The Mouse Kreisler (Krml1/MafB) Segmentation Gene Is Required for Differentiation of Glomerular Visceral Epithelial Cells

Virginia S. Sadl,*1 Fuzi Jin,*1 Joanna Yu,* Shiying Cui,* Douglas Holmyard,* Susan E. Quaggin,* Greg S. Barsh,† and Sabine P. Cordes*2

*Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, and †Department of Medical and Molecular Genetics, University of Toronto 600 University Avenue, Toronto, Ontario M5G 1X5 Canada; and ‡Department of Pediatrics and Genetics and the Howard Hughes Medical Institute, Beckman Center, Stanford, California 94305-5428

Molecular components of the glomerular filtration mechanism play critical roles in renal diseases. Many of these components are produced during the final stages of differentiation of glomerular visceral epithelial cells, also known as podocytes. While basic domain leucine zipper (bZip) transcription factors of the Maf subfamily have been implicated in cellular differentiation processes, Kreisler (Krml1/MafB), the gene affected in the mouse kreasler (kr) mutation, is known for its role in hindbrain patterning. Here we show that mice homozygous for the kr<sup>enu</sup> mutation develop renal disease and that Kreisler is essential for cellular differentiation of podocytes. Consistent with abnormal podocyte differentiation, kr<sup>enu</sup> homozygotes show proteinuria, and fusion and effacement of podocyte foot processes, which are also observed in the nephrotic syndrome. Kreisler acts during the final stages of glomerular development—the transition between the capillary loop and mature stages—and downstream of the Pod1 basic domain helix-loop-helix transcription factor. The levels of Podocin, the gene mutated in autosomal recessive steroid-resistant nephrotic syndrome (NPHS2), and Nephrin, the gene mutated in congenital nephrotic syndrome of the Finnish type (NPHS1), are slightly reduced in kr<sup>enu</sup>/kr<sup>enu</sup> podocytes. However, these observations alone are unlikely to account for the aberrant podocyte foot process formation. Thus, Kreisler must regulate other unknown genes required for podocyte function and with possible roles in kidney disease. © 2002 Elsevier Science (USA)

Key Words: Kreisler (Krml1/MafB); Pod1 (epicardin/capsulin); podocyte; kidney disease; cellular differentiation; proteinuria.

INTRODUCTION

In the kidney, the glomerular filtration apparatus consists of several highly differentiated cell types: mesangial cells, podocytes, and the specialized fenestrated capillary endothelium. Both podocytes and endothelial cells produce the glomerular basement membrane (GBM), which surrounds the mesangial cells and the capillaries (Lehtonen et al., 2000; Li et al., 2000). The mesangial cells provide structural support to the capillaries located immediately adjacent to them. The podocytes, or visceral glomerular epithelial cells, produce long slender cellular processes, known as pedicles or foot processes, that wrap around the GBM encased capillary and together with the GBM form a blood filtration barrier that maintains selective permeability to proteins. Damage to podocytes causes renal diseases, such as minimal change nephrosis (MCNS) or focal segmental glomerulosclerosis (FSGS). Aside from diabetes, FSGS is currently the main cause of renal insufficiency in humans. Kidney disease is often progressive. At the onset of renal pathogenesis, the GBM thickens and podocytes assume ultrastructural characteristics of less mature glomeruli. In glomerulosclerosis, mesangial cells and an apparently thickened GBM expand and begin to displace damaged...
podocytes, which have a dedifferentiated appearance, and ultimately kidney failure results. Such dedifferentiation of podocytes and loss of the molecular components of the filtration apparatus are associated with genetic renal diseases (Kriz et al., 1994; Pagtalunan et al., 1997). Recent advances in human genetics further underscore the importance of the podocyte. Mutations in genes that encode components of the specialized podocyte cytoskeleton can cause genetic renal disease. In mice and humans, the expression of these genes is highly podocyte-specific and must be regulated by podocyte-specific differentiation factors. However, only a few transcription factors with roles in glomerular development have been identified, and even fewer are known to regulate podocyte differentiation specifically (Lechner and Dressler, 1997). The identification of regulatory molecules that could reactivate proper expression of the key structural components of the podocytes, the filtering pedicles, and the GBM would be necessary to resuscitate injured podocytes or induce development of new ones.

Members of the Maf subfamily of basic domain leucine zipper transcription factors play key roles in cellular differentiation (Blank and Andrews, 1997). Loss-of-function mutations in mouse c-Maf and MafG interrupt terminal differentiation of lens fiber cells and megakaryocytes, respectively (Kim et al., 1999; Ring et al., 2000; Shavit et al., 1998). Furthermore, potential binding sites for Maf protein homodimers or heterodimers have been found in the promoters of important cell type-specific genes, such as β-globin, heme oxygenase, interleukin-4, and crystallins, and expression of these genes can be regulated by specific Maf proteins in transient transfection assays (Inamdar et al., 1996). In contrast to other Maf family members, Kreisler (Krml1/MafB; known as MafB according to mouse nomenclature) is known for its role in embryonic patterning rather than any functions in cellular differentiation. Kreisler was originally identified as the gene affected in the krenu (kr) mouse mutation, in which a primary defect in embryonic hindbrain patterning causes circling behavior and deafness in kr/kr embryos (Cordes and Barsh, 1994; Deol, 1964). The embryonic hindbrain is transiently subdivided into seven to eight segments, known as rhombomeres. Kreisler is expressed transiently in the fifth and sixth rhombomeres (Cordes and Barsh, 1994). In animals with mutations in Kreisler, the fifth and sixth rhombomeres do not form properly and rhombomere-specific expression of Hox genes is disrupted in r5 and r6 (Cordes and Barsh, 1994; Manzanares et al., 1997, 1999; Moens et al., 1998). In the mouse, there are two mutant alleles of Kreisler: the viable X-ray-induced kr allele and the

