Podocyte-Specific Deletion of Integrin-Linked Kinase Results in Severe Glomerular Basement Membrane Alterations and Progressive Glomerulosclerosis

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Alterations in glomerular podocyte cell–cell and cell–matrix contacts are key events in progressive glomerular failure. Integrin-linked kinase (ILK) has been implicated in podocyte cell–matrix interaction and is induced in proteinuria. For evaluation of ILK function in vivo, mice with a Cre-mediated podocyte-specific ILK inactivation were generated. These mice seemed normal at birth but developed progressive focal segmental glomerulosclerosis and died in terminal renal failure. The first ultrastructural lesions that are seen at onset of albuminuria are glomerular basement membrane (GBM) alterations with a significant increase in true harmonic mean GBM thickness. Podocyte foot process effacement and loss of slit diaphragm followed with progression to unselective proteinuria. No significant reduction of slit membrane molecules (podocin and nephrin), key GBM components (fibronectin, laminins, and collagen IV isoforms), or podocyte integrins could be observed at onset of proteinuria. However, α3-integrins were relocalized into a granular pattern along the GBM, consistent with altered integrin-mediated matrix assembly in ILK-deficient podocytes. As the increased GBM thickness precedes structural podocyte lesions and key components of the GBM were expressed at comparable levels to controls, these data suggest an essential role of ILK for the close interconnection of GBM structure and podocyte function.

Kidney function depends on an intact glomerular filtration barrier retaining macromolecules in the blood and allowing the excretion of potentially hazardous small molecules into the urine. The glomerulus is composed of three different cell types (mesangial cells, endothelial cells, and podocytes) that are anchored in the specialized extracellular matrix (ECM) of the glomerular basement membrane (GBM) and the mesangial matrix. The GBM is a gel-like meshwork of cell adhesive glycoproteins, including collagen IV, fibronectin, laminin, and proteoglycans (1). Endothelial cells and glomerular epithelial cells (podocytes) are attached via specific receptors, integrins, to the GBM. Integrins are heterodimeric transmembrane molecules that are assembled of α- and β-chains. Integrins mediate cell adhesion and link the ECM to the cell’s cytoskeleton. Each glomerular cell type expresses a specific combination of α- and β-subunits that determine the specificity to ECM ligands and define cellular phenotypes, including proliferation, differentiation, survival, and ECM assembly (2,3).

In the glomerular capillaries, the arteriolar intravascular pressure forces the plasma through a sequential sieve. The sieve consists of the endothelial fenestrae, the meshwork of the GBM and the slit diaphragm that connects interdigitating podocyte foot processes. A tightly controlled interaction of the podocyte foot processes with each other and with the matrix components of the GBM is crucial to maintain the filtration barrier against this high transcapillary pressure gradient. The cytoskeleton in the foot processes of podocytes is highly dynamic and serves as a central scaffold for the integrity of the filtration barrier (4). Molecules that regulate the interaction of the cytoskeleton with
the extracellular matrix via podocyte matrix receptors therefore are considered to be of critical importance for an intact glomerular filtration barrier (5).

Integrin-linked kinase (ILK) is a good candidate for regulating integrin function in podocytes. Initial evidence of ILK activation in glomerular filtration barrier failure was obtained by a mRNA expression screen of glomeruli from children with congenital nephrotic syndrome of the Finnish type (6). Furthermore, Wu et al. (7) found an activation of ILK in human diabetic glomeruli and in isolated glomerular mesangial cells that were cultured with high glucose medium. We previously reported an induction of ILK in murine models of glomerular disease in glomeruli and microdissected podocytes (6). In vitro studies demonstrated a role of ILK for focal contact assembly, cell attachment, cytoskeletal organization, proliferation, and survival of podocytes (6,8–10).

