

Role of distinct type IV collagen networks in glomerular development and function

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Role of distinct type IV collagen networks in glomerular development and function.

Background. In X-linked Alport syndrome, mutations in the COL4A5 gene encoding the $\alpha 5$ chain of type IV collagen result in progressive renal failure. This nephropathy appears to relate to the arrest of a switch from an $\alpha 1/\alpha 2$ to an $\alpha 3/\alpha 4/\alpha 5$ network of type IV collagen in the developing glomerular basement membrane (GBM; Kalluri et al, *J Clin Invest* 99:2470, 1997).

Methods. We examined the role of this switch in glomerular development and function using a canine model of X-linked nephritis with a COL4A5 mutation. The electron microscopic appearance and the expression of the $\alpha 1$ - $\alpha 6$ chains of type IV collagen in the GBM was correlated with glomerular function.

Results. In normal neonatal glomeruli, once capillary loops were present, there was staining of GBM for the $\alpha 1$ - $\alpha 5$ chains. Prior to this stage, only $\alpha 1$ and $\alpha 2$ chains were present, with rare glomeruli positive for the $\alpha 5$ chain. As glomeruli matured, the $\alpha 1$ and $\alpha 2$ chains tended to disappear from the GBM, with the $\alpha 3$ - $\alpha 5$ chains remaining. In affected male dogs, only the $\alpha 1$ and $\alpha 2$ chains were detected at any stage. GBM ultrastructure in these dogs remained normal until one month and proteinuria did not appear until two months.

Conclusion. Our results show that normal glomerular development involves a switch in type IV collagen networks. In affected male dogs, a failure of this switch results in an absence of the $\alpha 3/\alpha 4/\alpha 5$ network and a persistence of the $\alpha 1/\alpha 2$ network in GBM. GBM ultrastructure and glomerular function remain normal for one month, indicating that GBM deterioration in Alport syndrome begins as a postnatal process. Hence, only the $\alpha 1/\alpha 2$ network is essential for normal glomerular development, whereas the $\alpha 3/\alpha 4/\alpha 5$ network is essential for long-term maintenance of glomerular structure and function.

Key words: Alport syndrome, hereditary nephritis, collagen type IV, collagen network, developmental switch.

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Alport syndrome is a hereditary disorder characterized by progressive nephropathy that is frequently associated with sensorineural deafness and ocular abnormalities [1–4]. About 80% of affected families show X-linked inheritance, and the remainder are autosomal dominant or recessive [5, 6]. Patients usually present from childhood to early adulthood, with hematuria. Most male patients have near normal kidney function at birth that deteriorates over time, leading to end-stage renal disease by the end of the third decade [1–3]. The hallmark feature of the disorder is multilaminar splitting of the glomerular basement membrane (GBM), as shown by electron microscopy. The molecular defects underlying Alport syndrome are mutations in the genes for type IV collagen. How the gene mutations alter the structure of type IV collagen are largely unknown, as are the mechanisms underlying the progression of the nephropathy to end-stage renal disease.

Type IV collagen is a family of six distinct α (IV)-chains [7]. These are designated $\alpha 1$ to $\alpha 6$, and are encoded by six genes, COL4A1 to COL4A6. The GBM is comprised of five of these chains, $\alpha 1$ to $\alpha 5$ [8, 9]. These five chains are assembled in triple helical molecules comprised of three α chains, that self assemble to form supramolecular networks. Two distinct networks have recently been established at the biochemical level, an $\alpha 1/\alpha 2$ network and an $\alpha 3/\alpha 4/\alpha 5$ network [10]. This novel $\alpha 3/\alpha 4/\alpha 5$ network is characterized by loops and supercoiled triple helices that are stabilized by disulfide bonds.

In the X-linked form of Alport syndrome, over 200 mutations have been found in the COL4A5 gene [11]. These mutations lead to the assembly of an adult GBM that is abnormal with respect to morphology and composition of type IV collagen chains. In contrast to normal GBM, Alport GBM is comprised of only $\alpha 1$ and $\alpha 2$ chains, and is devoid of the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains of type IV collagen [8, 9, 12, 13]. The altered chain composition appears to reflect an arrest of an early developmental switch, wherein the

fetal $\alpha 1$ and $\alpha 2$ chains persist in adult GBM and are not replaced by the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains [14]. This switch can now be defined at the supramolecular level in which the fetal $\alpha 1/\alpha 2$ network is replaced by the $\alpha 3/\alpha 4/\alpha 5$ network in normal glomerular development [10], but in Alport syndrome the switch is arrested. The newly described $\alpha 3/\alpha 4/\alpha 5$ network provides a plausible explanation for the absence of the $\alpha 5$ chain as well as the $\alpha 3$ and $\alpha 4$ chains in Alport syndrome, wherein the $\alpha 5$ chain is required for the assembly of the $\alpha 3$ and $\alpha 4$ chains in the network [10, 15].

Although renal function is near normal in Alport patients at birth, it is unknown whether glomerular development and GBM morphology are normal at that time. Subsequent postnatal events such as proteolysis are presumed to cause progressive deterioration of the GBM and renal function owing to the absence of the $\alpha 3/\alpha 4/\alpha 5$ network [14]. Since patients with Alport syndrome are for practical purposes biopsied only when they develop disease, glomerular development and presymptomatic GBM morphology cannot be studied in these patients.

To circumvent this limitation, we utilized the Samoyed dog model of X-linked hereditary nephritis in the present study to investigate the timing and the role of the $\alpha 1/\alpha 2$ and $\alpha 3/\alpha 4/\alpha 5$ networks in glomerular development and maintenance of glomerular function. This model results from a nonsense mutation in the COL4A5 gene [16] and closely mimics human Alport syndrome at the clinical, morphological and immunohistochemical levels [17–20]. Our results confirm the existence of a developmental switch for type IV collagen networks and that the switch is arrested by the COL4A5 gene mutation. The findings establish that only the $\alpha 1/\alpha 2$ network is essential for normal glomerular development, whereas the $\alpha 3/\alpha 4/\alpha 5$ network is essential for long-term stability of the GBM and maintenance of glomerular function. Moreover, the findings reveal that GBM deterioration in Alport syndrome is a postnatal process.

