Maintenance of Glomerular Filtration Barrier Integrity Requires Laminin α5

Seth Goldberg,* Tracy L. Adair-Kirk,† Robert M. Senior,†‡ and Jeffrey H. Miner*‡

*Renal Division, †Division of Pulmonary and Critical Care Medicine, and ‡Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri

ABSTRACT

Mutation of the mouse laminin α5 gene results in a variety of developmental defects, including defects in kidney structure and function. Whereas the total absence of laminin α5 results in breakdown of the glomerular basement membrane (GBM) and failed glomerular vascularization, a hypomorphic *Lama5* mutation (the *Lama5*^{neo} allele) results in proteinuria, hematuria, polycystic kidney disease (PKD), and death 3 to 4 weeks after birth. Here, we examined the role of podocyte-derived laminin α5 via podocyte-specific inactivation of *Lama5* and podocyte-specific rescue of the *Lama5*^{neo} mutation. Podocyte-specific inactivation of *Lama5* resulted in varying degrees of proteinuria and rates of progression to nephrotic syndrome. The GBM of proteinuric mice appeared thickened and "moth-eaten," and podocyte foot processes became effaced. Podocyte-specific restoration of laminin α5 production using two distinct strategies in *Lama5*$^{neo/neo}$ mice resulted in the resolution of proteinuria, hematuria, and PKD. These results suggest that the development of normal GBM structure and function requires podocyte-derived laminin α5 during and after glomerulogenesis and present a unique mechanism for the pathogenesis of PKD in these mice.

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The glomerular capillary wall consists of podocytes with interdigitated foot processes bridged by slit diaphragms, fenestrated endothelial cells, and the intervening glomerular basement membrane (GBM)[1] that these two cell types together produce.[2] A defect in or injury to any one of these three components can cause pathologic leak of albumin and other plasma proteins into the urine. This and a wealth of other evidence suggest that the three layers interact to establish and maintain the glomerular filtration barrier to plasma macromolecules.

Most research into the mechanisms of glomerular disease is currently focused on the structure of the podocyte slit diaphragm and its interaction with the actin cytoskeleton (reviewed in reference 3). Yet the podocyte's intimate association with the GBM via specific receptors, coupled with the fact that mutations in four genes encoding GBM components cause glomerular disease in humans and mice,[4–7] make the GBM an attractive—if not necessary—target for research aimed at providing a complete understanding of the glomerular filtration barrier. Indeed, the GBM's prominence in the filtration barrier was hypothesized several decades ago,[8] and recent studies have provided additional support.[9–11]

Like all basement membranes, the GBM is comprised of four major extracellular matrix proteins: laminin, collagen IV, nidogen, and sulfated proteoglycans. Laminins are obligate α-β-γ heterotrimers[12] that self-polymerize in the extracellular matrix to provide the basis for basement membrane formation via interactions with cellular receptors.[13]

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Correspondence: Dr. Jeffrey H. Miner, Renal Division, Box 8126, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110. Phone: 314-362-8235; Fax: 314-362-8237; E-mail: minerj@wustl.edu

The major laminin in mature GBM is laminin-521 ($\alpha5\beta2\gamma1$), but during glomerulogenesis there are developmental transitions in which laminin-111 ($\alpha1\beta1\gamma1$) is replaced by laminin-511 ($\alpha5\beta1\gamma1$), which is then joined and eventually replaced by laminin-521.[14–16]

Here we focus on the laminin $\alpha5$ chain. Laminin $\alpha5$ is widely expressed,[17] and *Lama5*$^{-/-}$ mice die at late fetal stages with multiple developmental defects.[18] Kidneys are small and occasionally absent in *Lama5*$^{-/-}$ embryos, and in those mutant kidneys that do form, the GBM breaks down during glomerulogenesis, which prevents glomerular vascularization and filtration.[19] Studies of chimeric laminin chains in transgenic mice revealed that the $\alpha5$ COOH-terminal laminin globular domain is required for proper adhesion of mesangial cells to the GBM[20] and for a proper filtration barrier.[21] In addition, mice with a hypomorphic *Lama5* mutation (*Lama5*^{neo}) that reduces $\alpha5$ expression because of *neo* insertion causes proteinuria, hematuria, and a form of polycystic kidney disease (PKD).[22]

