Building the Glomerulus: A Matricentric View

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J Am Soc Nephrol 16: 857-861, 2005. doi: 10.1681/ASN.2004121139

The structural integrity of the glomerular capillaries stems from the stereotyped arrangement of the three constituent cell types: Podocytes (also called visceral epithelial cells), endothelial cells, and mesangial cells. The arrangement of these cells depends in part on their interactions with extracellular matrix, most notably the glomerular basement membrane (GBM). In this essay, I discuss the role of the GBM in glomerulogenesis and in kidney function and disease and provide a historical perspective regarding how our current state of knowledge was achieved.

Basement Membranes

Basement membranes are thin sheets of specialized extracellular matrix found in tissues throughout the body. They underlie all epithelial and endothelial cells and surround all muscle cells, fat cells, and axons in peripheral nerves. Basement membranes have been shown to influence cell proliferation, migration, and differentiation and to be involved in compartmentalization of tissues and in filtration.

All basement membranes contain four major components: Laminin, type IV collagen, nidogen/entactin, and sulfated proteoglycans. Each of these components actually describes a family of individual proteins that are differentially distributed in basement membranes. The initial biochemical studies of these components in the 1970s and 1980s were facilitated by the existence of the Engelbreth-Holm-Swarm (EHS) tumor, a tumor of mouse origin that produces large amounts of particular basement membrane protein isoforms that are easily purified. These isoforms are now known as laminin-1 (Figure 1), collagen IV $(\alpha1)_2(\alpha2)_1$, nidogen-1 or entactin-1, and perlecan.

Once these proteins were purified and biochemically characterized, they were used as immunogens to develop antibodies for localization in tissues. Because of the abundance of basement membranes in the kidney—indeed, each nephron and its attached collecting duct is entirely surrounded by a continuous basement membrane—the kidney was a common site for localizing basement membrane proteins. Antibodies to most of the EHS proteins stained all kidney basement membranes. However, some but not all laminin antibodies presented interesting exceptions to this.

Published online ahead of print. Publication date available at www.jasn.org.

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Laminin

Laminin from the EHS tumor (1), now known as laminin-1, is a large cruciform heterotrimeric glycoprotein with a mass of approximately 800 kD (Figure 1). The component chains were originally called A (approximately 400 kD), B1, and B2 (each approximately 200 kD) but are now called $\alpha1$, $\beta1$, and $\gamma1$ (2). Whereas antibodies to the B1 and B2 chains seemed to stain most, if not all, basement membranes in kidney, antibodies specific for the laminin A chain stained only a subset of tubular basement membranes (primarily proximal) and the glomerular mesangial matrix but not the GBM (3). This indicated that either GBM laminin lacked the A chain or there might be a different A-like chain present in the GBM laminin.

Meanwhile, several investigators were attempting to purify laminins from an abundant source of human basement membranes, the full-term placenta and its associated membranes. Unlike the EHS tumor, which secretes large amounts of easily extracted basement membrane proteins, proteases were sometimes necessary to liberate laminins from placental basement membranes (4). Nevertheless, human laminin subunits that seemed to be the same size as EHS laminin subunits were identified, and they were presumed to represent the human homologues of the mouse A, B1, and B2 chains (5). In some cases, novel laminin or laminin-like proteins were noted, on the basis of distinct migration patterns in SDS gels (6). Engvall *et al.* (7) made mAb to their human placental laminin preparation and noted that one antibody specific for the A chain (clone 4C7) recognized all basement membranes in human kidney, including the GBM (8). This presented somewhat of a conundrum, because antibodies to the mouse A chain did not stain GBM (3).

Molecular cloning of the genes encoding laminins revealed that the three chains were evolutionarily related to each other, and this established the laminins as a gene family. Expansion of the family occurred in the late 1980s, when two novel laminin chains were identified. The first, synaptic laminin (s-laminin), now known as the laminin $\beta2$ chain, is a homologue of the B1 chain and is concentrated in the synaptic basement membrane of the neuromuscular junction and in the kidney GBM (8,9). The second, the merosin M chain, now called laminin $\alpha2$, is a homologue of the A chain and is widely expressed in placenta, cardiac and skeletal muscle, and peripheral nerve (10). Within the next few years, additional laminin genes and chains were identified, and it was determined that laminin chains assembled with each other in a nonrandom manner to form specific heterotrimers with defined distributions in basement membranes. This led in 1994 to the adoption of a novel, systematic