**FIG. 1.** Mice homozygous for the krenu mutation die as neonates. (A) A schematic diagram shows the novel EarI recognition site generated by the krenu mutation in the DNA-binding domain of Kreisler and the location of primers used to amplify this region. (B) EarI digestion of the PCR product from normal Kreisler results in a 416-bp undigestable fragment present in +/+ and +/krenu mice, while EarI digestion of the PCR fragment generated from krenu DNA yields 289- and 127-bp fragments present in krenu heterozygous and homozygous animals. (C) The table shows the genotypes of complete litters from krenu/+ intercrosses at various stages of development. No decrease in viability of krenu/krenu embryos was observed throughout embryogenesis or at birth. However, within 24 h after birth, all krenu/krenu mice have died. No weanling or adult krenu homozygotes have ever been recovered. Only complete litters harvested are shown here. Additional krenu homozygotes and normal littermates were harvested at 14.5 and 15.5 dpc and as neonates for the experiments performed here.
lethal kr
enu allele, which we generated previously by chemical mutagenesis with ethylnitrosourea (ENU). The unexpected lethality of kr
enu homozygotes suggested that further analysis of the phenotypes of mice homozygous for the kr
enu allele would reveal additional essential functions of the Kreisler gene, possibly in cellular differentiation. Here we show that Kreisler is required specifically for podocyte differentiation.

**MATERIALS AND METHODS**

**Mice and Genotyping**

Fifteen CSJ/L male mice, which are homozygous for the A allele of the agouti coat color locus, were mutagenized with four weekly doses of 100 mg/kg ENU (Sigma). Upon recovery of fertility at 10–12 weeks postinjection, mutagenized males were bred to a kr/A Kr females, which had been maintained on the C3H/HeJ strain for more than 10 generations. We recovered 3 circling mice upon screening 597 weanling mice (Cordes and Barsh, 1994). Deafness was initially scored by lack of the Preyer's reflex. To determine whether the kr
enu mutation could be a new allele of Kreisler, we tested whether this mutation is linked to the agouti coat color locus. The original Agouti male recovered from this screen was bred to C57Bl/6J females; only the resulting Agouti offspring had deaf, circling progeny when mated to a kr/a kr males. Only in the presence of the original kr allele do animals carrying the krenu mutation exhibit the characteristic circling and deafness. As described previously, we determined that the krenu mutation indeed represents a new allele of Kr. The original A krenu/a kr male recovered from this screen was bred to C57Bl/6J females; only the resulting Agouti female offspring had deaf, circling progeny when mated to a kr/a kr males.

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**FIG. 3.** *kr^mu*/*kr^mu* neonatal kidneys are histologically indistinguishable from +/+ and +/*kr^mu* kidneys. Sagittal sections of +/*kr^mu* (A) and *kr^mu*/*kr^mu* (B) neonatal kidneys stained with hematoxylin and eosin do not reveal any histological abnormalities. The nephrogenic zone (nz), the birthplace of all glomeruli, is located on the outer rim of the cortex. Mature glomeruli are located more internally. Proximal (pt) and distal tubules are clearly visible just below the nephrogenic zone and are well-organized. The stroma (s) appears normal. Sagittal sections of mature glomeruli from +/*kr^mu* (C) and *kr^mu*/*kr^mu* (D) kidneys stained with toluidine blue show no abnormalities. Capillaries (c) and all glomerular cell types are present and appear morphologically normal. Podocytes (pc) are in normal positions and appear normal at this magnification. The scale bar in (A) indicates 60 μm, while that in (C) represents 20 μm. (B) is at the same magnification as (A), and (C) is at the same magnification as (D). (E-H) Lack of normal podocyte foot processes in *kr^mu*/*kr^mu* glomeruli. Electron microscopy reveals that all glomerular cell types—mesangial and endothelial cells as well as podocytes—are present in normal (E, G) and *kr^mu*/*kr^mu* (F, H) glomeruli. However, there are significant ultrastructural differences between glomeruli from *kr^mu*/*kr^mu* (E, G) and *kr^mu*/*kr^mu* (F, H) neonates. Discrete foot processes can be seen in normal glomeruli (E, G). In (F, H), *kr^mu*/*kr^mu* mice podocyte foot processes (fp) are fused and do not interdigitate. Where these foot processes should be located is indicated with “fp” in parentheses. (E, F) Lower magnification views. The scale for these panels is shown in (E) and represents 2 μm. Higher magnification shows that, while podocyte foot processes are absent, the GBM of *kr^mu*/*kr^mu* (G) and *kr^mu*/*kr^mu* (H) glomeruli appear indistinguishable. The scale for (G, H) is shown in (G) and represents 1 μm. E, erythrocytes; gbm, glomerular basement membrane; M, mesangial cells; pc, podocyte; fp, podocyte foot process.
Bevery, MA) results in 289- and 127-bp fragments diagnostic for the kr<sup>enu</sup> allele. In addition to the whole litters collected and tabulated in Fig. 1C, 21 kr<sup>enu</sup>/kr<sup>enu</sup> mice and corresponding 26 +/− and 27 +/kr<sup>enu</sup> litters were collected for phenotypic analyses described.