These data suggest an important role of ILK for the function of the filtration barrier, serving multiple roles at the cell contact sites of podocyte foot processes. With the plethora of ILK function shown in vitro, the role of ILK in podocytes had to be evaluated in the glomerular context using genetic models. As ILK deletion results in early embryonic lethality, the Cre-Lox system was used for a podocyte-specific excision of ILK. The resulting phenotype was evaluated on a functional, structural, and molecular level to define the sequence of events that lead to the progressive filtration barrier failure seen with ILK deletion in podocytes.

Materials and Methods
Targeted Inactivation of ILK in Podocytes

For selective deletion of ILK in glomerular podocytes, transgenic mice that express Cre-recombinase specifically in podocytes were crossed with “floxed” mice, which contain loxP sites upstream of exon 5 and downstream of exon 12 of the ILK gene (11). 2.5P-Cre mice (podocinCre/Cre) with the Cre-recombinase cassette under the regulation of a fragment of the human NPHS2 promoter of the podocin gene, leading to podocyte-specific expression of the Cre-recombinase, were obtained from L.B. Holzman (University of Michigan, Ann Arbor, MI) (12). ILKflox/flox mice were provided by S. Dedhar (University of British Columbia, Vancouver, BC, Canada) (11). The podocinCre/Cre mice were crossed with homozygous floxed ILK mice. The F1 podocinCre+/−/ILKflox/flox mice were crossed to homozygous ILKflox/flox/podocin−/− mice, generating homozygous podocinCre+/−/ILKflox/flox mice (podolLK−/−).

Genotyping was performed by PCR as described (11–13), using the following oligonucleotide primers for the floxed ILK locus: Fr-Lox 5′-CAAGGCTGACATCAATGC-3′ and Rv-Lox 5′-GTGCCACCTG-GACATCAATGC-3′. All animal experiments were conducted according to institutional and national guidelines.

Isolation of RNA and Real-Time Reverse Transcription–PCR Analysis

Steady-state mRNA expression levels were quantified with real-time reverse transcription–PCR (RT–PCR) as described (8,14) using the ΔΔCt technique (15). Controls that consisted of ddH2O were negative in all runs. All real-time RT–PCR reagents were supplied by Applied Biosystems (Foster City, CA). Except for 18S rRNA, the primers were cDNA specific and did not amplify genomic DNA. PCR oligonucleotide primer and probes are given in Supplementary Table 1.

Urine and Plasma Analysis

Urine protein excretion was detected by SDS-PAGE followed by Coomassie blue staining and quantified using the Bradford colorimetric assay (BioRad, Munich, Germany), expressed as ratios to urine creatinine. Serum values were obtained from EDTA blood samples at time of killing for blood urea nitrogen, total protein, albumin, and cholesterol using a Hitachi autoanalyzer (Hitachi, Tokyo, Japan).

Histology and Morphometry

Mice were killed by ether inhalation, and tissue was fixed for histology via orthograde vascular perfusion with 3% glutaraldehyde and processed for plastic embedded light microscopy (16) and scanning and transmission electron microscopy as described previously (17).

The GBM thickness was determined by the orthogonal intercept method (18) as described by Ramage et al. (19). Using a transparent grid, the shortest distance between the endothelial cytoplasmic membrane and the outer lining of the lamina rara externa underneath the cytoplasmic membrane of the epithelial foot processes was measured with a logarithmic ruler (19) where gridlines transected the GBM. The apparent harmonic mean thickness (lh) was calculated, from which the true harmonic mean thickness (Th) was estimated by the following equation

\[
Th = \frac{8}{3\pi} \times \frac{10^4}{M} \times lh
\]

where M represents the final print magnification. On average, 800 intercepts determined in six glomeruli per animal (range: 660 to 980) were measured.

The filtration slit frequency (FSF) was determined by counting the number of epithelial filtration slits divided by the length of the peripheral capillary wall at the epithelial interface. On average, 324 filtration slits (range 265 to 387) were counted per mouse. The mean glomerular volume was determined from the mean glomerular profile area according to the method developed by Weibel and Gomez using a Videoplan image analysis system (Zeiss-Kontron, Munich, Germany) (20–22).