Here we manipulated *Lama5* expression *in vivo* specifically in podocytes in three different ways to investigate the function of laminin $\alpha5$ in the GBM. (*1*) We used a podocyte-specific Cre mouse to mutate *Lama5* during glomerulogenesis, resulting in a new model of nephrotic syndrome. (*2*) We generated novel transgenic mice expressing FLP recombinase specifically in podocytes, which rescues the *Lama5*^{neo} mutation via FLPase-mediated removal of *neo*. This prevented proteinuria, hematuria, and, unexpectedly, cystogenesis. (*3*) We used a novel tetracycline-resistance operon (tetO)-regulated human laminin $\alpha5$ transgene and a podocyte-specific reverse tetracycline transactivator (rtTA)[23] to express human laminin $\alpha5$ in podocytes during and after glomerulogenesis in *Lama5*$^{neo/neo}$ mice. This resulted in deposition of human $\alpha5$ in the GBM, which ameliorated proteinuria, hematuria, and, again unexpectedly, the PKD. Together, these results implicate podocyte-derived laminin $\alpha5$ as a crucial component of the glomerular filtration barrier, and they show that in mice a disease with features of PKD can be causally related to a GBM defect.

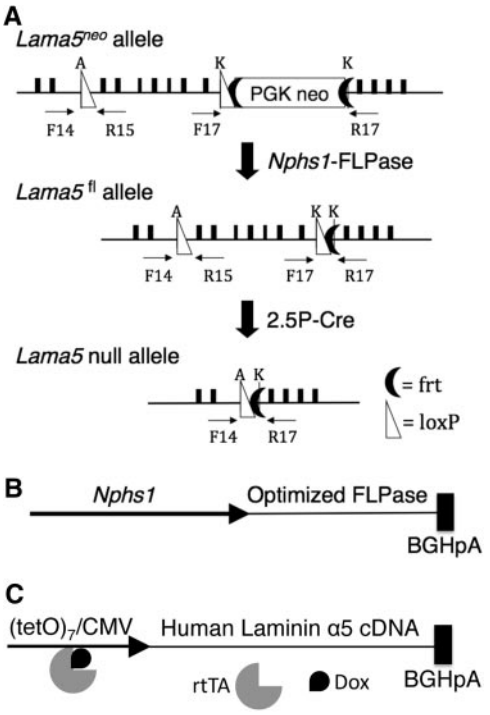

Figure 1. Schematic diagrams of *Lama5* alleles and new transgenes used in these studies. (A) The hypomorphic *Lama5*^{neo} allele is converted to the functional conditional *Lama5*^{fl} allele by FLPase-mediated removal of the FRT-flanked PGK-*neo* cassette. A *Lama5* null allele is generated by the activity of Cre recombinase, which removes the seven exons between the loxP sites. The primers used for PCR of genomic DNA are indicated. (B) The *Nphs1*-FLPo transgene contains the mouse nephrin (*Nphs1*) promoter driving FLPo and a bovine growth hormone polyA signal sequence. (C) The doxycycline-inducible human laminin $\alpha5$ transgene (tetO-LA5) contains seven copies of tetO with a CMV minimal promoter driving the human LAMA5 cDNA. The binding of doxycycline (Dox) to the rtTA promotes recruitment to and activation of the promoter.

RESULTS

Podocyte-Specific Inactivation of *Lama5* by Cre Recombinase

Lama5 was mutated during glomerulogenesis specifically in podocytes by crossing the podocyte-specific 2.5P-Cre transgene[24] onto the conditionally mutant *Lama5*$^{fl/fl}$ (Figure 1A) and *Lama5*$^{fl/-}$ genetic backgrounds.[25] PCR analysis of genomic DNA showed that excision of the seven floxed *Lama5* coding exons by Cre recombinase (Figure 1A) occurred in kidney but not in other tissues (Figure 2A and A′), consistent with the known specificity of the 2.5P-Cre transgene.[24] *Lama5*$^{fl/fl}$;2.5P-Cre and *Lama5*$^{fl/-}$;2.5P-Cre mice were viable and most presented with mild proteinuria early in life that eventually progressed to the nephrotic range (Figure 3, A and B). As the affected mice aged, they became edematous, hypoalbuminemic, hypercholesterolemic, hematuric, and their kidneys yellowed (data not shown). Light microscopy revealed protein casts in tubules and tubulointerstitial nephritis, but glomerular lesions, including glomerulosclerosis and mesangial matrix expansion, were not obvious until later stages of disease (Figures 3C through 3F and data not shown). Ultrastructural analysis revealed few abnormalities at early stages (approximately 3 weeks) despite mild proteinuria (data not shown). At later stages of disease the GBM was thickened, had a moth-eaten appearance, and there were irregular contours with frequent subepithelial outpocketings and extensive foot process effacement (Figures 3G through 3J and data not shown).