**Phenotypic Analysis**

**Urinanalysis.** Urine was expressed from neonatal mice between 6–24 h after birth and analyzed by spotting onto Multistix 8 SG Reagent strips for Urinanalysis (Miles Inc., Diagnostics Division, Elkhart, IN). Then, 60 s after "spotting," the urine was analyzed for the presence of protein by comparison with the standardized chart. Urine from normal litters (kr<sup>enu</sup>/+ and +/+) and from kr/kr mice contained approximately 30 mg/dL of protein, while that from kr<sup>enu</sup> homozygous neonates contained between 100 and 300 mg/dL. For protein analysis, 0.1 μL of urine from mutant and normal neonates was examined by electrophoresis on a 10% denaturing SDS-polyacrylamide minigel.

**Analysis of Expression Patterns**

Whole-mount RNA in situ analysis was performed as previously described by using sense and anti-sense probes generated from 1183 to 1411 of the Kreisler 3′UTR, and probes for Hoxa3, Hoxb3, and Hoxd3 (Cordes and Barsh, 1994; Frohman et al., 1993; Manley and Capecki, 1998). Sense and anti-sense probes for Nephrin and Pod1 were generated as previously described (Quaggin et al., 1999; Wong et al., 2000). Sense and anti-sense probes for Podocin (GenBank Accession No. A1552534) and CD2AP (GenBank Accession No. AA739155) were generated from ESTs obtained from the I.M.A.G.E. clone consortium (Genome Systems, Inc., Berkeley, CA). The RNA levels of kr<sup>enu</sup> animals at all stages of embryonic development and at birth, kr<sup>enu</sup> heterozygotes appeared indistinguishable from normal animals (Fig. 1C). Even though many kr<sup>enu</sup>/kr<sup>enu</sup> neonates suckled initially, all died severely dehydrated within 24 h of birth.

Several observations suggested that hindbrain-independence roles of Kreisler must be responsible for the lethality of kr<sup>enu</sup>/kr<sup>enu</sup> neonates. First, during later embryonic stages and throughout adulthood, Kreisler is expressed in a wide range of tissues in a cell type-specific manner. In the original viable kr mutation, a submicroscopic chromosomal inversion abrogates Kreisler expression in the fifth and sixth rhombomeres of the embryonic hindbrain. All other domains of Kreisler expression examined in 8.5- to 11.5- and 14.5-dpc embryos and in adult tissues by RNA in situ and Northern hybridization were equivalent in normal and kr/kr animals (data not shown) (Eichmann et al., 1997). Secondly, the defects in hindbrain patterning are less severe in kr<sup>enu</sup>/kr<sup>enu</sup> than in kr/kr embryos (V.S.S., A. Sing, L. Mar, F.J., and S.P.C., unpublished observations). Finally, we have shown previously that Kreisler activates transcription of Hoxb3 in the fifth rhombomere directly (Manzanares et al., 1997). Kreisler-dependent transcription of Hoxb3 in the fifth rhombomere cannot be detected in either kr/kr or kr<sup>enu</sup>/kr<sup>enu</sup> embryos (S.P.C., unpublished observations; Frohman et al., 1993). Thus, transcriptional activation that depends on the presence of active Kreisler homodimers appears to be abrogated or severely reduced in kr<sup>enu</sup> homozygotes.

**RESULTS**

**Neonatal Lethality of kr<sup>enu</sup>/kr<sup>enu</sup> Homozygotes**

The kr<sup>enu</sup> mutation was identified in a noncomplementation mutagenesis screen by the circling behavior and deafness of kr/kr<sup>enu</sup> mice. However, no circling deaf mice have ever been recovered from kr<sup>enu</sup>/+ intercrosses (Cordes and Barsh, 1994). We used an Earl restriction site, which was created by the A-to-G transition responsible for the kr<sup>enu</sup> mutation, in a polymerase chain reaction-based assay to unambiguously identify kr<sup>enu</sup>/kr<sup>enu</sup> and kr<sup>enu</sup>/+ mice (Figs. 1A and 1B). We observed the expected Mendelian ratios of +/+, kr<sup>enu</sup>/+, and kr<sup>enu</sup>/kr<sup>enu</sup> animals at all stages of embryonic development and at birth. kr<sup>enu</sup> heterozygotes appeared indistinguishable from normal animals (Fig. 1C). Even though many kr<sup>enu</sup>/kr<sup>enu</sup> neonates suckled initially, all died severely dehydrated within 24 h of birth.

**Morphologic Abnormality of kr<sup>enu</sup>/kr<sup>enu</sup> Kidneys**

We observed a variety of morphologic abnormalities, which include enlarged ventricles in the brain and abnor-
normal kidneys, that in concert would compromise neonatal viability of kr
kr neonates. Most strikingly, kr/kr neonates can be readily distinguished from normal and heterozygous littermates by the appearance of their kidneys (Figs. 2A and 2B). Kidneys from kr/kr homozygotes appear shrunken and have punctate cortical surfaces with obvious superficial areas of hemorrhaging (Fig. 2B). Within the kidney, Kreisler expression commences specifically in glomeruli at 14.5 dpc and is maintained in adults (Figs. 2C and 2D). The renal vesicle, from which the nephron arises, proceeds through four morphologically identifiable and defined developmental stages: the vesicle or comma-shaped body, the S-shaped body, the capillary loop, and the mature stages (Abrahamson, 1991). Kreisler mRNA is not detected in glomeruli at the comma- or S-shaped stages, which are located near the outer margin of the cortex in the nephrogenic zone (Figs. 2C and 2D) (Eichmann et al., 1997; Imaki et al., 2000). Kreisler is expressed exclusively in the podocytes of the more internally located capillary loop-stage and mature glomeruli. Because members of the Maf subfamily of bZIP transcription factors, such as Kreisler, play key roles in cellular differentiation, we examined kidneys from kr homozygotes further for possible defects in podocyte differentiation.