Immunohistology

Unfixed renal tissue was embedded in OCT compound (Miles Scientific, Naperville, CA), snap-frozen in a mixture of isopentane and dry ice, and stored at −80°C. Five- to 7-μm sections were placed on gelatin-coated slides and stored at −20°C until immunostaining. For fixations and primary antibodies used, see Supplementary Table 2 (references [41–48]). Confocal imaging was performed on a Zeiss LSM510 confocal microscope at ×600 magnification. Rabbit anti-chicken integrin α3 serum was a gift from Dr. C. Michael Dipersio (Albany Medical College, Albany, NY), and rat anti-mouse entactin clone ELM1 was purchased from Chemicon (Temecula, CA). Alexa-Fluor 488– and Cy3-conjugated goat anti-rabbit antibodies were used as secondary antibodies (Molecular Probes, Eugene, OR; and Invitrogen, Carlsbad, CA). Negative controls were performed concurrently by substituting buffer or isotype control immunoglobulins (rabbit primary antibody isotype control; Invitrogen) for the primary antibody.

Statistical Analyses

Data are given as mean ± SD. A minimum of four mice were used for each analysis, unless stated otherwise. Statistical analysis was performed by using Mann-Whitney U test (SPSS-PC Version 12; SPSS, Inc., Chicago, IL); significance was determined at P < 0.05.
Results
Podocyte-Specific Deletion of ILK

Because ILK null embryos die at time of implantation (23), mice with flanking loxP sites (floxed) of the ILK exons 5 to 12 (ILK^floxed/floxed) were crossed with mice that express the Cre-recombinase under the control of a podocyte-specific promoter (2.5P-Cre^{+/−}) for targeted ILK inactivation in podocytes (Figure 1A). Resulting bitransgenic mice were crossed with homozygous ILK^floxed/floxed mice to obtain mice with the genotype ILK^floxed/floxed/2.5P-Cre^{+/−} (called podoILK^{−/−}).

To evaluate the ability of the podocin-Cre recombinase to excise the floxed ILK alleles, we examined kidney cortex DNA by PCR, and the excised floxed ILK allele was detected only in kidney but not tail DNA of ILK^floxed/floxed/2.5P-Cre^{+/−} mice, confirming tissue-specific recombination of the loxP sites within the ILK locus (Figure 1B). In microdissected glomeruli from podoILK^{−/−} mice, steady-state ILK mRNA was reduced by 74% compared with Cre-negative litter mates (podoILK^{+/+} 2.29 ± 1.00 × 10 to 3 (n = 7) versus podoILK^{−/−} 0.59 ± 0.53 × 10 to 3 (n = 4); ratios to 18S rRNA; Figure 1C).

Because ILK has been shown to be expressed in glomerular mesangial cells and glomerular podocytes (6,7), intraglomerular ILK protein expression was localized by immunohistochemistry. In podoILK^{+/+} mice, an ILK signal is seen in the mesangium and along the outer aspects of the GBM that are derived from the podocyte foot processes that are attached to the GBM. In podoILK^{−/−} mice, the mesangial staining remained unchanged, but the GBM-associated signal was lost, confirming loss of podocyte ILK expression (Figure 1D).

PodoILK^{−/−} Mice Show Rapid Progression of Proteinuria to Terminal Renal Failure

Mice of all genotypes were born at the expected Mendelian frequency. Heterozygous mice for the floxed ILK allele (ILK^floxed/−/2.5P-Cre^{+/−}) had no overt phenotype, and urine analysis showed no albuminuria up to 15 mo of age.