The rate of disease progression was highly variable, because some *Lama5*$^{fl/fl}$;2.5P-Cre mice lived only a few weeks, whereas others showed little sign of disease even at 8 months of age (Figure 3A and data not shown). This variability may have stemmed from the mixed genetic background; from heterogeneity in the onset, level, or extent of Cre expression; or from a combination of these variables. The notion that Cre might be

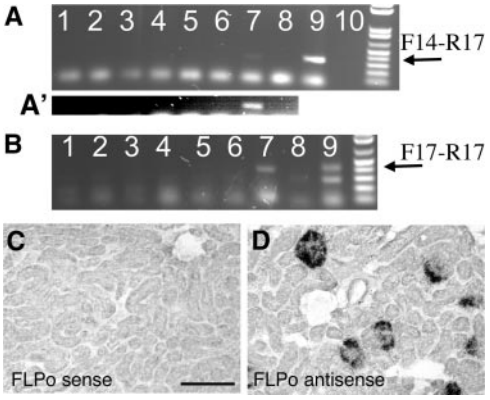

Figure 2. Expression of Cre or FLPo alters *Lama5* alleles. (A) PCR analysis of DNA extracted from multiple tissues of a *Lama5*$^{fl/+}$; 2.5P-Cre mouse only generates the appropriate product (arrow) in kidney. Lane 1, lung; 2, liver; 3, spleen; 4, intestine; 5, cerebrum; 6, cerebellum; 7, kidney; 8, no DNA; 9, positive control tail; 10, blank. (A′) The region of interest in lanes 1 to 8 was brightened to better reveal the kidney-specific product. (B) PCR analysis of DNA extracted from multiple tissues of a *Lama5*$^{neo/neo}$;*Nphs1*-FLPo mouse only generates the appropriate product (arrow) in kidney. Lanes are as in panel A; the positive control here is from a *Lama5*$^{fl/+}$ mouse. (C, D) *In situ* hybridization with a (C) sense or (D) antisense FLPo riboprobe on kidney sections from a *Nphs1*-FLPo transgenic mouse shows expression confined to podocytes.

limiting in some cases is supported by the fact that all of the *Lama5*$^{fl/-}$;2.5P-Cre mice, which only required one rather than two floxed *Lama5* alleles to be recombined by Cre to generate *Lama5*$^{-/-}$ podocytes, exhibited nephrotic levels of proteinuria (Figure 3A).

Immunofluorescence analysis of laminin α5 deposition in the GBM of proteinuric animals at an early stage showed similar levels in controls and mutants, but at later stages laminin α5 levels were reduced compared with control (data not shown). The residual laminin α5 in the GBM of mutants was likely synthesized by glomerular endothelial cells, which do not express the 2.5P-Cre transgene, and some may have been secreted by podocytes during and after glomerulogenesis before they became depleted of *Lama5* mRNA. Laminins α1 and α2 could be detected ectopically in the mature mutant GBM (Figure 3, K and L, and data not shown), presumably as attempted compensation for the reduced α5, but they were not sufficient to prevent proteinuria. This is consistent with our previous studies showing that replacing endogenous laminin α5 with a modified version carrying COOH-terminal globular domain segments of laminin α1 also resulted in proteinuria.[21] These studies show that podocyte-derived laminin α5 is required to maintain the structural integrity of the GBM and the glomerular filtration barrier.

Podocyte-Specific Rescue of the *Lama5*^{neo} Insertional Mutation

In the process of creating the conditional *Lama5*^{fl} allele, we generated mice carrying a FLP recognition target (FRT) site-