Renal Function in Neonatal kr/kr Mice

To further evaluate renal function in kr/kr mice, we carried out a series of clinical laboratory and histologic studies. A standard urinalysis revealed an absence of urinary sediment but the presence of proteinuria and occasional hematuria by the appearance of their kidneys (Figs. 2A and 2B). Kidneys from kr/kr homozygotes appear shrunken and have punctate cortical surfaces with obvious superficial areas of hemorrhaging (Fig. 2B). Within the kidney, Kreisler expression commences specifically in glomeruli at 14.5 dpc and is maintained in adults (Figs. 2C and 2D). The renal vesicle, from which the nephron arises, proceeds through four morphologically identifiable and defined developmental stages: the vesicle or comma-shaped body, the S-shaped body, the capillary loop, and the mature stages (Abrahamson, 1991). Kreisler mRNA is not detected in glomeruli at the comma- or S-shaped stages, which are located near the outer margin of the cortex in the nephrogenic zone (Figs. 2C and 2D) (Eichmann et al., 1997; Imaki et al., 2000). Kreisler is expressed exclusively in the podocytes of the more internally located capillary loop-stage and mature glomeruli. Because members of the Maf subfamily of bZIP transcription factors, such as Kreisler, play key roles in cellular differentiation, we examined kidneys from kr homozygotes further for possible defects in podocyte differentiation.

Podocyte Foot process Fusion in kr/kr Kidneys

Standard histologic analysis revealed no gross malformations at any stage of kidney development in kr/kr animals (Figs. 3A–3D). The nephrogenic zone, which is located near the external surface of the cortex and in which glomerular development begins, appeared normal in kr/kr animals. The stroma, proximal and distal tubules, ureter, and organization of nephrons in kr/kr kidneys appeared indistinguishable from those in normal kidneys (Figs. 3A and 3B). We had initially noticed the shrunken and spotted appearance of kidneys in kr homozygotes. In mice homozygous for mutations in either PDGFβ or the PDGFβ receptor, a “spotted kidney” phenotype is seen and is caused by the absence of mesangial cells and abnormal vasculogenesis (Heilstrom et al., 1999; Leveen et al., 1994; Soriano, 1994). However, in kr/kr animals, mesangial cells and capillaries within glomeruli themselves are present and appear ultrastructurally normal (Fig. 3). Furthermore, no hemorrhaging or accumulation of blood is seen within glomeruli. Podocytes appear histologically normal as well (Figs. 3C and 3D). Finally, no defects in branching morphogenesis of the ureteric bud epithelium of 13.5- or 15.5-dpc kidneys were detected by DBA-lectin staining (data not shown).

A glomerular protein leak, such as that observed in kr homozygotes, is most often caused either by defects in the formation or maintenance of the glomerular basement membrane or by effacement of podocyte foot processes. Therefore, we examined neonatal kr/kr kidneys for ultrastructural defects by electron microscopy (Figs. 3E–3H). In the normal glomerulus, the podocyte and the GBM are responsible for maintaining the filtration slit structure that prevents protein escaping from the capillaries. The podocyte performs these functions by virtue of a complex array of interdigitating foot processes, or pedicles, that create the large intercellular surface area required for glomerular filtration and are located distant from the podocyte cell body. During the early capillary loop stage, when Kreisler expression is first observed, the epithelial cytoplasm appears as a continuous, uninterrupted mass without any foot processes (Abrahamson, 1991; Kriz et al., 1994). As podocyte differentiation proceeds, the epithelium begins to interdigitate until the glomerular basement membrane is completely fringed by fully formed and distinct pedicles. Electron microscopy did not uncover any defects in the GBM. However, in kr homozygotes, podocytes were present, but podocyte foot processes were absent or “fused” (Figs. 3E–3H). The epithelial cytoplasm is continuous and uninterrupted, and in essence, the podocytes in mature kr glomeruli appear to be arrested in the capillary loop stage of normal development.

Podocytes Form Normally in Kidneys from kr Homozygotes

At times, point mutations can compromise either protein stability or protein localization. To examine whether Kreisler protein was expressed at normal levels and within the nucleus of mutant podocytes, we performed immunofluorescence with an antibody raised against full-length mouse Kreisler protein (Figs. 4A and 4B). These immunofluorescence experiments detected nucleyar localized Kre-
FIG. 4. Kreisler protein is present in krenu homozygotes, but absent in Pod1−/− kidneys. Immunofluorescence detects Kreisler protein in the nuclei of podocytes of +/+ (A) and krenu/krenu (B) mutant neonatal kidneys at the capillary loop stage. Immunofluorescence with an anti-integrin α3 antibody detects equivalent expression in the podocytes of normal (+/+)(C) and krenu/krenu (D) neonatal kidneys. Laminin β2 is present in the GBM of normal (+/+)(E) and krenu/krenu (F) glomeruli. PCNA cannot be detected in podocytes of +/+ (G) and krenu/krenu (H) neonatal kidneys at the capillary loop stage. Autofluorescence of erythrocytes is seen in both (G) and (H). (I) The DAPI counter-stained +/+ glomerulus seen in (G). (J) The DAPI counter-stained krenu/krenu glomerulus seen in (H) is shown. (L) Autofluorescence of erythrocytes can be seen in the absence of anti-PCNA antibody, as shown here in a +/+ glomerulus. The DAPI counter-stained glomerulus for (L) is shown in (K). Scale bars represent 20 μm. Each pair of panels is shown at the same magnification in normal and mutant animals.