PodoILK^{−/−} mice seemed normal at birth but showed a drastically reduced life span with a median age of death at 19 wk (Figure 2A). Sequential urine analysis for proteinuria by Bradford assay and SDS-PAGE revealed the appearance of selective albuminuria at 2 to 4 wk of age, progressing to unselective proteinuria at 4 to 12 wk (Figure 2, B and C). Biochemical analysis at 12 wk was consistent with massive proteinuria and impaired renal function (blood urea nitrogen 79 ± 62 versus 27 ± 5 mg/dl; albumin 2.5 ± 0.4 versus 3.4 ± 0.3 g/dl; total cholesterol 400 ± 190 versus 78 ± 8 mg/dl; podoILK^{+/+} versus podoILK^{−/−}, n = 4 for each group, respectively). Macroscopically, 16-wk-old podoILK^{−/−} mice showed nephrotic, end-stage kidneys with rough surface and yellow appearance (Figure 3A).

PodoILK^{−/−} Mice Develop Progressive Focal Segmental Glomerulosclerosis

Histologic examination of kidneys from 1- to 16-wk-old mice revealed the development of progressive glomerulosclerosis in podoILK^{−/−} mice (Figures 3, A and B, and 4). Kidneys from 1- and 12-d-old podoILK^{−/−} mice showed the typical gradient of postnatal renal development and were indistinguishable from those of podoILK^{+/+} mice, consistent with normal nephrogenesis in podoILK^{−/−} mice.
At the onset of selective albuminuria at 2 to 3 wk of age, podoILK−/− mice showed occasionally prominent single podocytes, predominantly in juxtamedullary glomeruli. Mean glomerular volume, as a parameter for overall glomerular architecture, was not different between podoILK−/− and podoILK+/+ littermates at this stage (podoILK+/+ 91,300 ± 15,600 versus podoILK−/− 78,500 ± 8600 μm³; NS). At 4 wk, juxtamedullary glomeruli of podoILK−/− mice demonstrated prominent podocytes and occasional segmental mesangial expansion with increased matrix deposition and distorted capillaries.

At 12 wk of age, glomerulosclerosis with severe podocyte lesions, tuft adhesions to Bowman's capsule, crescent formation, mesangial expansion with increased matrix deposition, distorted capillaries, and glomerular obsolescence were found. Tubulointerstitial lesions included tubular atrophy, interstitial fibrosis, and mononuclear infiltration. Magnifications: ×10 in A.
and segmental sclerotic lesions evolved and was associated with tubulointerstitial changes. End-stage kidney lesions were characterized by diffuse glomerulosclerosis and tubulointerstitial inflammation and fibrosis. ILK<sup>flx/flx</sup>/2.5P-Cre<sup>+/−</sup> and 2.5P-Cre-negative littermates of the different age groups showed no renal pathology.

**PodoILK<sup>−/−</sup> Mice Show GBM Alteration at Onset of Albuminuria**

Because albuminuria preceded the development of the focal segmental glomerulosclerosis (FSGS) lesions, the ultrastructure of the filtration barrier was evaluated starting at the first sign of altered glomerular function in podoILK<sup>−/−</sup> mice. At 3 wk of age, juxtamedullary glomeruli of podoILK<sup>−/−</sup> mice showed single prominent podocytes compared with podoILK<sup>+/+</sup> mice. At 4 wk, juxtamedullary glomeruli of podoILK<sup>−/−</sup> mice demonstrated prominent podocytes with protrusions and microvillous transformation of the epithelial surface. At 12 wk, podocytes of podoILK<sup>−/−</sup> mice were enlarged and showed protrusions and microvillous transformation. Foot processes were effaced and covered a broadened GBM. Magnification, ×100.

At 8 to 12 wk of age, the podocyte lesions had progressed further in podoILK<sup>−/−</sup> mice with vacuolization, microvillous transformation, widespread foot process effacement, and focal detachment from the basement membrane. The GBM exhibited diffuse and irregular thickening and showed electron-lucent areas (Figure 5).