ISSN: 1046-6673/1604-0857

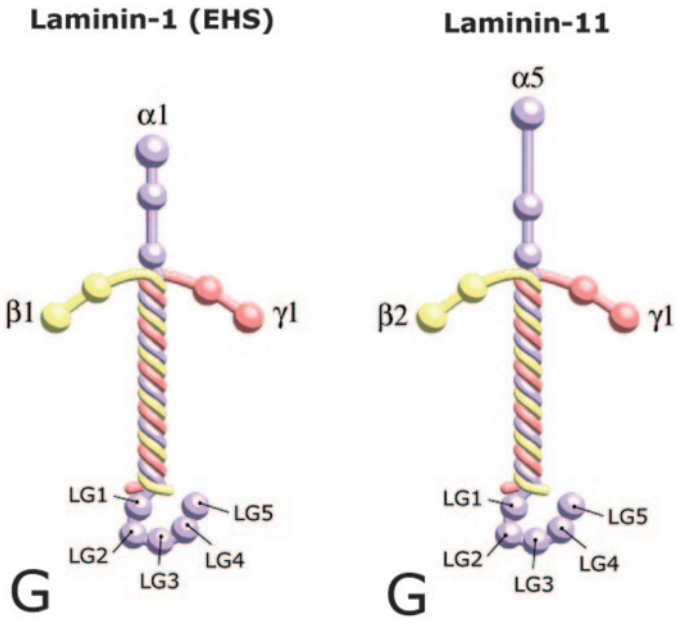

Figure 1. Accepted structure of "full-sized" laminin heterotrimers. Laminin-1, the trimer present in the nascent glomerular basement membrane (GBM), is identical to that made by the Engelbreth-Holm-Swarm (EHS) tumor and contains the α1, β1, and γ1 chains. Laminin-11, the trimer present in mature GBM, contains the α5, β2, and γ1 chains. Laminin-11 replaces laminin-1 in the GBM during glomerulogenesis. In the short arms, globular domains (circles) are separated by LE domains composed of laminin EGF-like repeats, the number of which vary between the short arms of α1 and α5. Trimers assemble and are stabilized via interchain interactions that form the coiled coil long arm. The COOH-terminal globular (G) domain specific to α chains is composed of five self-folding LG domains and contains binding sites for cellular receptors. Adapted from reference 37.

laminin nomenclature in which the heterotrimers were assigned Arabic numerals, and the four A-like, three B1-like, and two B2-like chains were changed to α1 to 4, β1 to 3, and γ1 to 2 chains, respectively (2). All laminins are thought to exist in basement membranes as $\alpha\beta\gamma$ heterotrimers.

The Missing GBM α Chain

Despite these discoveries of multiple new laminin chains, the identity of the α chain in the human GBM reacting with the 4C7 mAb still had not been determined in late 1994. In addition, the α chain present in the mature mouse GBM was not known. We hypothesized that a novel gene encoding orthologous human and mouse GBM α chains must exist. Because this was a time well before the sequencing of the human genome and before the existence of large expressed sequence tag databases, we searched for the novel mouse gene by using a then-state-of-the-art molecular method to clone all laminin α chains expressed in mouse kidney. On the basis of the reasoning that a novel laminin α chain would share amino acid stretches conserved between the laminin α1 and α2 chains, we aligned the most conserved domains of the α1 and α2 chains and synthesized several degenerate oligonucleotides that would encode conserved stretches of six to eight amino acids. These were used as primers for reverse transcription–PCR amplification of RNA isolated from 1-wk-old mouse kidney, and the resulting products were cloned and sequenced. This led to the identification of a novel α chain, which we called laminin α5 (11). It is interesting that comparison of α5 with α1 and α2 revealed that α5 was in a different class, in that its domain sizes were different from, although still homologous in sequence to, α1 and α2 (Figure 1).

A survey of laminin α5 expression by Northern blotting revealed widespread expression in adult mouse tissues (11). This was consistent with the expression pattern of the 4C7 antigen in human tissues, suggesting that 4C7 might recognize the human laminin α5 chain. Additional evidence supporting this was the reactivity of anti-mouse laminin α5 antibodies with kidney basement membranes, including the GBM, in a pattern similar to that of 4C7 on human kidney (12,13). Antibodies generated specifically to human α5 and additional biochemical analyses led to direct proof that 4C7 reacts with human laminin α5 (14). Thus, our cloning of laminin α5 solved an important mystery in basement membrane biology and had particular relevance to nephrology.