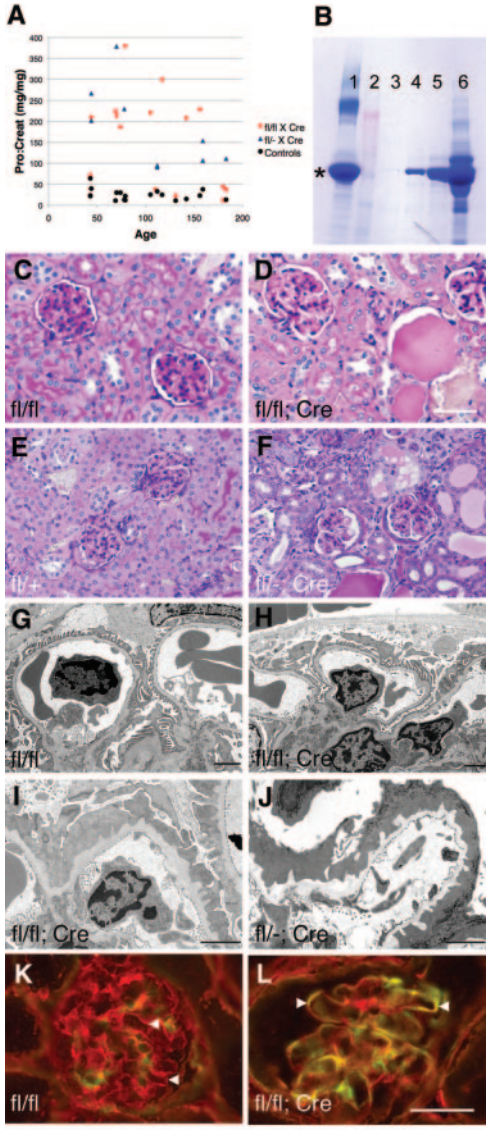

Figure 3. Podocyte-specific mutation of *Lama5* causes nephrotic syndrome. (A) Graph showing protein:creatinine ratios of mutant and control mice plotted against age. Note that all *Lama5*$^{fl/-}$; 2.5P-Cre mice became overtly proteinuric. (B) SDS-PAGE analysis of 1 μl of urine shows that albumin is the major protein. Lane 1, 10 μg BSA; 2, markers; 3, *Lama5*$^{fl/fl}$, postnatal day (P) 131; 4 and 5, *Lama5* $^{fl/fl}$;2.5P-Cre, P131; 6, *Lama5*$^{fl/fl}$;2.5P-Cre, P69. * denotes albumin. The three P131 mice were littermates. (C through F) Periodic acid–Schiff staining of kidney sections from mice of the indicated genotypes at (C, D) P215 and (E, F) P78. (G through J) Ultrastructural analysis of the glomerular capillary wall of mice of the indicated genotypes at (G through I) P215 and (J) P78. Panels H and I are from the same mouse, which was already albuminuric at P131 (lane 5 in panel B), demonstrating significant variation in the ultrastructural changes. (K, L) Immunofluorescence analysis of laminin α5 (red) and laminin α1 (green) deposition in the GBM (arrowheads) of P156 mice of the indicated genotypes. Note the lack of green in (K) the control GBM but frequent green and yellow staining in (L) the mutant. Bar in D: 40 μm for C and D, 53 μm for E and F; bars in G through J: 2 μm; bar in L, 33 μm for K and L.

flanked *neo* insertion in intron 21. This genetic alteration leads to proteinuria, hematuria, PKD, and death at 3 to 4 weeks of age in *Lama5*$^{neo/neo}$ homozygotes. Molecular analyses revealed that the insertion causes aberrant *Lama5* RNA splicing and reduced laminin α5 protein deposition.[22] We concluded from these data that reduced laminin α5 expression in glomerular cells caused GBM defects and impaired permselectivity, whereas reduced expression in tubular cells caused cystogenesis because of aberrant cell/matrix interactions. Alternatively, because the loss of laminin α5 from tubular basement membranes (TBMs) appeared more dramatic than the loss from the GBM (Figure 4F and reference 22), the possibility existed that the proteinuria stemmed in part from tubular cell dysfunction, consistent with concepts proposed by Comper and colleagues.[26,27] To distinguish the pathologic effects of laminin α5 reduction in the GBM from those stemming from reduction in TBMs, we devised two different strategies to specifically rescue the *Lama5*^{neo} insertion mutation in podocytes.