FIG. 5. Expression of podocyte-specific genes in normal and mutant kidneys. Digoxigenin RNA in situ hybridization experiments were performed on cryosections from +/+ (A, C, E, G, K) and krenu/krenu (B, D, F, H, L) neonatal kidneys. α-Actinin 4 is expressed in normal (A) and mutant (B) animals starting at the S-shaped stage. S-shaped-stage glomeruli are outlined with a dashed line. Nephrin is expressed at equivalent levels in podocytes of normal (C) and krenu/krenu (D) neonatal kidneys. CD2AP is expressed in both +/+ (E) and krenu/krenu (F) podocytes. Podocin expression appears slightly lower in krenu/krenu (H, J) podocytes than in normal (G, I) podocytes. Pod1 is expressed equivalently in +/+ (K) and krenu/krenu (L) neonatal kidneys. Experiments were performed on 15.5-dpc and neonatal kidneys. Neonatal kidneys are shown here. All experiments were performed in triplicate on independent +/+, +/krenu, and krenu/krenu kidneys. Scale bars represent 20 μm in all panels except for (I) and (J). In (I) and (J), the scale bars represent 100 μm.
isler protein at equivalent levels in normal and mutant podocytes at the capillary loop stage. Thus, the kr\textsuperscript{enu} mutation does not destabilize Kreisler protein.

The basal membrane of podocyte foot processes (the "sole") attaches to the extracellular matrix of the GBM (Abrahamson, 1991). Numerous proteins, including Integrin α3β1, are localized to the "soles" of podocyte foot processes and are essential for the maturation of podocytes. Integrin α3-deficient mice, for instance, do not form foot processes (Kreidberg et al., 1996). In kr\textsuperscript{enu}/kr\textsuperscript{enu} podocytes, Integrin α3 is expressed and localized to the cell membrane much as in normal podocytes as detected by immunofluorescence with anti-Integrin α3 antibody (Figs. 4C and 4D).

The "soles" of the podocyte foot processes attach to the GBM, and defects in the GBM can cause podocyte foot process effacement. In vivo labeling and ultrastructural studies indicate that dual membranes produced by the podocyte and the glomerular endothelial cells fuse to form the GBM during the capillary loop stage. In mice lacking Collagen IV or Laminin α5, defects in the ultrastructural appearance of the GBM coincide with the fusion and loss of podocyte foot processes (Cosgrove et al., 1996; Lu et al., 1999; Miner and Li, 2000). In the absence of some GBM components, such as Laminin α5, an intact GBM is never formed, and hence, podocyte foot processes can never attach to the GBM. Other molecules, such as Collagen COL4A3, and Laminin β2, appear to be required not for the initial formation of the GBM, but for its maintenance. For example, at birth, both the GBM and podocyte foot processes appear normal in mice lacking either Col4A3 or Laminin β2. GBM defects and pedicle loss only become apparent at 4 weeks of age in mice with loss-of-function

![FIG. 6. Nephrin and Podocin are expressed at slightly lower levels in neonatal kr\textsuperscript{enu}/kr\textsuperscript{enu} kidneys. (A) Levels of mRNA of murine homologues of kidney disease genes were examined by RNA slot blot analysis. Each well contains 10 \textmu g of total RNA from the following tissues: lane 1, neonatal +/+ kidney; lane 2, neonatal kr\textsuperscript{enu}/+ kidney; and lane 3, kr\textsuperscript{enu}/kr\textsuperscript{enu} neonatal kidney. Nephrin, Podocin, and α Actinin 4 are all expressed in normal, heterozygous, and homozygous kr\textsuperscript{enu} mice. (B) Densitometry was used to quantify RNA levels. Levels of Podocin and Nephrin were slightly lower in kidneys from kr\textsuperscript{enu}/kr\textsuperscript{enu} animals relative to their +/+ and kr\textsuperscript{enu}/+ littermates. Levels of α Actinin 4 are approximately equivalent in normal and mutant kidneys. (C–F) Localization of Podocin and Nephrin proteins was examined by immunofluorescence. Podocin protein is present in podocyte membranes from normal (+/+) (C) and kr\textsuperscript{enu}/kr\textsuperscript{enu} (D) kidneys as detected by immunofluorescence with an anti-Podocin antibody. Nephrin is localized to the membranes of normal (E) and kr\textsuperscript{enu}/kr\textsuperscript{enu} (F) podocytes as detected with immunofluorescence with an anti-Nephrin antibody. The scale bar represents 20 \textmu m. All panels are shown at the same magnification. The Role of Kreisler (Krml1/MafB) in Kidney Disease © 2002 Elsevier Science (USA). All rights reserved.](image-url)
mutations in collagen COL4A3, a mouse model for human autosomal Alport syndrome, and at 2 weeks in mice lacking Laminin β2 (Cosgrove et al., 1996; Noakes et al., 1995).

In kr<sup>enu</sup>/kr<sup>enu</sup> kidneys, the GBM appears normal when examined by transmission electron microscopy (Figs. 3G and 3H). Notably, at present, only in mice lacking Laminin β2 is foot process effacement observed prior to any visible ultrastructural abnormalities in the GBM. In all other known “GBM” mutants, foot processes appear normal as long as the GBM appears ultrastructurally intact, as it does in kr<sup>enu</sup>/kr<sup>enu</sup> kidneys. Laminin β2, which is expressed starting at the capillary loop stage, can be detected in the GBM of both normal and mutant glomeruli by immunofluorescence (Figs. 4E and 4F). Current analyses of mouse mutants in GBM components have not identified any single mutation with a phenotype identical to that observed in kr<sup>enu</sup>/kr<sup>enu</sup> glomeruli.

