notch2del1/Notch2del1 embryos exhibited a pronounced asymmetry (Fig. 8F,G), which was apparently caused by the retrolenticular hyperplasia.

Heart defects in Notch2del1/Notch2del1 embryos

Quantitative analysis of intercross progeny from Notch2del1 heterozygous mice revealed that, at gestational stages later than E16.5, only 12% of the progeny were Notch2del1/Notch2del1 homozygous mutants (Table 1). We therefore isolated mutant embryos at earlier stages in order to determine the cause of embryonic lethality prior to E16.5. Through E10.5 we detected no obvious mutant phenotype in Notch2del1/Notch2del1 embryos. However, at E11.5, approximately 40% of the homozygous mutant embryos exhibited growth retardation, pericardial effusion and widespread hemorrhaging (Fig. 9A and Table 1). Mutant embryos that survived this stage displayed a variety of vascular defects, including myocardial hypoplasia, hemorrhaging, and edema (Fig. 9B-E, and Table 1). Beginning at E13.5, edema and hemorrhaging vessels near the surface of the skin were observed in approximately 50% of Notch2del1/Notch2del1 homozygous mutant embryos (Fig. 9C, and data not shown). Myocardial hypoplasia and reduced myocardial trabeculation were observed in all mutant embryos E12.5 and older (Fig. 9D,E). We analyzed Notch2 expression in the heart by in situ hybridization to determine whether the observed phenotype, particularly the myocardial hypoplasia, correlated with a domain of Notch2 expression. We detected no Notch2 message in the heart at either E11.5 or E14.5. However, we did detect Notch2 expression in the outflow tract of the heart at these stages (data not shown).

DISCUSSION

The Notch2del1 allele causes a probable hypomorphic mutation due to alternative splicing

The Notch2del1 allele described here is a probable hypomorphic allele, owing to unexpected in-frame alternative splicing. This results in the production of novel Notch2 mRNAs that can
encode Notch2 proteins that may be partially functional. The novel mRNAs have excised the coding sequence for either one or two of the 36 EGF repeats present in the Notch2 protein. The shorter protein isoform joins the first half of EGF12 to the second half of EGF14, while the longer isoform joins the first half of EGF13 to the second half of EGF14. These excised EGF repeats may hinder Notch2 ligand-binding ability owing to their proximity to the EGF11 and EGF12 repeats, which are essential for ligand binding by the *Drosophila* Notch and

![Fig. 5. Analysis of molecular markers for glomerular differentiation and patterning.](image)

Fig. 5. Analysis of molecular markers for glomerular differentiation and patterning. (A,B) PECAM1 immunohistochemistry of E18.5 wild-type (A) and Notch2del1/Notch2del1 mutant (B) kidneys. Some PECAM1-positive endothelial cells can be seen migrating into the glomerular cleft in both wild-type and mutant S-shape bodies. (C,D) Desmin immunohistochemistry of E18.5 wild-type (C) and mutant (D) kidneys. Wild-type glomeruli contain desmin-expressing mesangial cells (arrows) while no desmin-positive cells were expressed in mutant glomeruli. (E,F) Pdgfrb RNA expression in E16.5 kidneys. In the wild-type kidney, as the glomerulus reaches the capillary loop stage, the center becomes populated with Pdgfrb expressing mesangial cells (arrows). In the mutant, Pdgfrb expressing cells remain on the outside of the glomerulus. Boundaries of the glomerulus are indicated by broken lines. (G,H) Vegf RNA expression in E16.5 kidneys. In the wild-type kidney, Vegf-expressing podocytes formed a cup-shaped semicircle (arrow in G). In the mutant, kidney, Vegf-expressing cells were clustered (arrow in H). (I,J) Wt1 RNA expression in E16.5 kidneys. The Wt1 expression pattern resembled the Vegf expression pattern in both wild-type (I) and mutant (J) kidneys.

![Fig. 6. Cell proliferation and cell death in Notch2del1/Notch2del1 kidneys.](image)

Fig. 6. Cell proliferation and cell death in Notch2del1/Notch2del1 kidneys. (A,B) Cell proliferation was assayed by detecting incorporation of BrdU into newly synthesized DNA. Proliferating cells have brown nuclei. Large numbers of proliferating cells were detected in both wild-type (A) and Notch2del1/Notch2del1 mutant (B) kidneys at E15.5. (C,D) Cell death was assayed by TUNEL assay to identify apoptotic cells in E15.5 kidneys. TUNEL-positive cells have brown nuclei. In wild-type kidneys (C), few TUNEL-positive cells were detected (arrows). In mutant kidneys (D), large numbers of TUNEL-positive cells (arrows) were detected. TUNEL-positive cells were present in higher numbers at the periphery of the kidney, immediately beneath the renal capsule.