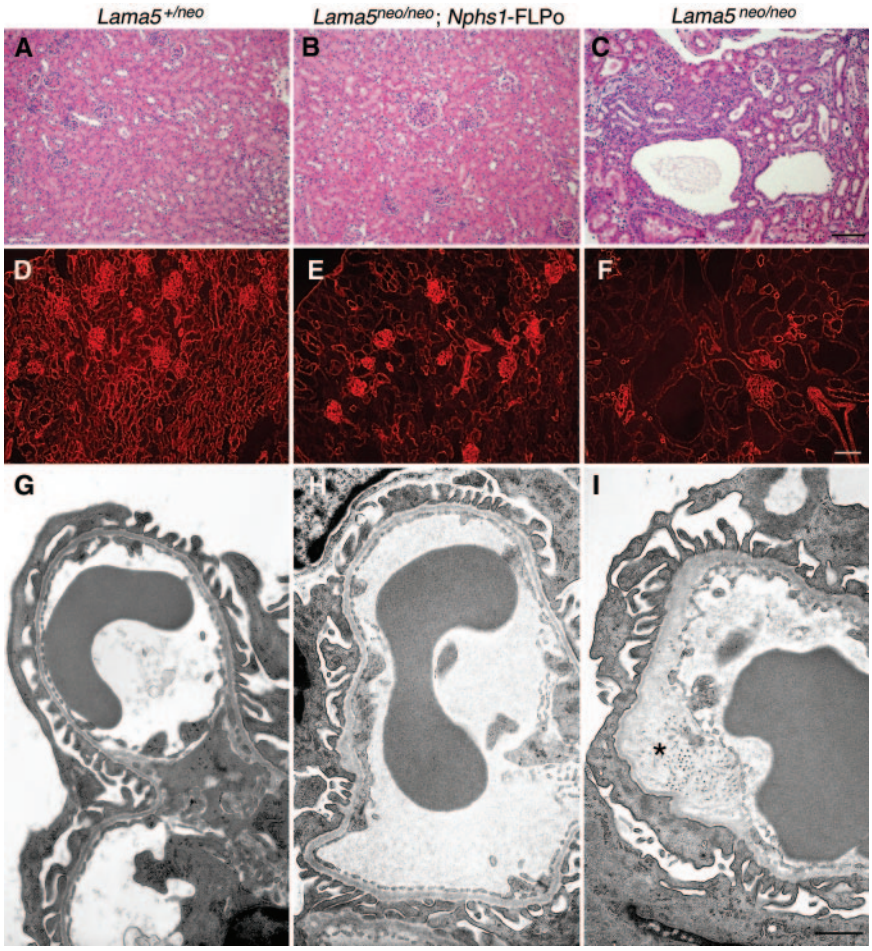

Figure 4. Podocyte-specific expression of FLP recombinase rescues renal defects in *Lama5*$^{neo/neo}$ mice. (A through C) Hemotoxylin and eosin staining of P21 kidney sections of the indicated genotypes reveals overall renal architecture. Note the lack of any histopathology in (B) the *Lama5*$^{neo/neo}$ kidney expressing FLPo in podocytes and (C) the presence of cysts and tubulointerstitial disease in the nonrescued mutant littermate. (D through F) Immunofluorescence analysis of laminin α5 deposition in kidney sections from the same mice. Note the reduced TBM signals in panels E and F as compared with panel D. (G through I) Ultrastructural analysis of glomerular capillary loops from the same mice. GBM thickening (*) and adjacent foot process effacement is evident in (I) the mutant but not in (G) the control or in (H) the rescued mutant. Bar in C, 100 μm for A through C; bar in F, 80 μm for D through F; bar in I, 1.1 μm for G through I.

Strategy 1: Podocyte-Specific Removal of the *neo* Insertion

To remove the pathogenic *neo* insertion specifically in podocytes, we generated transgenic mice designed to express a codon-optimized FLP recombinase (FLPo)[28] under the control of the 4.2-kb mouse nephrin (*Nphs1*) promoter[29] (Figure 1B). FLP recombinase activity should excise the FRT site-flanked *neo* insertion during glomerulogenesis and convert the hypomorphic *Lama5*^{neo} allele to the fully functional *Lama5*^{fl} allele (Figure 1A).

We generated eighteen *Nphs1*-FLPo transgenic founders and bred seven different integrants onto the *Lama5*$^{+/neo}$ background. PCR using *Lama5* primers flanking the 2-kb *neo* insertion was used to look for evidence of excision of the *neo* in DNA extracted from kidney cortex without excision in other tissues. Successful removal of *neo* was observed for three of the lines (Figure 2B and data not shown), and *in situ* hybridization with a FLP riboprobe demonstrated podocyte-specific expression of FLPo in all three (Figure 2, C and D, and data not shown). Each of these three transgenes was then bred onto the *Lama5*$^{neo/neo}$ background.

Surprisingly, all three rescued not only proteinuria and hematuria but also prevented the development of cysts that appeared in *Lama5*$^{neo/neo}$ littermates lacking *Nphs1*-FLPo (Figures 4A through 4C and data not shown). The resulting *Lama5*$^{neo/neo}$;*Nphs1*-FLPo mice were viable and fertile, with no signs of proteinuria or kidney disease even at several months of age. Immunohistochemical analysis of laminin α5 deposition showed normal levels in the *Lama5*$^{neo/neo}$;*Nphs1*-FLPo GBM and, in one line (line 5), the expected low level in TBMs (Figures 4D through 4F). However, in the other two lines (lines 1 and 3), which showed higher podocyte FLPo expression, the TBM levels of laminin α5 appeared normal (data not shown). This suggests that the FLPase was expressed in some tubular epithelial cells despite the absence of *in situ* signals in tubules (Figure 2C). Alternatively, FLPase may have been secreted by podocytes and taken up by tubular cells, but there is no definitive evidence for this.