Previously, disruption of the cMaf gene has been shown to arrest differentiation of lens fiber cells. The absence of cMaf protein results in the failure of lens fiber cells to withdraw from the cell cycle (Ring et al., 2000). In kr<sup>enu</sup> homozygotes, we did not detect mitotic figures in any podocytes examined by transmission electron microscopy. Furthermore PCNA is expressed in actively dividing cells, but cannot be detected in podocytes from either normal or kr<sup>enu</sup>/kr<sup>enu</sup> neonates by immunofluorescence (Figs. 4G–J). Thus, in contrast to lens fiber cells in cMaf knock-outs, kr<sup>enu</sup>/kr<sup>enu</sup> podocytes do not re-enter the cell cycle appropriately. These observations suggest that the kr<sup>enu</sup> mutation disrupts the ability of Kreisler to transcribe molecules required specifically for podocyte differentiation, but does not affect any potential role that the Kreisler protein may play in cell cycle withdrawal during podocyte differentiation.

Expression of Murine Orthologues of Human Kidney Disease Genes in kr<sup>enu</sup>/kr<sup>enu</sup> Podocytes

Recently, advances in human genetic kidney diseases have identified additional molecules required for podocyte function and the formation or maintenance of pedicles. Mutations in ACTN4, the gene encoding α-Actinin 4, cause dominant familial focal segmental glomerulosclerosis (FSGS) (Kaplan et al., 2000). α-Actinin 4 is an actin filament cross-linking protein thought to regulate the actin cytoskeleton of podocytes. In vitro the dominant mutations identified in familial FSGS bind filamentous actin more tightly than normal α-Actinin 4 protein does. It is not known whether α-Actinin 4 is required to maintain foot processes and the slit diaphragm or whether increased rigidity of the foot processes might make them more vulnerable to damage. Expression of α-Actinin 4 commences in podocytes of the late S-shaped body and early capillary loop stages well before that of Kreisler. We examined expression of murine α-Actinin 4 in kr<sup>enu</sup>/kr<sup>enu</sup> kidneys by RNA in situ hybridization and RNA slot blot analyses. We found no difference in the temporal and spatial expression nor in the level of expression of α-Actinin 4 between normal and kr<sup>enu</sup>/kr<sup>enu</sup> kidneys (Figs. 5A, 5B, and 6A). Thus, although it is possible that Kreisler acts in parallel with the genes that regulate α-Actinin 4, we believe that Kreisler acts at a slightly later stage, and in the future may serve as a marker for the mid-capillary loop to mature stages.

A highly unique feature of podocyte foot processes is the presence of the “slit diaphragm.” The slit diaphragm is inserted between foot processes just above their “sole” and thus joins podocyte foot processes laterally. A complex that contains three known proteins, Nephrin, Podocin, and CD2 adapter protein, is required for slit diaphragm function and assembly. Nephrin, the gene mutated in congenital nephrotic syndrome of the Finnish type (NPHS1), encodes a transmembrane protein of the Immunoglobulin superfamily that is localized at the slit diaphragm joining the interdigitated pedicles (Kestila et al., 1998). Mutations in Nephrin cause severe proteinuria and usually lead to death within the first 2 years of human life (Donoviel et al., 2003; Kestila et al., 1998). Nephrin expression in humans and mice has been detected in podocytes of the capillary loop stage glomeruli, while in rats, Nephrin mRNA is already present in S-shaped bodies (Kawachi et al., 2000; Putaala et al., 2000). In addition, in the mouse, CD2 adapter protein (CD2AP) has been identified as another component of the slit diaphragm. CD2AP is a Src homology 3 domain-containing protein that interacts directly with Nephrin and is required for assembly of the slit diaphragm in the mouse (Lehtonen et al., 2000; Li et al., 2000). In mutant mice lacking CD2AP, podocyte foot processes form initially, but begin to efface starting 1 week after birth. Podocin was identified as the gene mutated in autosomal recessive steroid-resistant nephrotic syndrome (NPHS2) on chromosome 1q25–31 and is first expressed at the capillary loop stage. The Podocin protein is structurally related to the integral membrane phosphoprotein stomatin.

We examined expression of these components of the slit diaphragm in +/+ , +/kr<sup>enu</sup>, and kr<sup>enu</sup>/kr<sup>enu</sup> kidneys by RNA in situ hybridization and RNA slot blot analyses. We found no difference in the temporal and spatial expression of Nephrin, Podocin, or CD2AP between normal and kr<sup>enu</sup>/kr<sup>enu</sup> kidneys (Figs. 5C–5I). Although the level of Podocin expression was indistinguishable in the cranial ganglia of normal, heterozygous, and homozygous mutant embryos at 15.5 dpc (data not shown), Podocin expression consistently appeared lower in kr<sup>enu</sup>/kr<sup>enu</sup> kidneys. In RNA in situ hybridization experiments, Podocin expression can normally be detected after 6–8 h of color development in neonatal kidneys, but could be detected only upon 12–18 h of color development in RNA in situ hybridization in mutant kidneys, and even then the observed level appears reduced (Figs. 5G–5J). We examined mRNA levels of Podocin, Nephrin, and α Actinin 4 in RNA slot blots to determine whether these were affected in kr<sup>enu</sup>/kr<sup>enu</sup> mutants (Figs. 6A and 6B). The levels of Nephrin and Podocin mRNA appear slightly reduced in kr<sup>enu</sup>/kr<sup>enu</sup> kidneys. When we quantified the levels of Nephrin and Podocin mRNA by
densitometry, Podocin and Nephrin mRNA levels are reduced to 76 ± 7 and 62 ± 9%, respectively, in kr<sup>enu</sup> homozygotes relative to their heterozygous and +/+ littermates. Levels of α-Actinin 4 RNA in neonatal kidneys appear equivalent between kr<sup>enu</sup> homozygotes, heterozygotes, and unaffected littermates. By immunofluorescence with anti-Podocin and anti-Nephrin antibodies, we determined that Podocin and Nephrin proteins are localized to the membranes of both normal and kr<sup>enu</sup>/kr<sup>enu</sup> podocytes (Figs. 6C–6E). Thus, transcription of Podocin and Nephrin does not depend on active Kreisler homodimers, but indirectly or directly, Kreisler may have a small effect on the transcription or stability of Podocin and Nephrin mRNAs. In the future, identification of direct Kreisler targets during podocyte differentiation may reveal additional proteins important in glomerular function and possibly in kidney disease.