METHODS

Dogs used in this study were from the Samoyed hereditary nephritis pedigree. For electron microscopy, samples of renal cortex from six-day-old, three-week-old and one-month-old dogs were fixed in a 4% paraformaldehyde-1% glutaraldehyde mixture, post-fixed in osmium tetroxide, and embedded in an Epon-Araldite mixture. Sections were cut at 50 nm, stained with uranyl acetate and lead citrate and viewed on a Philips 400 electron microscope. For immunostaining, samples of renal cortex from six-day-old and one-month-old dogs were embedded in OCT, snap-frozen in liquid nitrogen and sectioned at 5 μ . Frozen sections were fixed in acetone and stained with antibodies specific for each of the six α chains of collagen type IV. The antibodies used were rat monoclonals raised against peptide sequences specific for each of the $\alpha 1$ – $\alpha 6$ chains of collagen type IV. Their specificity and the reactive epitopes (Table 1) have been previously established [9, 21]. These

Table 1. Specificity and reactive epitopes of the monoclonal antibodies for each of the $\alpha 1$ – $\alpha 6$ chains of collagen type IV

Collagen chain	Epitope	Source	Canine sequence	Amino acid match
$\alpha 1$	KKPTPSTL	Human	KKPTPSTL	8/8
$\alpha 2$	DTLKAGLIR	Human	DTLKAGL???	6/6?
$\alpha 3$	IPSTVKA	Human	IPSTTKA	6/7
$\alpha 4$	PAPDTLKE	Human	PSPDTLKE	7/8
$\alpha 4$	FSSAP	Human	FSSAP	5/5
$\alpha 5$	SKQSETL	Human	SKQSETL	8/8
$\alpha 5$	IDVEF	Human	IDVEF	5/5
$\alpha 6$?	Murine		
$\alpha 6$?	Bovine		

antibodies were shown in a previous study to react with $\alpha 3$ – $\alpha 5$ chains of dog glomeruli by Western blot [20]. The epitopes are given above with the comparable canine sequence; the epitopes for the $\alpha 6$ chains have not yet been mapped. Kidney sections were pretreated for 10 minutes with an acid-KCl solution (pH 1.5) to expose epitopes and then blocked with 1.5% normal rabbit serum (Sigma, St. Louis, MO, USA). An ABC immunoperoxidase technique was used: the primary antibody (1:100 dilution for 1.5 hr) was followed by a biotinylated rabbit anti-rat antibody (1:200 dilution for 1 hr; Vector Laboratories, Burlingame, CA, USA), then a peroxidase-conjugated avidin-biotin complex for 30 minutes (Santa Cruz Biotechnology, Santa Cruz, CA, USA), with a five minute incubation in diaminobenzidine as a chromagen. Sections were then counterstained with hematoxylin.

For double immunostaining, sections of normal and affected dog kidney were prepared as above. The rat monoclonal anti- $\alpha 3$ antibody was applied followed by an FITC-conjugated goat-anti rat antibody (1:30 dilution for 1 hr; Organon Teknika, West Chester, PA, USA). Following this, a mouse anti-collagen type IV antibody (Dako, Glostrup, Denmark) was applied (1:10 dilution for 1 hr) followed by a rhodamine conjugated goat anti-mouse antibody (1:30 dilution for 1 hr; Jackson Immunoresearch, West Grove, PA, USA). The mouse anti-collagen type IV antibody is a monoclonal raised against collagen type IV peptides isolated from placenta, and shows identical reactivity in tissue sections to the rat monoclonal anti- $\alpha 1$ and anti- $\alpha 2$ antibodies.

RESULTS

Neonatal dog glomeruli

At six days of age, glomeruli could be distinguished at different stages of development. Three different stages were evaluated: the pre-capillary stage (at six days of age, these glomeruli are largely in the S phase); the early capillary loop stage (when capillary loops are first seen in a simplified vascular tuft) and the late capillary loop stage (when the more complex vascular tuft of a mature glomerulus is present). No histologic differences were apparent at

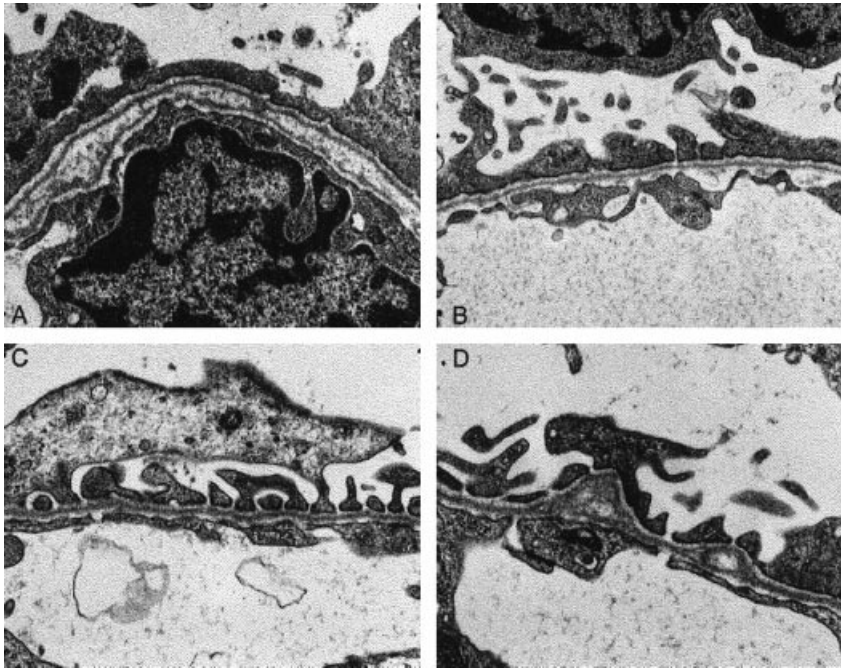


Fig. 1. Ultrastructural appearance of the glomerular basement membrane (GBM). At six days of age, immature glomeruli (A) show a bilaminar GBM with distinct endothelial and epithelial basement membranes separated by an electron lucent region. In the more mature glomeruli (B), these two basement membranes become apposed into a single GBM with the classic trilaminar appearance. This appearance persists in both normal and affected dogs up to three weeks of age (C). Only by one month can affected dogs be distinguished, at which time foci of bilaminar 'splitting' became apparent in GBM (D), while the appearance of the normal dog GBM remains normal (result not shown).

the light microscopic level between normal and affected dogs.

Ultrastructural appearance of the glomerular basement membrane

No differences were apparent between normal and affected dogs until one month of age (see Fig. 1). In the neonatal period, immature glomeruli showed a bilaminar appearance to the GBM with separate endothelial and epithelial basement membranes separated by an electron lucent region (Fig. 1A). In the more mature glomeruli, these two basement membranes became apposed into a single GBM with the classic trilaminar appearance of lamina dense bordered on both the endothelial and epithelial sides by a lamina rara (Fig. 1B). This appearance persisted in both normal and affected dogs (Fig. 1C) until one month of age, at which time foci of bilaminar 'splitting' became apparent in affected dog GBM (Fig. 1D), while the appearance of the normal dog GBM remained unchanged (result not shown).