Consistent with the lack of proteinuria, ultrastructural analysis of the glomerular capillary wall revealed no defects in *Lama5*$^{neo/neo}$;*Nphs1*-FLPo kidneys, in contrast to the GBM thickening and occasional splitting observed in *Lama5*$^{neo/neo}$ glomeruli (Figures 4G through 4I). From these studies of *Lama5*$^{neo/neo}$;*Nphs1*-FLPo mice we conclude that restoration of *Lama5* expression to normal levels specifically in podocytes during glomerulogenesis is sufficient to rescue GBM architecture and the integrity of the glomerular filtration barrier.

Strategy 2: Podocyte-Specific Expression of a Laminin α5 Transgene

To express a full-length laminin α5 cDNA specifically in podocytes during and after glomerulogenesis using a doxycycline-inducible system, we produced a new transgenic mouse line carrying the human *LAMA5* cDNA under the control of the tetO-cytomegalovirus (CMV) regulatory element (tetO-LA5; Figure 1C). tetO-LA5 mice were crossed to 2.5P-rtTA mice expressing rtTA under control of the human *podocin* (*NPHS2*) promoter.[23] Next, we mated both transgenes onto the *Lama5*$^{neo/neo}$ background to generate *Lama5*$^{neo/neo}$;2.5P-rtTA;tetO-LA5 mice. Doxycycline was administered when pregnancy became obvious (usually embryonic day 12 to 15) and continuously thereafter such that human laminin α5 synthesis should occur in podocytes from when they first begin to differentiate.

Similar to the results presented above, the *Lama5*$^{neo/neo}$;2.5P-rtTA;tetO-LA5 mice treated with doxycycline did not develop proteinuria or hematuria. They were fertile and developed no signs of kidney disease as adults, at least to 6 months of age. Light microscopy revealed normal glomeruli, no cysts, and normal renal architecture (Figure 5, A and B). Immunohistochemical analysis revealed an apparent reduction of mouse laminin α5 in the GBM and the expected low levels in the TBMs (Figure 5, C and D). Human laminin α5 deposition was restricted to the GBM and was doxycycline-dependent (Figure 5, E and F, and data not shown). Electron microscopic analysis revealed normal architecture of the glomerular capillary wall in the rescued mutant (Figure 5, G and H), consistent with the lack of proteinuria. Thus, human laminin α5 can compensate for the reduced levels of mouse α5 in the mutant GBM, allowing for the establishment and maintenance of an intact glomerular filtration barrier.

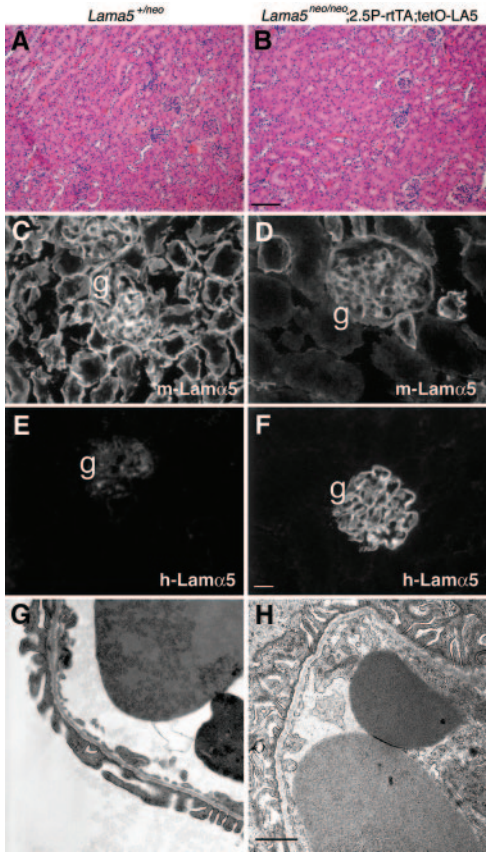

Figure 5. Podocyte-specific expression of human laminin α5 induced by doxycycline rescues renal defects in *Lama5*$^{neo/neo}$ mice. (A, B) Hemotoxylin and eosin staining of P42 kidney sections of the indicated genotypes reveals normal renal architecture. (C, D) Immunofluorescence analysis of mouse laminin α5 deposition shows reduced levels in the rescued mutant GBM (g) and in most TBMs (D) as compared with control (C). (E, F) Immunostaining for human laminin α5 shows deposition in the GBM (g) but not in TBMs of the rescued mutant (F) but only background fluorescence in the control glomerulus, perhaps because of low-level accumulation of mouse IgG1 in the mesangium (E). (G, H) Ultrastructural analysis of a glomerular capillary loop from the same mice reveals normal GBM and foot process architecture. Bar in B, 100 μm for A and B; bar in F, 20 μm for C through F; bar in H, 1.1 μm for G and H.