![Fig. 7. Kidney defects in Notch2del1/+ Jag1DSL/+ double heterozygotes.](image)

Fig. 7. Kidney defects in Notch2del1/+ Jag1DSL/+ double heterozygotes. Kidneys were isolated at P6 from wild-type and Notch2del1/+ Jag1DSL/+ double heterozygous mice. (A) The double heterozygous kidney (right) is smaller than the wild-type littermate kidney (left). While the control kidney glomeruli exhibit a normal capillary tuft (arrow in B), some double heterozygous glomeruli (arrows in C) exhibit capillary aneurysm-like structures similar to those observed in Notch2del1/Notch2del1 homozygotes.
Analysis of a hypomorphic Notch2 mutation

Xenopus Notch1 proteins (Rebay et al., 1991). We hypothesize that the phenotypes we observe in Notch2 del1 /Notch2 del1 mutants are most likely caused by a decrease in Notch2 signaling. An alternative possibility is that the phenotypes we observe in Notch2 del1 /Notch2 del1 mutants are due to deleterious indirect effects such as ligand sequestering. However, the fact that Notch2 del1 /+ heterozygotes have no visible phenotype argues against this hypothesis.

Notch2 is required for glomerular morphogenesis and patterning

Mice homozygous for the Notch2 del1 mutation exhibit a complex phenotype, with at least two stages of lethality. All Notch2 del1 /Notch2 del1 homozygous mutants that survive until birth die within the first 24 hours. These homozygotes probably die from renal insufficiency due to defects in glomerular differentiation and patterning, although heart defects (i.e., myocardial hypoplasia) may also contribute to the lethality observed in the Notch2 del1 /Notch2 del1 neonates.

In the majority of abnormal glomeruli in the Notch2 del1 /Notch2 del1 mutants, neither mesangial cells nor endothelial cells could be detected using molecular markers. Some mutant
glomeruli appeared to consist of a disordered group of podocytes, as the clustered cells expressed the podocyte markers Vegf and Wt1. However, the mutant podocytes had not formed the cup-shaped epithelial layer observed in the control littermate glomeruli. This phenotype is reminiscent of the defects in development of epithelial tissues in *Drosophila* embryos mutant for Notch or for other components of the Notch signaling pathway (Hartenstein et al., 1992; Goode et al., 1996). In the second type of abnormal glomeruli observed in Notch2del1/Notch2del1 mutants, the glomerular capillary tuft was replaced by a capillary aneurysm-like structure similar to that observed in mice mutant for the Pdgfb gene (Levéen et al., 1994; Lindahl et al., 1998) or the Pdgfrb gene (Soriano, 1994). It has been demonstrated for the Pdgfb and Pdgfrb mutants that these glomerular capillary aneurysms arise because of defects in mesangial cell differentiation.

It is important to point out that there is also a substantial quantitative effect on glomerular differentiation in Notch2del1/Notch2del1 mutants. The total number of glomeruli is markedly reduced in the mutants, compared with heterozygous and wild-type littermates. This suggests that Notch2 signaling may be required at multiple stages during glomerular differentiation. Notch2-mediated signaling may be required for cellular differentiation of the mesenchymal vesicles, as well as for providing the proper signals for migration of endothelial and mesangial precursors into the glomerular cleft, and for vascularization and epithelialization of the developing glomerulus.

**The Jag1 protein is a probable ligand for the Notch2 receptor during glomerulogenesis**

Expression analysis indicated that the Notch2 and Jag1 genes were expressed in adjacent cells during glomerular differentiation. Notch2 was expressed at the periphery of the glomerulus in differentiating podocytes, while Jag1 was expressed in the interior of the glomerulus, in a position consistent with expression in differentiating mesangial and/or endothelial cells. In addition, Notch2del1/+ Jag1+/-/ double heterozygotes displayed a kidney phenotype similar to, although somewhat less severe than, that of Notch2del1/Notch2del1 homozygotes. These data indicate that the Jag1 gene encodes a physiological ligand for the Notch2 receptor during glomerulogenesis. In support of this model, in vitro cell culture studies have confirmed that the Jag1 protein can act as a signaling ligand for the Notch2 protein, although either protein may bind to other ligands or receptors as well (Noziger et al., 1999; Shimizu et al., 1999; Shimizu et al., 2000).

**Heart and vascular defects in Notch2del1/Notch2del1 mice**

The second stage of lethality observed in Notch2del1/Notch2del1 embryos was from E11 through E16. Abnormal mutant embryos at these stages exhibited symptoms of cardiovascular dysfunction. These embryos were edematous, and histological analysis revealed myocardial hypoplasia and reduced myocardial trabeculation. Many of these embryos also exhibited hemorrhages of small blood vessels in the skin.