Expression and Function of Laminin α5 in Developing Kidneys

We and others determined the expression pattern of laminin α5 in the developing mouse kidney (12,13). At the onset of metanephrogenesis, laminin α5 is prominent in the Wolffian duct and in the ureteric bud basement membranes. Uninduced metanephric mesenchyme expresses little if any laminin α5. Once the mesenchyme condenses and begins to form an epithelium, there is a burst of laminin α1 expression. As the nascent renal vesicle then begins to change its morphology to form comma- and S-shaped bodies, laminin α5 becomes detectable in the surrounding basement membrane. At the S-shape stage, the developing GBM adjacent to the differentiating podocytes is positive for both laminin α1 and α5, but as the capillaries begin to form, α1 is eliminated by an unknown mechanism, leaving α5 as the only GBM laminin α chain. Thus, there is a laminin α1 to α5 developmental transition in the GBM, and this occurs approximately concurrently with the laminin β1 to β2 and collagen α1,2 to α3,4,5 developmental transitions (15,16). With respect to laminin trimers, those involved are laminin-1 ($\alpha1\beta1\gamma1$) + laminin-10 ($\alpha5\beta1\gamma10$) in immature GBM and laminin-11 ($\alpha5\beta2\gamma1$; Figure 1) in mature GBM. This transition was actually predicted by the work of Abrahamson and St. John (17), who showed that a laminin epitope now known to represent α1 disappeared from the GBM as it matured.

Are these developmental transitions in laminin deposition actually important for glomerulogenesis? When the *Lamb2* gene encoding β2 was mutated by targeted insertion, glomerulogenesis proceeded normally, but proteinuria was observed by 1 wk of age and the mice died at 3 wk of age, exhibiting defects in both glomerular filtration and neuromuscular junction differentiation (18,19). We concluded that the GBM's integrity was maintained because the laminin β1 chain, which is normally

eliminated as glomeruli mature, was retained in the GBM. Thus, β1 substituted structurally for β2, but the massive albuminuria that ensued indicated that it was a poor functional substitute (18). These data may have implications for the GBM's role as a component of the filtration barrier, an issue that we are currently pursuing in the laboratory. In addition, mutations in human *LAMB2* have recently been reported in a rare syndrome (Pierson syndrome) with congenital nephrosis, diffuse mesangial sclerosis, and blindness (20), demonstrating that *Lamb2* −/− mice are a model for a human disease.

In mice that lack laminin α5 as a result of targeted mutation, there are two interesting kidney developmental defects (21), as well as fetal lethality at 14 to 19 d of gestation likely as a result of placental insufficiency, in addition to other developmental defects (22,23). A small subset (approximately 20%) of *Lama5* −/− embryos lacked one or both kidneys, and in some cases, one kidney was significantly smaller than the other (21). Because laminin α5 is detectable only in the ureteric bud basement membrane at early stages of metanephrogenesis (12,13), a defect in ureteric bud outgrowth or branching, rather than an intrinsic mesenchymal defect, is the most plausible explanation for renal agenesis. Indeed, when E13.5 metanephroi from control and *Lama5* −/− embryos were placed into organ culture, ureteric bud branching was less robust in the mutant (21).

In kidneys that did form in *Lama5* −/− embryos, nephrogenesis seemed to proceed in the usual manner. However, we observed a novel defect in glomerulogenesis that results in failed vascularization. Shortly after the stage at which laminin α5 would normally replace laminin α1 in the GBM, there was a breakdown in the integrity of the GBM. This was associated with the on-schedule elimination of laminin α1 from the GBM as the capillary loops began to form, so neither laminin α1 nor α5 was present (21). GBM breakdown occurred despite the presence of laminin α4, a truncated α chain (reviewed in 24). This is consistent with the concept that a full-sized laminin trimer, *i.e.*, one with full-length short arms, is necessary to maintain basement membrane integrity (25). As a consequence of GBM breakdown, neither endothelial cells nor mesangial cells were able to maintain their positions within the developing glomerulus, and the podocytes failed to maintain their normal single-cell layer arrangement and lacked foot processes and slit diaphragms (21). Histologically, the endothelial and mesangial cells seemed to be extruded from the interior of the glomerulus, and the end product was a cluster of podocytes adjacent to a cluster of endothelial and mesangial cells (Figure 2). An intact GBM therefore is necessary for maintenance of the podocyte epithelium and for proper adhesion of endothelial cells and the capillaries that they form adjacent to the podocytes (21). This was the first demonstration of a developmental role for the GBM and suggests that one important function of laminin α5 is to maintain the integrity of the GBM during glomerulogenesis.