The $\alpha 1$ and $\alpha 2$ chains of collagen type IV

In normal dogs, the staining distribution for the $\alpha 1$ and $\alpha 2$ chains of collagen type IV was identical (Table 2 and Figs. 2 A-C and 3 A-C). The pre-capillary stage (Figs. 2A and 3A) showed positive staining around the epithelial component destined to become Bowman's epithelium and podocytes. The ingrowing mesenchymal component destined to become endothelial and mesangial cells was also positive. The early capillary loop stage (Figs. 2B and 3B) showed positive staining of capillary loops, mesangial regions and Bowman's capsule. In the late capillary loop

Table 2. Stage and location of staining

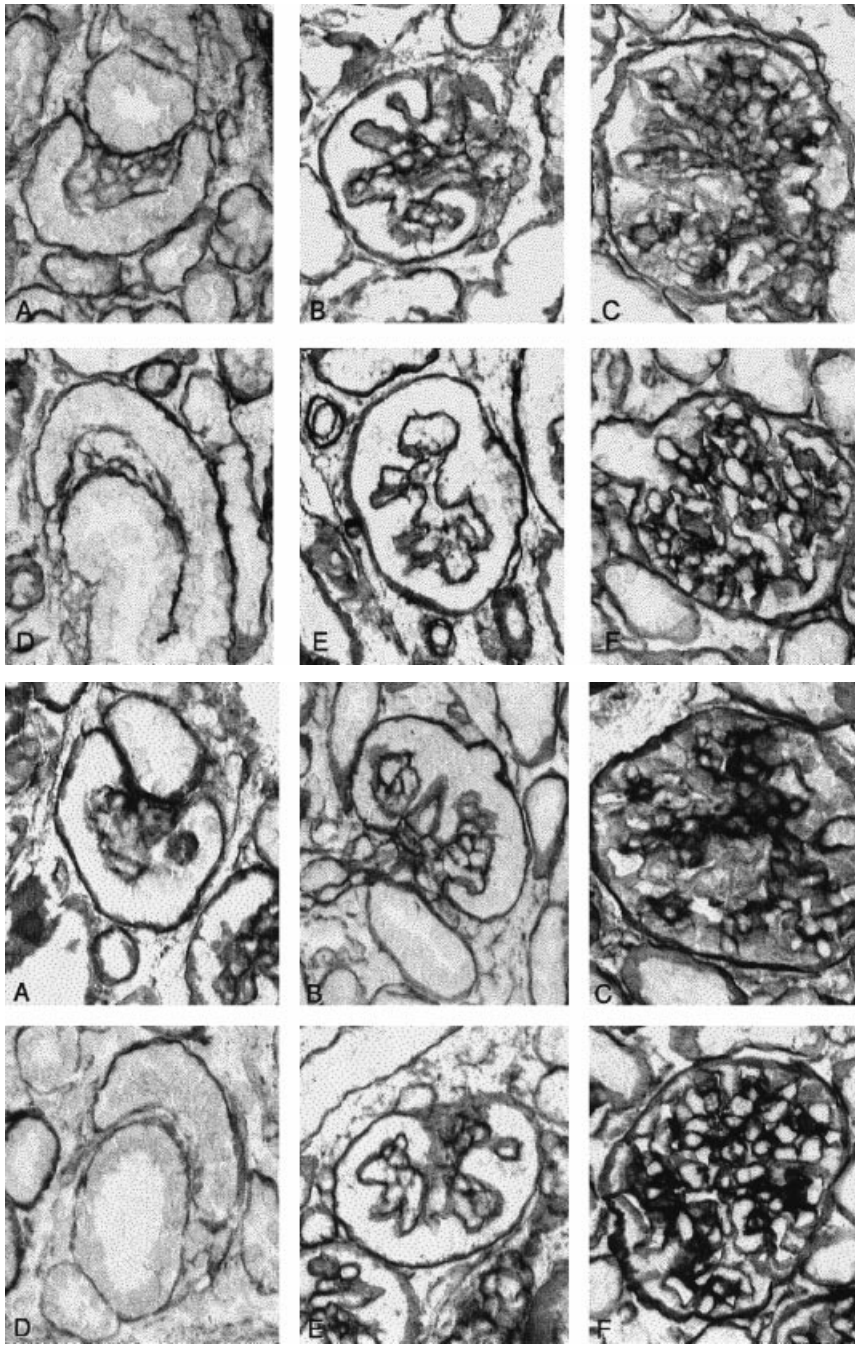
Status	Stage	Component	$\alpha 1$	$\alpha 2$	$\alpha 3$	$\alpha 4$	$\alpha 5$	$\alpha 6$
Normal	Pre-cap Early	GBM	+	+	-	-	-/+	-/+
		Mesangium	+	+	-	-	-	-
		Bowman's C	+	+	-	-	+	+
	Late	GBM	\pm	\pm	+	+	+	-
		Mesangium	+	+	-	-	-	-
		Bowman's C	+	+	-	-	+	+
Affected	Pre-cap Early	GBM	+	+	-	-	-	-
		Mesangium	+	+	-	-	-	-
		Bowman's C	+	+	-	-	-	-
	Late	GBM	+	+	-	-	-	-
		Mesangium	+	+	-	-	-	-
		Bowman's C	+	+	-	-	-	-

Symbols are: +, positive; -, negative; \pm , weakly positive; -/+, some negative and some positive.

stage (Figs. 2C and 3C), the mesangial region and Bowman's capsule remained positive, while the staining of capillary loops diminished. Staining for the $\alpha 1$ and $\alpha 2$ chains of collagen type IV in affected dog glomeruli was similar to normal dogs with some notable differences (Figs. 2 D-F and 3 D-F). The pre-capillary (2 days and 3 days) and early capillary loop (Figs. 2E and 3E) stages were identical to those in normal dog. In the late capillary loop stage (Figs. 2F and 3F), however, the capillary loops remained positive along with the mesangial region and Bowman's capsule.

The $\alpha 3$ and $\alpha 4$ chains of collagen type IV

In normal dogs, the pattern of staining for the $\alpha 3$ and $\alpha 4$ chains of collagen type IV was identical (Table 2 and Figs.