At 3 wk of age, juxtamedullary glomeruli of podoILK<sup>−/−</sup> mice showed single prominent podocytes compared with podoILK<sup>+/+</sup> mice. At 4 wk, juxtamedullary glomeruli of podoILK<sup>−/−</sup> mice demonstrated prominent podocytes with protrusions and microvillous transformation of the epithelial surface. At 12 wk, podocytes of podoILK<sup>−/−</sup> mice were enlarged and showed protrusions and microvillous transformation. Foot processes were effaced and covered a broadened GBM. Magnification, ×100.

**Slit Diaphragm and Associated Molecules Are Altered with Progressive Proteinuria in podoILK<sup>−/−</sup> Mice**

Because the podocyte slit diaphragm is considered to be a key element of the filtration barrier, electron microscopic analysis focused on slit diaphragm alterations at onset of albuminuria (3 wk) and could not detect ultrastructural alteration at this stage (Figures 5 and 6A). With progression to unselective proteinuria, loss of slit diaphragm and foot process effacement could be encountered. To further evaluate the slit diaphragm, we evaluated the expression level and localization of two key SD components, nephrin and podocin, in glomeruli from podoILK<sup>−/−</sup> mice with selective albuminuria compared with podoILK<sup>+/+</sup> littermates (1655.47 ± 107.21 versus 1714.18 ± 81.57 filtration slits/mm GBM; n = 4 mice in each group; NS).

With progressive proteinuria, podocyte foot process architecture deteriorated. Analysis of the mice with unselective proteinuria by scanning electron microscopic analysis revealed progressive podocyte changes with age, including multiple luminal microvilli and widespread flattening of the cell bodies and major processes, thereby covering major parts of the filtration area with rarely interdigitating foot processes (Figure 6B).

**Podocyte Matrix Receptors in podoILK<sup>−/−</sup> Mice**

Because ILK has been reported to be involved in matrix assembly via transmembrane matrix receptors (24), concentration and distribution of integrin-α3 and -β1 were evaluated. By immunofluorescence studies, no difference in the distribution...
of integrin-β1 in mesangial or endothelial cells and podocytes could be observed (data not shown). Confocal microscopy analysis found in wild-type mice a partial overlap of the podocyte-derived integrin-α3 signal with entactin staining marking the GBM. In 3-wk-old podoILK−/− mice, overall signal intensity for integrin-α3 seemed unchanged, but co-localization with entactin was lost, consistent with an ILK-dependant redistribution of this integrin at the onset of albuminuria (Figure 8).

GBM Composition in podoILK−/− Mice

To define the alterations in the molecular composition of the GBM, we evaluated the expression levels and distribution of the major GBM components by real-time RT-PCR and immunofluorescence. Analysis of laminin-α 1, 2, 4, and 5; laminin-β 1 and 2; collagen type IV α 1 through 6; agrin; perlecán; nidogen-1; and fibronectin did not reveal an increase on the mRNA and/or protein level for any of these molecules in 3-wk-old mice with selective albuminuria (Table 1, Figure 9). Also, no difference in GBM staining for murine IgA, IgG, or IgM was found (data not shown). However, in mice with progressive filtration barrier failure and glomerular scarring (12 wk of age), increased levels of fibronectin and collagen type I α 1 could be found, consistent with induction of these molecules during mesangial expansion and glomerulosclerosis (Table 1). The mRNA for the GBM molecules collagen IV α3 through 5, in contrast, were reduced in these advanced stages of glomerular damage.

Discussion

For maintaining an intact filtration barrier against the high transcapillary pressure gradient of the glomerulus, an intimate molecular cross-talk between podocyte foot processes and the GBM is crucial (25). α3/β1-Integrins are the main transmembrane matrix receptors of podocytes, and collagen IV and laminins are their GBM ligands. Integrins regulate cell function and
in 3-wk-old podoILK−/− mice, the true harmonic mean GBM thickness increased by 22.6% compared with podoILK−/− mice at onset of albuminuria. In orthogonal intercepts, the true harmonic mean GBM thickness increased by 22.6% in 3-wk-old podoILK−/− mice with selective albuminuria compared with podoILK+/+ (n = 4 mice in each group; P < 0.05, top). In contrast, in the same glomeruli, no significant difference of the FSF, determined as number of slit diaphragms encountered per millimeter of GBM, could be detected (n = 4 mice in each group; NS, bottom). In vivo.