DISCUSSION

Together with our prior results, the data presented here show that expression of laminin α5 by podocytes and its secretion into the GBM is essential for establishing and maintaining the glomerular filtration barrier. We previously showed that with no laminin α5 the GBM breaks down[19]; with only endothelial laminin α5, the GBM is intact, but podocytes do not form normal foot processes[30]; and with reduced laminin α5 throughout the kidney because of a *neo* insertion in *Lama5*, there is proteinuria, hematuria, and development of multiple cysts.[22] Here we demonstrated that knockout of *Lama5* in podocytes during glomerulogenesis causes a variable, delayed onset nephrotic syndrome. This variability likely stems from the interplay of variabilities in the spatial and temporal expression of Cre from the podocin promoter, in the level of Cre expression, in the efficiency of recombination, and the potentially long half-lives of laminin α5 mRNA and protein. Despite the variability, we conclude that ongoing expression of laminin α5 in podocytes is a necessity.

To further explore the role of podocyte-derived laminin α5 in the filtration barrier, we used two different podocyte-specific strategies to attempt to rescue the glomerular defects in

mice carrying the *neo* insertion. Removing the pathogenic *neo* insertion with FLP recombinase or expressing a human laminin α5 transgene prevented proteinuria and hematuria and restored the architecture of the glomerular capillary wall to normal. In both cases, cystogenesis in the tubules was also prevented, despite the glomerulus-specific nature of the rescues. This suggests that under the right circumstances a glomerular defect can cause cystic kidney disease. Although an attractive explanation for this finding is the notion that cysts begin in the glomeruli because of filtration defects and then propagate down the tubule, glomerular cysts were rarely, if ever, observed.

Regarding the etiology of cyst development in this model, we had initially hypothesized that the reduced laminin α5 in TBMs of *Lama5*$^{neo/neo}$ mice led to impaired cell-matrix interactions and subsequent cystogenesis,[22] despite the fact that there are no reports of laminin α5 alterations in PKD. Furthermore, a recent report showed that laminin α5 in the hair follicle basement membrane is important for cilia structure and function.[31] However, here a direct effect of laminin α5 reduction on tubular epithelial cell behavior seems not to be the complete story; neither mode of glomerular rescue should affect the TBMs, yet cystogenesis was inhibited. There are no precedents for congenital nephrotic syndrome and/or hematuria causing early cystogenesis in humans or in mice.

In human autosomal dominant PKD, cysts form in only a small percentage of tubules despite the equal genetic susceptibility of all nephrons, giving rise to the theory that a "second hit" is necessary for cystic transformation. What might be unique about the *Lama5*$^{neo/neo}$ mice is the combination of GBM and TBM defects. Perhaps a factor that is filtered at higher than normal levels in all proteinuric conditions mildly injures tubular epithelial cells, but in *Lama5*$^{neo/neo}$ mice these cells are more sensitive to injury because of impaired cell-matrix interactions (the second hit), resulting in cystic transformation. We are testing this hypothesis by attempting to selectively rescue the TBMs without affecting the abnormal GBM.

Alternatively, it is possible that despite the selective nature of the podocyte rescue, a small amount of laminin α5 may have found its way from glomeruli to TBM at sufficient levels to prevent cystogenesis. This would be difficult to demonstrate in the podocyte-specific FLP recombinase rescue because mouse laminin α5 is already weakly detectable in the TBMs of *Lama5*$^{neo/neo}$ mice.[22] However, the restriction of human laminin α5 deposition to the GBM in the doxycycline-inducible system, with complete absence from the TBMs, makes this mechanism for tubular rescue unlikely.