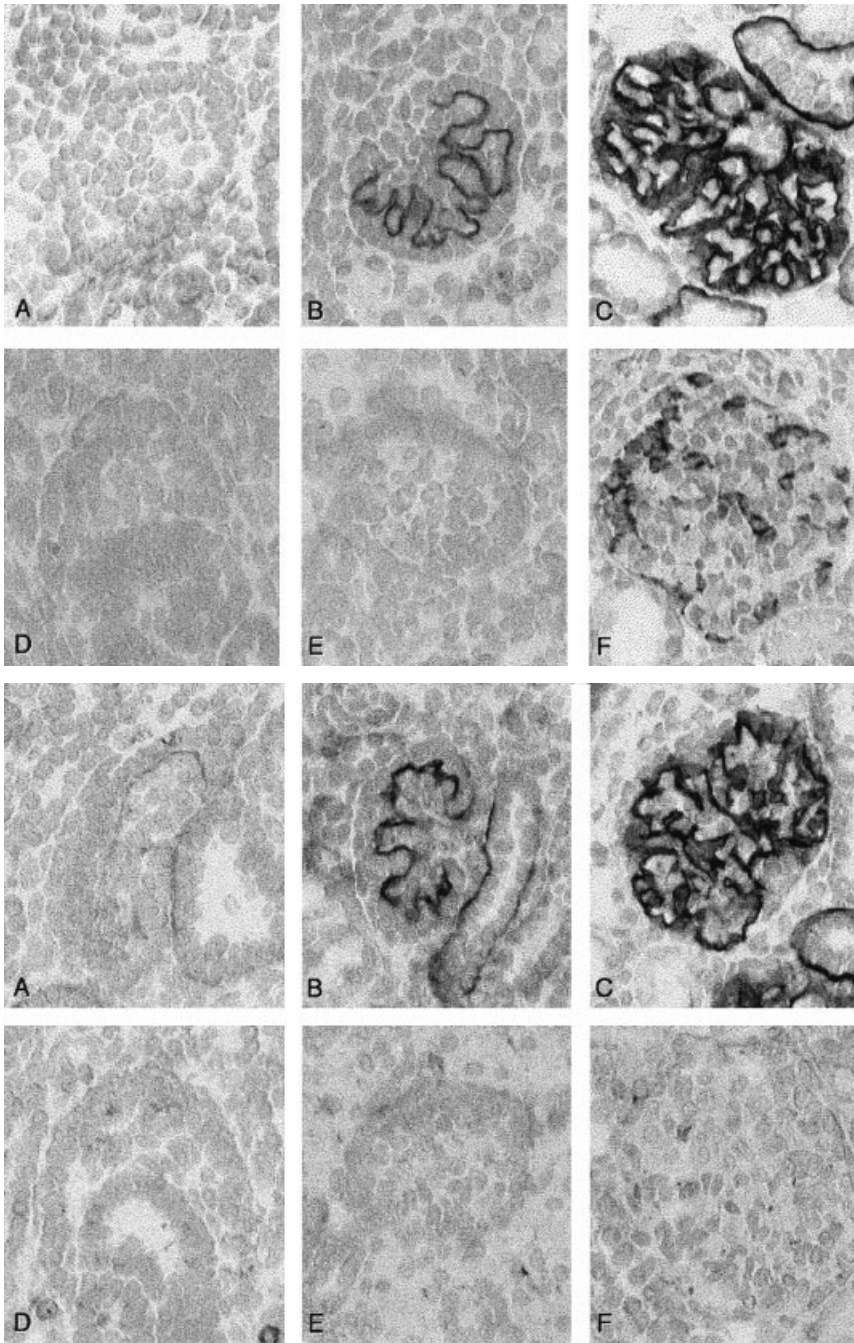


Figs. 2 and 3. Staining for the $\alpha 1$ chain (Fig. 2) and $\alpha 2$ chain (Fig. 3) of collagen type IV in normal (A-C) and affected (D-F) dog glomeruli. In normal dogs, the pre-capillary stage (A) shows positive staining around the epithelial component destined to become Bowman's epithelium and podocytes. The ingrowing mesenchymal component destined to become endothelial and mesangial cells is also positive. The immature GBM stage (B) shows positive staining of capillary loops, mesangial regions and around Bowman's capsule. In the mature GBM stage (C), the mesangial region and Bowman's capsule remain positive, while the staining of capillary loops diminishes. In affected dog glomeruli the pre-capillary (D) and immature GBM (E) stages are identical to those in normal dog. In the mature GBM stage (F), however, the capillary loops remain positive along with the mesangial region and Bowman's capsule.

4 A-C and 5 A-C). The pre-capillary stage (Figs. 4A and 5A) showed no staining for either chain. The early capillary loop stage (Figs. 4B and 5B) showed positive staining of capillary loops only, with mesangial regions and Bowman's capsule negative. In the late capillary loop stage (Figs. 4C and 5C), this pattern of staining persisted. In the case of affected dog glomeruli, there is no staining of any basement membrane at any stage for both the $\alpha 3$ and $\alpha 4$ chains (Figs. 4 D-F and 5 D-F). However, in the late capillary loop stage (Fig. 4F and 5F), there is positive staining of podocytes for the $\alpha 3$ chain but not the $\alpha 4$ chain.

The $\alpha 5$ chain of collagen type IV

In normal dog glomeruli (Table 2 and Fig. 6 A-D), two different patterns of staining were observed in the pre-capillary stage. A minority showed no staining of any basement membrane (Fig. 6A), while the majority showed positive staining for the $\alpha 5$ chain around the epithelial component destined to become Bowman's epithelium and podocytes (Fig. 6B). The ingrowing mesenchymal component remained negative. At the early capillary loop stage (Fig. 6C), there was positive staining of capillary loops and Bowman's



Figs. 4 and 5. Staining for the $\alpha 3$ chain (Fig. 4) and $\alpha 4$ chain (Fig. 5) of collagen type IV in normal (A-C) and affected (D-F) dog glomeruli. In normal dogs, the pre-capillary stage (A) shows no staining. The immature GBM stage (B) shows positive staining of capillary loops only, while mesangial regions and Bowman's capsule are negative. In the mature GBM stage (C), this pattern of staining persists. In affected dog glomeruli, there is no staining of any basement membrane at the pre-capillary (D), immature GBM (E) or mature GBM (F) stages. In the mature GBM stage, however, there is positive cytoplasmic staining of podocytes for the $\alpha 3$ chain.

capsule, while mesangial regions was negative. This pattern of staining persists in the late capillary loop stage (Fig. 6D). There was no staining of any basement membrane at any stage in affected dog glomeruli (Fig. 6 E-G).