The function of laminin α5 in the *intact* GBM obviously cannot be investigated in this context. However, we were determined to find out whether laminin α5 might have a more specific developmental role in the GBM that, for example, the other full-sized α chains (α1, α2, and α3B) could not perform.

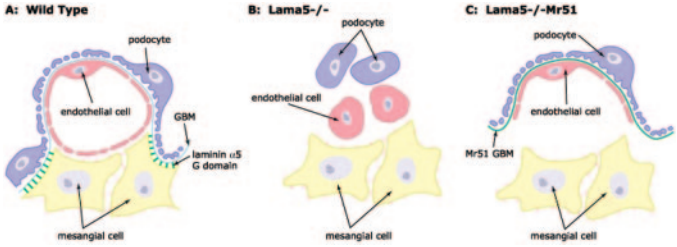

Figure 2. Structure of glomerular capillary loops in wild-type, *Lama5* −/−, and *Lama5* −/−; Mr51 kidneys. (A) In wild-type, podocytes sit atop the GBM in the urinary space and elaborate foot processes, fenestrated endothelial cells line the vascular surface of the GBM, and mesangial cells sit at the base of the capillary loops and bind the GBM to maintain the loop's structure. (B) In the absence of laminin α5, glomerular vascularization fails: The GBM breaks down, podocytes become clustered, and endothelial cells and mesangial cells fail to establish capillary loops. (C) In the presence of the Mr51 chimeric α chain, in which the G domain of α5 replaced with that of α1, the GBM's integrity is restored, podocytes and endothelial cells behave as in wild-type, but mesangial cells fail to maintain adhesion to the GBM, resulting in capillary loop distension. This is because mesangial cells organize the glomerular capillaries by adhering to the G domain of α5, which is missing from the Mr51 chimera.

Studies from several groups, including ours, had already shown that the large (approximately 100 kD) COOH-terminal globular (G) domains of α chains harbor binding sites for integrin and nonintegrin cellular receptors and that these receptors exhibited different affinities for the different α chains (26–31), which might provide the basis for functional differences. To investigate whether the laminin α5 G domain imparts a unique role to α5 during glomerulogenesis, we decided to express in mice novel laminin α5 variants in which all or part of the mouse α5 G domain was replaced with the analogous region of human laminin α1. This particular swap was chosen because laminin α1 and α5 not only are the two major chains in the developing GBM but also can be viewed as representing the two ancestral α chains present in *Drosophila melanogaster* and *Caenorhabditis elegans* (24).

We constructed chimeric cDNA encoding full-sized α5/α1 chimeric proteins and placed them under control of the miw regulatory element, which exhibits widespread expression during embryogenesis. This element contains a Rous sarcoma virus long terminal repeat inserted into the chicken β-actin promoter (32). Our approach was to make transgenic mice that express these chimeric proteins and mate the encoding transgenes onto the *Lama5* −/− background such that the transgene-derived proteins would replace the missing endogenous α5. In a proof-of-principal experiment, transgenic expression of the full-length mouse α5 cDNA under the control of miw (Mr5) on the *Lama5* −/− background fully rescues all known developmental defects, and *Lama5* −/−; Mr5 mice are viable and fertile.