It is interesting that despite the widespread expression of *Lama5* during development and the many defects observed in its absence,[32] the GBM manifests the most sensitivity to the reduced levels in *Lama5*$^{neo/neo}$ mice. The death of these mice at 3 to 4 weeks of age from renal failure may have masked abnormalities in other tissues, such as the lung.[25] Yet restoration of laminin α5 expression only to podocytes led to viable, fertile mice, suggesting that only the glomerular filter is defective. It is notable that some of these rescued mice were smaller than their control littermates, perhaps because of placental insufficiency, as observed in *Lama5*$^{-/-}$ mice.[18]

Finally, as part of these studies we generated a new tool for manipulation of podocyte gene expression that should be useful for the nephrology community. Expression of FLPo recombinase driven by the nephrin promoter was demonstrated to be podocyte-specific in the kidney. Combined with available Cre transgenic mice and inducible systems, the design of increasingly complex genetic experiments now becomes feasible.

CONCISE METHODS

Genetically Altered Mice

All animal experiments conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. *Lama5*$^{-}$,[18] *Lama5*^{fl},[25] *Lama5*^{neo},[22] 2.5P-Cre,[24] and 2.5P-rtTA[23] mice have been described previously and were maintained on mixed, primarily B6/CBA/129 genetic backgrounds. *Nphs1*-FLPo transgenic mice were generated by microinjection of the isolated transgene (Figure 1B) into the pronuclei of B6CBAF2/J single-celled embryos. The 4.2-kb mouse nephrin promoter,[29] a gift from Susan Quaggin (Samuel Lunenfeld Research Institute, Toronto, Canada), was placed upstream of FLPo recombinase with a 3′ bovine growth hormone polyadenylylation signal sequence[28] (Addgene Inc., Cambridge, MA). Transgenic mice were identified by PCR using the FLPo primers 5′-CCACCTTCATGAGCTACAACACCATC-3′ and 5′-ACTTGTTCTGCACCAGCTTGAAGCTC-3′.

tetO-LA5 transgenic mice were generated by microinjection of the isolated transgene (Figure 1C) into the pronuclei of C57BL/6NTac single-celled embryos. The full-length human laminin α5 cDNA with a bovine growth hormone polyadenylylation signal sequence (a gift from Kiyotoshi Sekiguchi, Osaka University) was placed under the control of the $(tetO)_7$-CMV regulatory element (a gift from Jeffrey Whitsett, University of Cincinnati). tetO-LA5 transgenic mice were identified by PCR using human *LAMA5* primers 5′-TGCATCGAGATGGACACG-3′ and 5′-GCTTCAGGAAGAAGAGCAC-3′. To induce expression of tetO-LA5 in podocytes in animals also carrying the 2.5P-rtTA transgene, mice were treated with 1 mg/ml doxycycline in drinking water containing 5% sucrose. Of fifteen founders produced, the offspring of two expressed the transgene in the desired fashion, and one of these lines was used herein.

PCR Analysis of Genomic DNA

Crude DNA extracts from multiple tissues were obtained as described.[33] To assay for mutation of the *Lama5*^{fl} allele by Cre-mediated excision of the floxed exons, primers F14 (5′-ACCATGGGTATCCCGACTGTCACG-3′ and R17 (5′-GTTGAAGCCAAAGCGTACAGCG-3′) were used. These typically lie 2.5 kb apart, a segment normally too large to amplify, but after excision by Cre this segment shrinks to approximately 330 bp (Figures 1A and 2A). To assay for excision of the *neo* insertion from the *Lama5*^{neo} allele by FLP recombinase, primers F17 (5′-GTGCCGCCCTAACACCCAAGG-3′) and R17, which flank the *neo*, were used. Once the approximately 2-kb *neo*

gene is removed, these primers amplify a 425-bp product, which represents the functional *Lama5*^{fl} allele; a 300-bp product representing the wild-type *Lama5*$^{+}$ allele was also amplified (Figures 1A and 2B).

Renal Chemistry Assays

Analysis of blood and urine was performed on a Roche Cobas Mira Plus Analyzer. In some cases, 1 μl of urine was analyzed on SDS-PAGE gels stained with Coomassie brilliant blue.

Antibodies and Histology

Rabbit anti-mouse laminin α5,[14] mouse anti-human laminin α5 clone 4C7[34] (Chemicon, Temecula, CA), rat anti-mouse laminin α1 clone 8B3,[35] rat anti-human laminin α2 clone 4H8-2 (Alexis Biochemicals/Enzo Life Sciences, Plymouth Meeting, PA), and fluorescence secondary antibodies (Molecular Probes/Invitrogen, Carlsbad, CA) were used to immunostain frozen sections of unfixed kidneys as described.[36] For light and electron microscopy, tissues were fixed and processed for paraffin and plastic sectioning by standard methods. Sections were stained and viewed as described previously.[36] *In situ* hybridizations were performed as described.[37]

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DISCLOSURES

None.

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