The $\alpha 6$ chain of collagen type IV

In normal dog glomeruli (Table 2 and Fig. 7 A-D), again two different patterns of staining could be observed in the

pre-capillary stage. Some glomeruli showed no staining for the $\alpha 6$ chain (Fig. 7A), while in others there was staining around the epithelial component destined to become Bowman's epithelium and podocytes (Fig. 7B). The ingrowing mesenchymal component was consistently negative. At the early capillary loop stage (Fig. 7C), there was positive staining limited to Bowman's capsule. In the late capillary loop stage (Fig. 7D), staining for the $\alpha 6$ chain remained limited to Bowman's capsule. In affected dog glomeruli, no

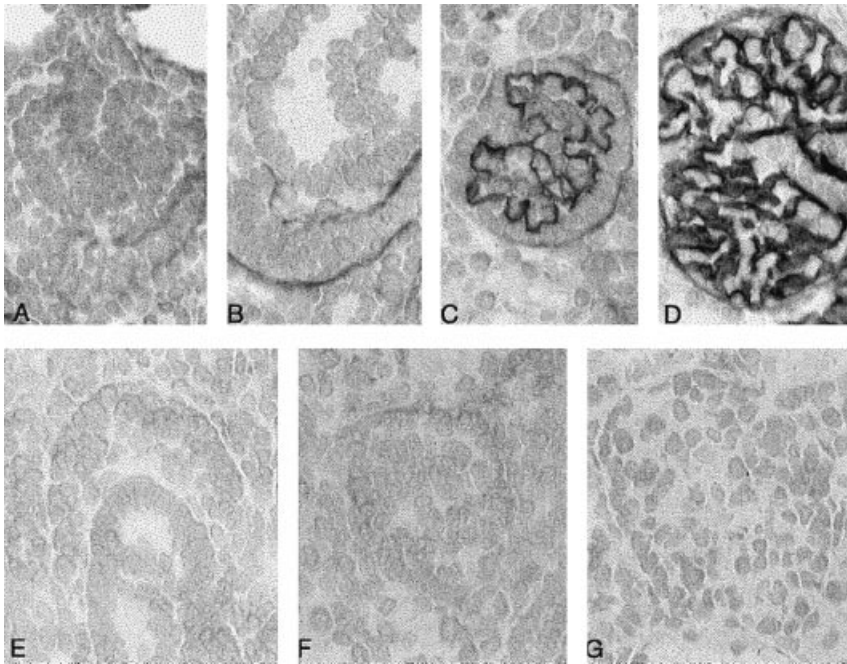


Fig. 6. Staining for the $\alpha 5$ chain of collagen type IV in normal (A-D) and affected (E-G) dog glomeruli. The early pre-capillary stage (A) shows no staining, but later in this stage (B) there is staining around the epithelial component destined to become Bowman's epithelium and podocytes. The ingrowing mesenchymal component is negative. The immature GBM stage (C) shows positive staining of capillary loops and Bowman's capsule, while mesangial regions are negative. In the mature GBM stage (D), this pattern of staining persists. In affected dog glomeruli, there is no staining of any basement membrane at any of the pre-capillary (E), immature GBM (F) and mature GBM (G) stages.

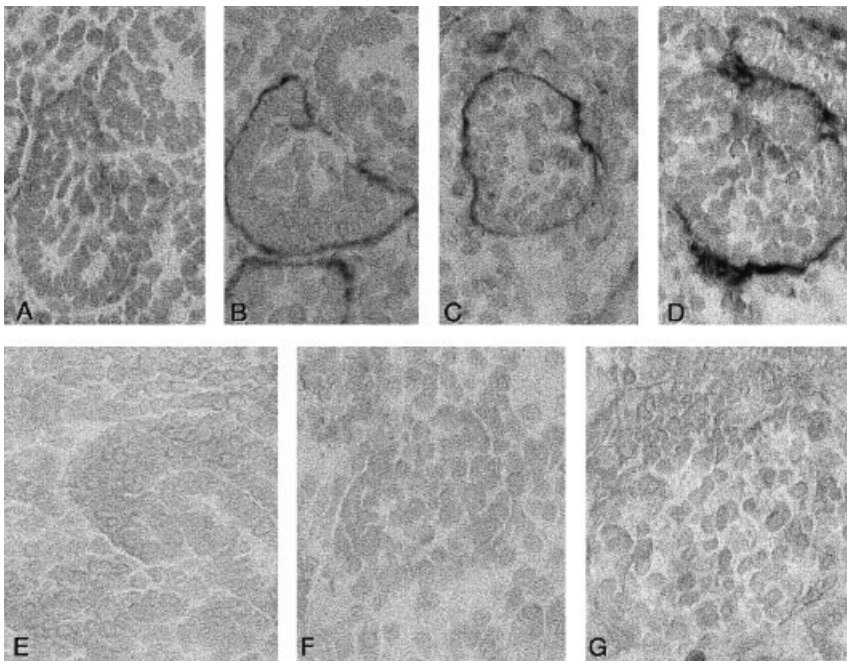


Fig. 7. Staining for the $\alpha 6$ chain of collagen type IV in normal (A-D) and affected (E-G) dog glomeruli. The early pre-capillary stage (A) shows no staining, but later in this stage (B) there is staining around the epithelial component destined to become Bowman's epithelium and podocytes. The ingrowing mesenchymal component is negative. The immature GBM stage (C) shows positive staining limited to Bowman's capsule. In the mature GBM stage (D), this pattern of staining persists. In affected dog glomeruli, there is no staining of any basement membrane at any of the pre-capillary (E), immature GBM (F) and mature GBM (G) stages.

staining of any basement membrane was detected at any stage (Fig. 7 E-G).

Double immunostaining for the $\alpha 1$ and $\alpha 3$ chains

This was performed to establish that the localization of the $\alpha 3$ chain with respect to the GBM in normal and affected dogs (Fig. 8). The results of this procedure confirmed, within the same glomerulus, the results described

above for the $\alpha 1$ chain and $\alpha 3$ chains separately, in both normal and affected dog kidney.

One-month-old dogs

Immunostaining results for the $\alpha 1$ - $\alpha 6$ chains were essentially the same at those seen in the mature glomeruli of neonatal dogs (results not shown). In normal dog glomeruli, the mesangial matrix contained the $\alpha 1$ and $\alpha 2$ chains,