Function had to be addressed in the glomerular environment (6,7), the physiologic role of ILK for podocyte adhesion and extracellular matrix assembly (6,9). Because glomerular filtration barrier loss has been linked to alterations in the slit diaphragm and podocyte foot process effacement, this crucial unit of the filter was studied at disease onset. In 3-wk-old mice with selective albuminuria, ultrastructural and molecular analysis could not detect visible alterations of the slit diaphragms (Figure 5). Elements of the podocyte cytoskeleton also were found to be normally expressed without ultrastructural evidence of significant podocyte foot process effacement as determined by FSF at the GBM (Figure 6A). These results, however, do not exclude a simultaneous role of ILK at the slit diaphragm, as interference with slit diaphragm function resulting in proteinuria has been demonstrated without out visible changes in ultrastructure (30). After progression to nonselective proteinuria, slit diaphragm loss and foot process effacement became evident (Figure 5). No detachments of podocytes from the GBM were seen, and transferase-mediated dUTP nick-end labeling staining did not reveal an increased staining of podocytes in podoILK−/− at 3 and 4 wk of age (data not shown), consistent with ILK’s not being essential for podocyte survival in vivo.

Figure 6. Ultrastructural analysis of the glomerular filtration barrier in podoILK−/− mice. (A) True harmonic mean GBM thickness but not filtration slit frequency (FSF) is altered in podoILK−/− mice at onset of albuminuria. In orthogonal intercepts, the true harmonic mean GBM thickness increased by 22.6% in 3-wk-old podoILK−/− mice with selective albuminuria compared with podoILK+/+ (n = 4 mice in each group; P < 0.05, top). In contrast, in the same glomeruli, no significant difference of the FSF, determined as number of slit diaphragms encountered per millimeter of GBM, could be detected (n = 4 mice in each group; NS, bottom). (B) Scanning electron micrographs of glomeruli from 12-wk-old mice. View of a capillary loop from Bowman’s space. Bar = 1 μm. (Left) PodoILK+/+. (Middle and Right) PodoILK−/−. Podocytes demonstrated a spectrum of changes from irregular foot process interdigitation (middle) to complete foot process effacement, cell body attenuation, and microversus transformation (right). Magnification, ×10,000 in B.

matrix assembly via a protein complex that is associated with their cytoplasmic tail in focal adhesion plaques (26). ILK has emerged as a multifunctional protein in this complex. In cultured podocytes, ILK has been shown to orchestrate a wide array of functions, including focal adhesion plaque assembly (6); F-actin cytoskeletal organization (6,9); membrane proximal initiation of signal transduction via Akt, GSK-3β, and β-catenin (6,8); regulating cell phenotype and survival (6,8,9); and integrin binding affinity and avidity that are responsible for podocyte adhesion and extracellular matrix assembly (6,9). Because ILK levels have been found to be induced in progressive glomerular damage (6,7), the physiologic role of ILK for podocyte function had to be addressed in the glomerular environment in vivo to understand the consequences of ILK induction in disease. Recent studies using genetic models have demonstrated a high degree of context dependence of ILK function. ILK deletion in Caenorhabditis elegans resulted in a phenotype that resembled some aspects of β1-integrin deletion, underscoring ILK as a key adaptor between the cytoskeleton and integrins (27). Complete knockout of ILK in chondrocytes (11,28) and endothelial cells in mice and zebra fish (29) demonstrated key roles of ILK for integrin-mediated cell adhesion and spreading, actin stress fiber formation, cell survival, and proliferation (11,28,29).