We did not detect Notch2 RNA in the embryonic heart, and it has been reported by others that Notch2 is not expressed in the heart (Weinmaster et. al., 1992; Hamada et al., 1999). We did observe Notch2 expression in the cardiac outflow tract. Jag1 expression is also observed in the cardiac outflow tract (Loomes et al., 1999), and various heart defects are observed in humans with Alagille syndrome, an inherited disease syndrome caused by Jag1 haploinsufficiency (Li et al., 1997; Oda et al., 1997; Krantz et al., 1999).

It is intriguing that several phenotypes observed in Notch2del1/Notch2del1 homozygotes are similar to phenotypes observed in mice homozygous for targeted mutations of Pdgfb (Levéen et al., 1994; Lindahl et al., 1998) and Pdgfrb (Soriano, 1994). These shared phenotypes include formation of glomerular capillary aneurysms, myocardial hypoplasia and hemorrhages of blood vessels in the skin. Since Pdgfb and Pdgfrb are not expressed in the same cells that express the Notch2 gene during glomerulogenesis, any interaction between these two signaling pathways is probably indirect, at least with respect to glomerular differentiation. Future studies will examine in more detail possible interactions between these signaling pathways.

**Defects in the eye vasculature of Notch2del1/Notch2del1 homozygotes**

The hyaloid artery forms part of the hyaloid vascular system, a transient network of intraocular blood vessels that nourish the growing lens during embryogenesis (Jack, 1972; Balazs et al., 1980). During late embryonic and early postnatal stages in wild-type mice, the hyaloid vessel system atrophies. The retrolenticular hyperplasia present in the eyes of Notch2del1/Notch2del1 embryos resembles a congenital human disease syndrome termed persistent hyperplastic primary vitreous (PHPV) (Reese, 1955; Haddad et al., 1978). In individuals with PHPV, the hyaloid vascular system does not atrophy but instead persists postnatally. This can lead to retinal detachment, cataracts, glaucoma and degeneration of the eye. While most human cases of PHPV are unilateral and show no obvious hereditary predisposition, approx. 10% are bilateral, and some cases of familial inheritance have been reported (cited by Haddad et al., 1978; Stades, 1983). Moreover, a genetic basis for the development of PHPV is supported by the frequent occurrence of PHPV in two dog breeds, the Doberman pinscher (Stades, 1983; Boevé et al., 1988) and the Staffordshire bull terrier (Curtis et al., 1984; Leon et al., 1986). However, the genetic loci causing predisposition for development of PHPV has not been identified in either dog model of the disease. Notch2del1/Notch2del1 mutant mice may provide a useful, genetically defined model for study of the retrolenticular hyperplasia associated with the early embryonic stages of PHPV.

**What is the Notch2 null phenotype?**

It is clear that the phenotype we observe in Notch2del1/Notch2del1 homozygous mutants does not represent the Notch2 null phenotype. Hamada et al. constructed and analyzed a Notch2 mutant allele (Notch2lacZ) in which the *E. coli* β-galactosidase-coding region replaces all but one of the ankyrin repeats in the intracellular domain of the mouse Notch2 protein (Hamada et al., 1999). Embryos homozygous for the Notch2lacZ mutant allele exhibit a more severe phenotype than Notch2del1/Notch2del1 homozygotes. All Notch2lacZ/Notch2lacZ homozygous mutant embryos die before 11.5 days of gestation (Hamada et al., 1999). The design of the Notch2lacZ mutant allele is similar to the design of constructs
that exhibit dominant-negative effects when expressed in Drosophila. Reyb et al. found that dominant negative phenotypes resulted from overexpression of a Notch protein lacking most intracellular sequences (Reyb et al., 1993). Hamada et al. argue that they are not observing dominant negative phenotypes in Notch2lacZ/Notch2lacZ homozygotes, since Notch2lacZ/Notch2lacZ embryos arrested at a slightly later stage than Notch1+/− mutant embryos (Swiatek et al., 1994; Conlon et al., 1995), and no abnormalities were observed in Notch2lacZ/+ heterozygotes. However, it is not clear whether the phenotype of Notch2lacZ/Notch2lacZ homozygotes represents the Notch2 null phenotype. While the Notch2lacZ mutant allele probably prevents Notch2-mediated signaling, dominant negative effects of this allele (such as sequestration of Notch family ligands) could contribute to the phenotype observed in the Notch2lacZ/Notch2lacZ homozygotes. Only creation of another Notch2 mutant allele that totally abrogates production of Notch2 protein will answer the question of what the Notch2 null phenotype really looks like.

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