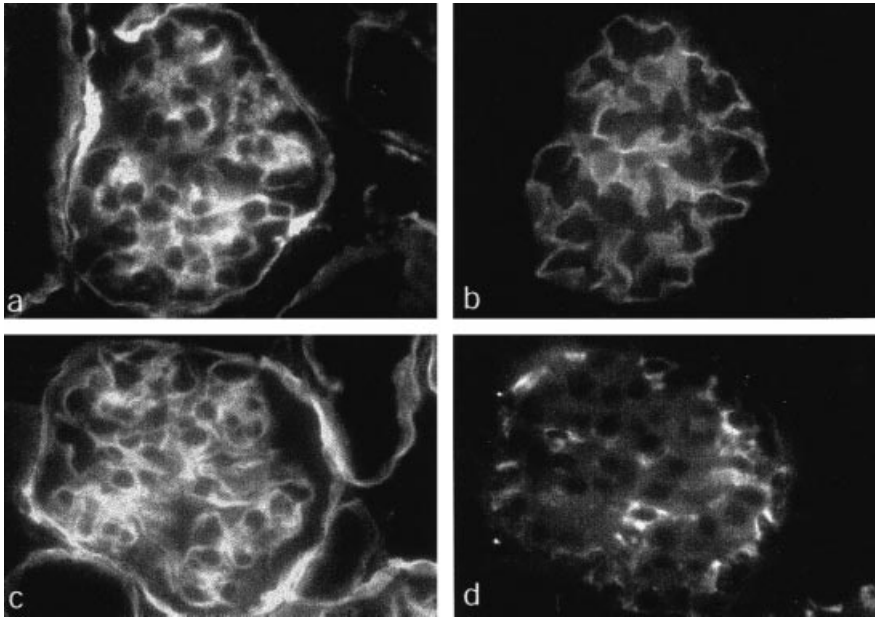


Fig. 8. Double immunostaining for the $\alpha 1$ chain (a and c) and $\alpha 3$ chain (b and d) of collagen type IV in normal (a and b) and affected (c and d) dog glomeruli. In normal dogs, the $\alpha 1$ chain localizes to the mesangial region and Bowman's capsule, with weak staining of capillary loops (a). The same glomerulus shows linear staining of capillary loops for the $\alpha 3$ chain, with no staining of the mesangial region or Bowman's capsule (b). In affected dog glomeruli, the capillary loops are positive for the $\alpha 1$ chain as well as the mesangial region and Bowman's capsule (c). In contrast, staining for the $\alpha 3$ chain in the same glomerulus is seen within podocyte cytoplasm and not along any of the capillary loops (d).

while the GBM contained mainly the $\alpha 3$ - $\alpha 5$ chains, with weak staining for the $\alpha 1$ and $\alpha 2$ chains. Bowman's capsule contained the $\alpha 1$, $\alpha 2$, $\alpha 5$ and $\alpha 6$ chains. In affected dog glomeruli, there was positive staining only for the $\alpha 1$ and $\alpha 2$ chains in the mesangial matrix, GBM and Bowman's capsule. Staining for the $\alpha 3$ chain was just barely detectable in podocytes of affected male dogs, representing a reduction from that noted in the neonatal affected dogs.

DISCUSSION

Our studies in normal dogs have shown that as soon as capillary loops are present in glomeruli, there is positive staining of GBM for the $\alpha 3$ - $\alpha 5$ chains of collagen type IV. Prior to this stage, the $\alpha 1$ and $\alpha 2$ chains are the predominant chains present, although the $\alpha 5$ and $\alpha 6$ chains begin to appear in Bowman's capsule. The formation of capillary loops corresponds to the appearance of GBM distinct from mesangial matrix. As glomeruli mature, the $\alpha 1$ and $\alpha 2$ chains tend to disappear from the GBM, while the $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains remain, implying there is active remodeling of the GBM during glomerular development. This involves the turning on of the genes producing the $\alpha 3$ - $\alpha 5$ chains, which can be termed a 'developmental switch' changing a 'fetal' GBM to an 'adult' GBM. In developing dog glomeruli, production of the $\alpha 5$ chain precedes the $\alpha 3$ and $\alpha 4$ chains. The idea that GBM collagens might undergo a developmental switch was first suggested in rodents [22], in which developing glomeruli go through a similar sequence of changes in collagen type IV chains as we have observed in the dog, including the $\alpha 5$ chain appearing before the $\alpha 3$ and $\alpha 4$ chains. Since that initial observation, this switch has been described in human fetal glomeruli [14] and rat testis [23].

The molecular basis for the switch is unknown, but must involve a mechanism to link the expression and incorporation of the $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains into a network because mutations in the COL4A5 gene cause disruption of the assembly of the $\alpha 3$ and $\alpha 4$ chains, as well as the $\alpha 5$ chain. This latter finding was first demonstrated by immunohistochemistry [8, 9, 12, 13] and later by biochemical analysis [14]. These observations suggested that the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains might comprise a distinct network separate from the classical network of $\alpha 1$ and $\alpha 2$ chains [12]. This hypothesis has now been recently confirmed by studying both seminiferous tubule basement membrane and glomerular basement membrane [10, 15] in which networks containing the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains could be separated from the classic $\alpha 1$ - and $\alpha 2$ -containing network. These findings established, to our knowledge for the first time, a structural linkage between the $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains.

The developmental switch mechanism could operate at the protein, mRNA and/or the gene levels. At the protein level, events at both triple helix formation and supramolecular assembly need to be considered. Should the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains form heterotrimers, then an abnormal $\alpha 5$ chain could lead to faulty heterotrimer assembly resulting in absence of these chains in Alport GBM. Should the $\alpha 3$ and $\alpha 4$ chains be in trimers distinct from those containing the $\alpha 5$ chain, then the $\alpha 5$ chain may be necessary for incorporation of $\alpha 3$ - and $\alpha 4$ -chain containing trimers. Hence, an abnormality of $\alpha 5$ -containing trimers could lead to absence of the $\alpha 3$ - and $\alpha 4$ -containing trimers from GBM. At the translational/transcriptional level, should the expression of the $\alpha 3$, $\alpha 4$, or $\alpha 5$ chains be coordinated, then in X-linked Alport syndrome the transcription or translation of the $\alpha 3$ and $\alpha 4$ chains might be impaired secondary

to a mutation in the $\alpha 5$ gene. Evidence that the mechanism of the switch affects the gene expression level comes from our study of the expression of the α (IV) chains in the kidney in canine X-linked nephritis [20]. The canine nephritis is caused by a nonsense mutation in the COL4A5 gene that results in a $\geq 77\%$ reduction of mRNA levels for not only the $\alpha 5$ chain but also the $\alpha 3$ and $\alpha 4$ chains. Additional support for this concept comes from the finding that expression of the COL4A5 gene precedes that of the COL4A3 and COL4A4 genes in developing rat kidney [22], rat testis [23], murine kidney [24] and, in this paper, developing dog kidney. Furthermore, in the COL4A3 knockout mouse model, loss of expression of the COL4A3 does not reduce the level of expression of the COL4A5 gene [25].