A podocyte-specific Cre-lox system was used to evaluate ILK function in the glomerular context in vivo (13). ILK excision was detectable at birth as mature glomeruli are found in the juxtaglomerular region of newborn mouse kidneys. At 3 wk of age, a loss of the ILK signal from podocyte foot processes at the GBM could be shown with unchanged ILK expression in the mesangium (Figure 1). ILK mRNA levels were reduced by 74%, consistent with detectability of the mesangial ILK mRNA in the glomerular preparation after Cre excision. PodoILK−/− mice developed the first functional alteration (selective albuminuria) 2 to 3 wk after birth (Figure 2). Albuminuria quickly progressed to nonselective proteinuria and progressive FSGS with terminal renal failure in all mice examined to date. This well-defined and easy-to-monitor onset of podocyte damage allowed the dissection of early events after ILK loss and to segregate them from late, unspecific lesions that were associated with FSGS. The delay between ILK genomic deletion and initially detectable functional alterations could be a consequence of a long ILK protein half-life in podocytes. Alternatively, ILK-negative podocytes could display a stable phenotype postpartum but decompensate with increasing demands on the filtration barrier in adolescence.

Because glomerular filtration barrier loss has been linked to alterations in the slit diaphragm and podocyte foot process effacement, this crucial unit of the filter was studied at disease onset. In 3-wk-old mice with selective albuminuria, ultrastructural and molecular analysis could not detect visible alterations of the slit diaphragms (Figure 5). Elements of the podocyte cytoskeleton also were found to be normally expressed without ultrastructural evidence of significant podocyte foot process effacement as determined by FSF at the GBM (Figure 6A). These results, however, do not exclude a simultaneous role of ILK at the slit diaphragm, as interference with slit diaphragm function resulting in proteinuria has been demonstrated without out visible changes in ultrastructure (30). After progression to nonselective proteinuria, slit diaphragm loss and foot process effacement became evident (Figure 5). No detachments of podocytes from the GBM were seen, and transferase-mediated dUTP nick-end labeling staining did not reveal an increased staining of podocytes in podoILK−/− at 3 and 4 wk of age (data not shown), consistent with ILK’s not being essential for podocyte survival in vivo.

The first lesions that consistently were found at onset of albuminuria were GBM alterations with an increase in thickness, followed by splitting and massive extension at later stages (Figure 5). Possible mechanisms for this surprising GBM phenotype could be an in-
Increased matrix synthesis or decreased degradation, deposition of circulating proteins in the GBM, or alterations in GBM assembly.

**Table 1. Glomerular gene expression of GBM, podocyte slit membrane molecules, and cell–matrix receptors**

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<th>Target</th>
<th>4 Wk</th>
<th>12 Wk</th>
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<tr>
<td></td>
<td>PodoILK+/+ (n = 6)</td>
<td>PodoILK−/− (n = 5)</td>
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<tr>
<td></td>
<td>Mean  SD</td>
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* mRNA levels were determined by real-time mRNA quantification from 4- and 12-wk-old mice. Significant expression induction is displayed in bold; significant expression repression is displayed in italics. At 4 wk in proteinuric mice, no significant differences are seen. At 12 wk, in mice that showed progressive glomerulosclerosis, repression of podocyte-specific molecules and induction of the matrix molecules Col I α1 and fibronectin are detected. GBM, glomerular basement membrane; podoILK+/+, ILKflox/flox/2.5P-Cre+/−; podoILK−/−, podocinCre+/−/ILKflox/flox mice.

An analysis of key components of the GBM at early disease stage did not demonstrate an increased concentration of GBM component transcripts or—accepting the inherent limitations of immunofluorescence studies for drawing quantitative conclusions—protein level. Also, no persistence of a developmental pattern of collagen IV or laminin isoforms could be found (Figure 8, Table 1). These findings do not indicate an increased synthesis of matrix molecules or decreased production of matrix-modifying enzymes, despite that ILK has been reported to be involved in these processes in in vitro models (10). The significant induction of fibronectin during the later stages of progression in the podoILK−/− has been observed in several animal models and human diseases and might be part of an ILK-independent scarring process (31).