**Kreisler Acts Downstream of the Pod1 Transcription Factor**

To begin to dissect the transcriptional hierarchy that governs podocyte differentiation, we examined the expression of Kreisler in mice homozygous for a mutation in the basic helix-loop-helix transcription factor Pod1 (epicardin/capsulin). Pod1 is expressed in mesenchymal tissues of the developing kidney and in podocytes (Hidai et al., 1998; Lu et al., 1998; Quaggin et al., 1998; Robb et al., 1998). Disruption of Pod1 causes podocytes to remain columnar shaped and to appear as though they are arrested in the S-shaped stage. A few rare primitive foot processes can be detected (Quaggin et al., 1999). This phenotype is significantly more severe than that observed in kr<sup>enu</sup>/kr<sup>enu</sup> kidneys, where overall glomerular differentiation appears to be unaffected and podocytes do not appear columnar-shaped, but are simply not developing foot processes. At present, it is unclear whether the more severe phenotype in Pod1 mutants is a consequence of the podocyte-specific role of Pod1 or because of a defect in mesenchymal cells that surround the glomerulus (Quaggin et al., 1998). In kr<sup>enu</sup>/kr<sup>enu</sup> animals, Pod1 was expressed normally in the podocytes of 15.5-dpc and neonatal kidneys (Figs. 5K and 5L). However, when we examined glomeruli from Pod1<sup>−/−</sup> mutant mice, we could not detect appreciable amounts of Kreisler protein in the podocytes (Fig. 7). Taken together, these observations suggest that Pod1 acts upstream of Kreisler in podocyte differentiation and that Pod1 may have a podocyte-specific cell-autonomous role.

**DISCUSSION**

Aside from Kreisler, a variety of other transcription factors play important roles in glomerular development. All of these appear to act at earlier stages of kidney development or podocyte differentiation than Kreisler does. Genetic experiments have implicated the Wilms's tumor suppressor gene, WT1, in podocyte development (Dressler et al., 1993). Patients with Denys-Drash syndrome, which is associated with specific heterozygous mutations of WT1, are not only at increased risk for developing Wilms's tumor, but also develop a nephropathy associated with grossly abnormal podocyte morphology (Péletier et al., 1991). Mice homozygous for a null allele of WT1 fail to develop kidneys; therefore, a possible later role of WT1 in podocyte development has not yet been assessed (Kreidberg et al., 1993). WT1 protein is detected in podocytes of the early comma-shaped glomerulus well in advance of Kreisler expression and is maintained throughout podocyte development and in mature podocytes. Our results would predict that the dedifferentiated tumorigenic state in Wilms’s tumors and in Denys-Drash syndrome may be associated with down-regulation of Kreisler. Assays of Kreisler expression might, in the future, serve as prognosticators of tumor progression. In contrast to humans, no increased occurrence of renal tumors is observed in mice heterozygous for a WT1 null mutation, and similarly we have not observed tumors or cysts in kidneys from adult kr<sup>enu</sup> heterozygotes.

As a bZip protein, Kreisler acts by forming complexes with other transcription factors and other bZip proteins in particular. At present, the only other bZip protein with a known role in kidney development is EYA1, a mammalian homologue of the eyes absent (eya) gene from Drosophila melanogaster (Abdelhak et al., 1997). In humans, mutations in EYA1 are responsible for the autosomal dominant branchio-oto-renal (BOR) syndrome, which is characterized by varying combinations of branchial, outer, middle, and inner ear, and renal anomalies. In the mouse, Eya1<sup>−/−</sup> animals show renal abnormalities and a conductive hearing loss equivalent to human BOR syndrome, while Eya1<sup>+/−</sup> mice lack ears and kidneys due to defective inductive tissue interactions and apoptotic regression of the organ primordia (Johnson et al., 1999; Xu et al., 1999). Eya1 expression is observed in the condensing mesenchymal cells of the kidney and consistent with the excretory and collecting system anomalies of BOR syndrome. We and others have not detected expression of EYA1 in mouse podocytes (data not shown), nor have specific podocyte defects been reported in BOR syndrome patients (Kalatzis et al., 1998). This lack of coexpression in podocytes precludes a biologically relevant interaction during glomerular development, and thus, Kreisler is likely to interact with other as yet unidentified proteins to regulate podocyte specific transcription.