The protein assembly and the translational/transcriptional mechanisms need not be mutually exclusive in that the incorporation of the $\alpha 5$ chain into the extracellular matrix could be required to modulate the transcription of the $\alpha 3$ and $\alpha 4$ chains. This idea would be consistent with our observation that the $\alpha 5$ chain appears earlier in canine glomerular development than the $\alpha 3$ and $\alpha 4$ chains. A novel observation in our studies was that podocytes of affected dogs showed cytoplasmic staining for the $\alpha 3$ chain, instead of in the GBM. Similar staining was not seen in normal dogs. This result suggests an inability to export the $\alpha 3$ chain into the extracellular matrix in the absence of the $\alpha 4$ and/or $\alpha 5$ chains. Since collagen chains are only exported after formation of a triple helix [reviewed in 26], this observation would imply the $\alpha 3$ chain is contained within the same triple helix as the $\alpha 4$ and/or $\alpha 5$ chain, instead of existing as an $\alpha 3$ homotrimer. The cytoplasmic staining for the $\alpha 3$ chain is also detectable in affected dogs at one month of age but not at four months, which could reflect a down-regulation of $\alpha 3$ chain production with time. In support of this hypothesis, we have shown the mRNA level for the $\alpha 3$ chain is reduced $\geq 77\%$ at four months of age [20].

In affected male dogs, only the $\alpha 1$ and $\alpha 2$ chains are detected in GBM, and this pattern of staining persists in the most mature glomeruli. The $\alpha 3$ - $\alpha 6$ chains are not detected in any basement membrane in the kidney. These results corroborate at the immunohistochemical level our earlier observations by Western blotting [20]. In other words, in affected male dogs, there is persistence of an fetal collagen network composed only of $\alpha 1$ and $\alpha 2$ chains. This can be seen as a failure of the developmental switch mentioned above, secondary to a COL4A5 mutation. Increased staining of the GBM in Alport syndrome for the $\alpha 1$ and $\alpha 2$ chains had been noted before [27]. This observation was later incorporated into a hypothesis that there was a failure of a developmental switch in Alport syndrome, based on work with normal fetal kidney [14]. This could not be confirmed experimentally since renal tissue from developing human glomeruli in Alport syndrome is not available.

Our canine model has provided the opportunity to explore and confirm this hypothesis.

Despite this failure of the developmental switch, and the resulting absence of the $\alpha 3/\alpha 4/\alpha 5$ network in GBM, the affected dog GBM has a normal appearance by electron microscopy up to one month of age as noted in earlier work [17] and reaffirmed in the present study. Furthermore, affected dogs do not develop proteinuria (the presenting sign of their disease) until about two months of age [19]. Similarly, the COL4A3 knockout mice, which also lack the $\alpha 3/\alpha 4/\alpha 5$ network in their GBM, do not develop proteinuria until two to three months of age [25]. These results are seemingly at variance with the earlier observation that progressive replacement of the $\alpha 1$ and $\alpha 2$ chains in the rat GBM by the $\alpha 3$ chain was associated with acquisition of permselectivity [28]. Instead, it would appear from our results that an $\alpha 1/\alpha 2$ network is capable of forming a morphologically normal GBM (as assessed at the EM level), which confers normal renal function for a limited period of time. This implies that only the $\alpha 1/\alpha 2$ network is essential for normal glomerular development whereas the $\alpha 3/\alpha 4/\alpha 5$ network is essential for the long-term stability of the GBM and maintenance of glomerular function.

A plausible hypothesis is that the $\alpha 3/\alpha 4/\alpha 5$ network confers long-term stability to GBM by protecting against proteolytic degradation [14]. The $\alpha 3$ and $\alpha 4$ chains are more cysteine rich in their 7S and triple helical regions than the $\alpha 1$ and $\alpha 2$ chains leading to a loop structure of the triple-helix covalently crosslinked by disulfide bonds. In this regard, the $\alpha 1/\alpha 2$ network is more easily extracted by pseudolysin than the $\alpha 3/\alpha 4/\alpha 5$ network [10, 15]. It has been shown that glomerular epithelial and mesangial cells produce enzymes such as metalloproteinases, neutral protease, and cathepsins that are capable of degrading basement membrane components [29–32]. Recent work identified a differential sensitivity of normal and Alport renal basement membrane to endopeptidase degradation [14]. Alport basement membrane was more readily degraded by bacterial collagenase, pseudolysin, and cathepsin B and G compared to control renal basement membranes. Additionally, the normal NC1 hexamer of type IV collagen had a greater resistance to proteolysis than the hexamer from Alport kidney.

Overall our results, in conjunction with the work of others, allow us formulate the following hypothesis (presented in diagram form in Fig. 9). Progression to end-stage renal disease in X-linked Alport syndrome evolves from a congenital malformation of the GBM, which alone is insufficient to cause disease, but which is permissive to one or more postnatal processes that deteriorate the GBM, causing glomerular dysfunction. Specifically, the congenital malformation of the GBM involves COL4A5 mutations that arrest a developmental switch from the fetal $\alpha 1/\alpha 2$ network to the adult $\alpha 3/\alpha 4/\alpha 5$ network, and the persistence of this fetal network predisposes the GBM to proteolytic