Five independent transgenic lines expressing a chimera in which the entire α5 G domain was replaced with the human α1 G domain (Mr51) all were mated onto the *Lama5* −/− background to generate *Lama5* −/−; Mr51 embryos (33). Despite

widespread incorporation of transgene-derived protein into basement membranes, there was little change in the overall *Lama5* mutant phenotype, and *Lama5* −/−; Mr51 fetuses still died before birth. However, close examination of kidney glomeruli revealed significant alterations in phenotype: Instead of a breakdown in the GBM and a failure of glomerular vascularization, as occurs in *Lama5* −/− kidneys, in *Lama5* −/−; Mr51 kidneys, the GBM were intact, endothelial cells were adherent, podocytes elaborated foot processes and assembled slit diaphragms, and red blood cells were present in glomeruli. Rather than there being a collection of typical glomerular capillary loops, each glomerulus instead exhibited a single large ballooned vessel (33) (Figure 2). This phenotype is similar to that observed in the total absence of mesangial cells (34). Mesangial cells, which exhibit smooth muscle cell–like contractile properties, adhere to the GBM at the bases of the capillary loops, maintain loop structure, and regulate loop diameter (35). Desmin immunostaining and ultrastructural analysis revealed that mesangial cells were present in *Lama5* −/−; Mr51 glomeruli, but they apparently failed to adhere to the GBM (33). We hypothesized that because the α5 G domain is the only missing component in the *Lama5* −/−; Mr51 glomeruli, it must represent the GBM component to which mesangial cells normally adhere to maintain capillary loop structure. Inherent in this conclusion is the assumption that mesangial cells either do not adhere or adhere less well to the α1 G domain, which is present in the *Lama5* −/−; Mr51 GBM.

If mesangial cells preferentially adhere to laminin α5 over α1, then they should express cell surface receptors that confer these properties. We wished to define which receptors are involved in mediating mesangial cell adhesion to laminin α5 in the GBM and began this analysis by using human mesangial cells in adhesion assays on laminin-1 (α1β1γ1) and on laminin-10/11 (α5β1/β2γ1). Mesangial cells adhered to both laminin preparations, but at lower laminin concentrations, adhesion to the α5-containing laminin was significantly more robust (33). We then used a panel of integrin-blocking antibodies in the adhesion assays and found that anti-integrin α3 and anti-integrin β1 antibodies inhibited most but not all of the adhesion. Including a soluble version of the Lutheran glycoprotein, which we had previously shown to bind the α5 G domain (27), along with the integrin blocking antibody, inhibited almost all of the adhesion. We therefore concluded that on the basis of these *in vitro* studies, mesangial cells maintain the glomerular capillary loops by using integrin α3β1 and the Lutheran glycoprotein to adhere to the G domain of laminin α5 in the GBM (33). It is possible that additional receptors on mesangial cells may be involved.

Conclusion

In this review, I have emphasized the importance of laminin α5 and the GBM in glomerulogenesis. Without any laminin α5, glomerular vascularization fails; without its G domain, the capillary loops become distended and eventually ballooned. In neither case can the kidney function as a filter. That the GBM did not breakdown in *Lama5* −/−; Mr51 glomeruli suggests that α5 has at least two and probably three separable functions in the GBM during glomerulogenesis. One involves its ability to assemble with β and γ chains to make a trimer; this can be attributed to properties of the coiled coil domain of the long arm. Another involves its ability to promote laminin trimer polymerization, which is required to maintain the integrity of the GBM as laminin α1 is eliminated; this can be attributed to the short arm and is presumably dependent on the laminin N-terminal domain (36). Finally, the α5 G domain is required for proper mesangial cell adhesion to the GBM but, perhaps surprisingly, does not seem to be necessary for podocyte or endothelial cell adhesion or differentiation. Our future studies are aimed at determining whether α5 and its G domain play any role in glomerular filtration by using a recently generated conditional *Lama5* allele to mutate the gene in defined spatial and temporal patterns.

Acknowledgments

First and foremost, I thank Joshua R. Sanes, my postdoctoral mentor, for allowing me to work on the kidney in his neurobiology laboratory. I thank Marc Hammerman, my Division Chief, for providing a supportive environment in which I could start my own laboratory to continue studying the GBM. I thank all the members of my laboratory over the past 8 yr for their contributions—Ellen Ryan, Daryl Kenoyer, Kaya Andrews, Jacqueline Mudd, Gloriosa Go, Jennifer Richardson, Brendan Shannon, Guang Xu, Meei-Hua Lin, Scott Harvey, George Jarad, Xin Zhen—and especially those who contributed to the work described in this review—Jeanette Cunningham, Cong (Connie) Li, Casey Moulson, and Yamato Kikkawa. Finally, I acknowledge the Damon Runyon Cancer Research Foundation, the National Institutes of Health, the March of Dimes, and an American Heart Association Established Investigator Award for funding my research over the past 12 yr.

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