Mutations in Lmx1b, which in humans are responsible for Nail patella syndrome, cause thickening and gaps in the GBM and an apparent lack of foot processes (Chen et al., 1998; Dreyer et al., 1998). Patients with nail patella syndrome, which is localized on chromosome 9q34, have a significantly thickened GBM, and renal insufficiency with proteinuria occurs in 30% of all patients. Lmx1b expression commences in the S-shaped body, and thus, prior to that of Kreisler. In the rod plate of the neural tube, Kreisler is downstream of Lmx1a, which is mutated in the classical mouse dreher mutation (Millonig et al., 2000). Taken
Kreisler protein is absent in kidneys in the podocytes of Pod1−/− mice. While Kreisler protein is present in podocytes from mice heterozygous for a mutation in Pod1 (A, C), it cannot be detected in podocytes from Pod1−/− mice (B, D) by immunofluorescence with anti-Kreisler antibody. All panels are shown at the same magnification. The scale bar represents 20 μm. Normal (C) and krenu/krenu (D) glomeruli that are shown in (A) and (B), respectively, were counter-stained with DAPI.

Schematic diagram of podocyte differentiation. At the S-shaped stage of glomerular development, podocytes appear cuboidal and in their appearance resemble those observed in Pod1−/− mice. At this stage, Pod1 and Lmx1b are expressed. α-Actinin 4 expression is also seen at this stage. During the transition from the S-shaped to the capillary loop stage, the composition of the GBM changes. Lmx1b regulates the expression of some of the components of the GBM. At the capillary loop stage, the GBM has fused, for the most part, into one thick membrane. During maturation of the podocyte, the foot processes form on the outer GBM, until the GBM is completely fringed by foot processes. During the capillary loop stage, podocytes begin to express Nephrin, Podocin, and CD2AP. In kidneys from mice homozygous for the krenu mutation, foot processes cannot form because of a defect in podocyte differentiation. However, the GBM appears electron-microscopically normal and Nephrin, Podocin, CD2AP, and α-actinin 4 are expressed in these animals. The severity of the defect, the appearance of the podocytes, and the time of expression all suggest that Kreisler acts directly or indirectly downstream of Pod1 and Lmx1b, and indeed Kreisler protein can no longer be detected at normal levels in podocytes from Pod1−/− mice. Electron microscopic cross-section through a mature podocyte shows the foot processes extending from the podocyte cell body. P, podocyte (blue); c, capillary; m, mesangial cell (solid black); blue denotes early GBM, which differs in its composition from the fused glomerular basement membrane in the capillary loop stage, shown in red.
together, these observations suggest that Kreisler acts at a later stage of podocyte differentiation and may be downstream, possibly directly, of Lmx1b. It is quite possible that some of the roof plate-specific and podocyte-specific regulatory elements of Kreisler may overlap.

Our analyses have revealed that Kreisler acts downstream of the Pod1 bHLH transcription factor. In Pod1−/− mice, glomerular maturation is arrested well before the podocyte-specific expression of Kreisler would normally commence. In Pod1−/+ mice, the podocytes appear columnar-shaped and no foot processes form (Quaggini et al., 1999). In kr<sup>enu</sup> homozygotes, the morphology of podocytes appears normal, but foot processes do not extend. Taken together, these observations suggest that Kreisler is more likely to be an indirect rather than a direct target of the Pod1 transcription factor. Furthermore, Kreisler is required for the final stage in podocyte differentiation, the progression through the capillary loop stage to the mature glomerulus. Thus, Kreisler regulates podocyte differentiation during the capillary loop stage and governs such processes as either the extension or attachment of the foot processes or possibly the assembly of the filtration apparatus.

While we could assess the role of Kreisler in initial podocyte differentiation, we could not analyze its role in maintenance of functional podocytes or in recovery from podocyte damage. For example, in neonatal kidneys, Kreisler is not required for the initial production or fusion of the GBM, and the GBM appears ultrastructurally normal. However, Kreisler may play a role in the later podocyte-specific generation of GBM in mature podocytes. Such a role would only be uncovered during maturation and aging of the animals. In that case, inactivation of Kreisler in adult mice might cause severe nephrotic syndrome within 4–8 weeks or prevent animals from recovering after short-term podocyte insults. Thus, conditional or inducible kidney-specific alleles of Kreisler would be invaluable for further understanding of podocyte development and renal disease.

The levels of two known components of the filtration apparatus, Nephrin and Podocin, may be slightly reduced in kr<sup>enu</sup> homozygotes, but these genes are not likely to be direct targets of Kreisler. This is especially true of Nephrin, which has been detected in other animals in podocytes of the S-shaped stage, and thus before the appearance of Kreisler expression. The slightly reduced expression of Podocin and Nephrin may reflect the reduced overall membrane surface area of podocytes arrested at the capillary loop stage. Thus, a dynamic feedback loop that senses the surface area of the membrane may help regulate the amount of these proteins.

The podocyte-specific target genes of Kreisler remain to be identified. We expect most of these to be distinct from those regulated by Kreisler in the hindbrain. For example, we could not detect expression of Hoxa3 or Hoxb3, two known Kreisler target genes in the hindbrain, in podocytes at any stage of glomerular development (S.P.C., unpublished observations; Mansour et al., 1988; Manzanares et al., 1997). Conversely, we and others have not detected expression of podocyte-specific genes in the fifth and sixth rhombomeres of the embryonic hindbrain. The list of known genes with podocyte-specific expression patterns has been expanding rapidly, and testing each known podocyte-specific gene for its expression in kr<sup>enu</sup> mutants is rapidly becoming a daunting task. Hence, podocyte-specific targets of Kreisler may perhaps best be identified in future subtractive hybridization or microarray experiments. Overall, our results suggest that such experiments would identify additional components important for podocyte function and in human kidney disease.

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