A second alternative would be passive deposition of circulating molecules into the GBM. This is a frequent event in autoimmune renal disease, resulting in subendothelial or subepithelial immune complexes at the GBM (32). However, immune complex deposits do show a typical ultrastructure that is not observed in the podoILK−/− mice. In addition, direct staining for Ig was not able to reveal a difference between control and podoILK−/− GBM (data not shown).

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**Figure 7.** Slit membrane–associated proteins in podoILK−/− mice. In 4-wk-old mice, two key elements of the glomerular filtration barrier, podocin and nephrin, were evaluated by immunofluorescence studies and showed comparable staining intensities and signal distribution along the GBM. For corresponding mRNA analysis, see Table 1. Magnification, ×40.
An alternative explanation for the GBM phenotype could be an altered matrix assembly with impaired integrin function (33). Matrix molecules could be less densely packed, causing a broadened and split GBM parallel to the consequences of some of the collagen IV mutations in Alport syndrome (34). Podocytes are crucial for GBM dynamics and maintenance in health and disease (35,36). In *in vitro* systems, ILK has been implicated in matrix assembly via modulation of integrin function by altering focal contact structure, integrin activity state, and cell migration (7,24,29,37–39). ILK depletion in endothelial cells *via* RNA interference impaired the ability to recruit α5/β1-integrins to fibrillar adhesions (as defined by Geiger *et al.* [26]) and the maturation of the adhesions to competent matrix-forming structures (39). Fibronectin matrix production remained unchanged in ILK knockdown, but the integration of fibronectin into a complex, focal adhesion–associated matrix was impaired. A potential mechanism of integrin-mediated matrix adhesions is the unmasking of self-assembly sites in the fibronectin molecule by activated integrins, allowing spontaneous polymerization into densely packed fibers (26,40). The hypothesis of a GBM matrix assembly defect in ILK−/− podocytes is difficult to test. As initial evidence of altered integrin GBM interaction in ILK-deficient glomeruli, dissociation of integrin-α3 signal from the GBM was detected (Figure 8). Using a small molecular ILK kinase inhibitor, preliminary data indicate impaired integrin function *in vitro* with reduced migratory ability of differentiated cultured podocytes in transfilter migration assays after ILK inhibition (M.K. *et al.*, 2005, unpublished observations).

**Conclusion**

The gradual onset of the glomerular phenotype in podoILK−/− mice allowed the dissection of the sequence of structural lesions that leads to filtration barrier failure. The GBM phenotype preceded subsequent podocyte damage, progressive glomerulosclerosis, and death. Because the key components of the GBM are expressed at comparable levels to controls, an alteration in matrix assembly subsequent to ILK deletion *via* modified integrin function seems to be an attractive hypothesis for this phenotype.

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*Figure 8. GBM components and matrix receptors in podoILK−/− mice. Immunofluorescence of α3-integrin. High-resolution confocal fluorescence microscopic images of integrin-α3 (red) and entactin as a GBM marker (green). In wild-type mice (podoILK+/+, left) a partial co-localization of integrin-α3 with the GBM staining is seen, resulting in the yellow signals in the merge. In podoILK−/− mice (right), a separation of the two signals is observed, consistent with re-localization of integrin-α3 at the earliest stages of disease onset in podoILK−/− mice.*
References

Figure 9. GBM components and matrix receptors in podoILK−/− mice. Immunofluorescence of GBM molecules. The distribution and signal intensity of the major components of the murine GBM was evaluated and did not show any consistent difference between podoILK+/+ and proteinuric podoILK−/− mice at 4 wk of age. Stainings for developmental and mature laminins and collagen type IV minor chains are shown. For corresponding mRNA expression analysis see Table 1. Magnification, ×40.