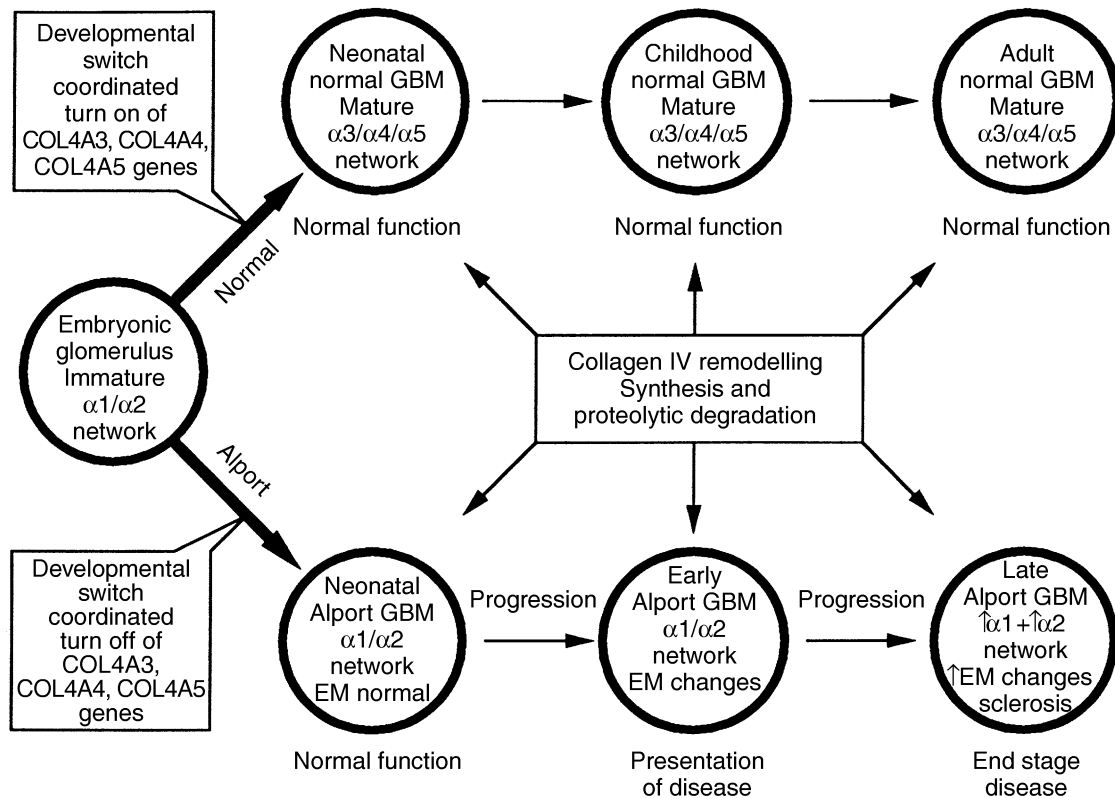


Fig. 9. A hypothetical scheme for the development of normal GBM and abnormal GBM in Alport syndrome. The scheme is a compilation of knowledge obtained about type IV collagen structure and degradation from the bovine species, a developmental switch from rodents and human beings, and coordinated gene regulation of $\alpha3$ - $\alpha5$ chains of type IV collagen from the canine model of X-linked nephritis.

degradation. The canine model of X-linked Alport syndrome will provide us with a unique opportunity to investigate this hypothesis and to pursue the mechanism of the progression of the GBM abnormality in this disease.

Finally, there are implications of this work with respect to using gene therapy to treat Alport syndrome. Since an $\alpha1/\alpha2$ network is capable of forming a GBM that maintains normal renal function for a limited period of time, this provides a 'time window' during which one could administer gene therapy before GBM damage begins. Although the duration of this window of time in humans remains to be determined, *in utero* gene therapy should not be necessary for the treatment of Alport syndrome.

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REFERENCES

- HABIB R, GUBLER M-C, HINGLAIS N, NOËL L-H, DROZ D, LEVY M, MAHIEU P, FOIDART J-M, PERRIN D, BOIS E, GRÜNFELD J-P: Alport's syndrome: Experience at Hôpital Necker. *Kidney Int* 21(Suppl 11): S20-S28, 1982
- GRÜNFELD J-P: The clinical spectrum of hereditary nephritis. *Kidney Int* 27:83-92, 1985
- KASHTAN CE, MICHAEL AF: Alport syndrome. *Kidney Int* 50:1445-1463, 1996
- FEINGOLD J, BOIS E, CHOMPRET A, BROYER M, GUBLER M-C, GRÜNFELD J-P: Genetic heterogeneity of Alport syndrome. *Kidney Int* 27:672-677, 1985
- REEDERS S: Molecular genetics of hereditary nephritis. *Kidney Int* 42:783-792, 1992
- LEMMINK HH, MOCHIZUKI T, VAN DEN HEUVEL LPWJ, SCHRÖDER CH, BARRIENTOS A, MONNENS LAH, VAN OOST BA, BRUNNER HG, REEDERS ST, SMEETS HJM: Mutations in the type IV collagen $\alpha3$ (COL4A3) gene in autosomal recessive Alport syndrome. *Hum Mol Genet* 3:1269-1273, 1994
- HUDSON BG, REEDERS ST, TRYGGVASON K: Type IV collagen: Structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis. *J Biol Chem* 268:26033-26036, 1993
- PEISSEL B, GENG L, KALLURI R, KASHTAN C, RENNKE HG, GALLO GR, YOSHIOKA K, SUN MJ, HUDSON BG, NEILSON EG, ZHOU J: Comparative distribution of the $\alpha1(IV)$, $\alpha5(IV)$, and $\alpha6(IV)$ collagen chains in normal human adult and fetal tissues and in kidneys from X-linked Alport syndrome patients. *J Clin Invest* 96:1948-1957, 1995
- NINOMIYA Y, KAGAWA M, IYAMA K, NAITO I, KISHIRO Y, SEYER JM, SUGIMOTO M, OOHASHI T, SADO Y: Differential expression of two

- basement membrane collagen genes, COL4A6 and COL4A5, demonstrated by immunofluorescence staining using peptide-specific monoclonal antibodies. *J Cell Biol* 130:1219–1229, 1995
10. GUNWAR S, BALLESTER F, NOELKEN ME, SADO Y, NINOMIYA Y, HUDSON BG: Identification of a novel disulfide-cross-linked network of $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains of type IV collagen and its implications for the pathogenesis of Alport syndrome. *J Biol Chem* 273:8767–8775, 1998
 11. KNEBELMANN B, BREILLAT C, FORESTIER L, ARRONDEL C, JACASSIER D, GIATRAS I, DROUOT L, DESCHÈNES G, GRÜNFELD J-P, BROYER M, GUBLER M-C, ANTIGNAC C: Spectrum of mutations in the COL4A5 collagen gene in X-linked Alport syndrome. *Am J Hum Genet* 59:1221–1232, 1996
 12. KLEPPEL MM, FAN WW, CHEONG HI, MICHAEL AF: Evidence for separate networks of classical and novel basement membrane collagen. Characterization of $\alpha 3(\text{IV})$ -Alport antigen heterodimer. *J Biol Chem* 267:4137–4142, 1992
 13. YOSHIOKA K, HINO S, TAKEMURA T, MAKI S, WIESLANDER J, TAKEKOSHI Y, MAKINO H, KAGAWA M, SADO Y, KASHTAN CE: Type IV collagen $\alpha 5$ chain: Normal distribution and abnormalities in X-linked Alport syndrome revealed by monoclonal antibody. *Am J Pathol* 144:986–996, 1994
 14. KALLURI R, SHIELD FC, TODD P, HUDSON BG, NEILSON EC: Isoform switching of type IV collagen is developmentally arrested in X-linked Alport syndrome leading to increased susceptibility of renal basement membranes to endoproteolysis. *J Clin Invest* 99:2470–2478, 1997
 15. KAHSAI TZ, ENDERS GE, GUNWAR S, BRUNMARK C, WIESLANDER J, KALLURI R, ZHOU J, NOELKEN ME, HUDSON BG: Composition and organization of type IV collagen chains, the linkage of the $\alpha 3(\text{IV})$ and $\alpha 5(\text{IV})$ chains. *J Biol Chem* 272:17023–17032, 1997
 16. ZHENG K, THORNER PS, MARRANO P, BAUMAL R, MCINNES RR: Canine X chromosome-linked hereditary nephritis: A genetic model for human X-linked hereditary nephritis resulting from a single base mutation in the gene encoding the $\alpha 5$ chain of collagen type IV. *Proc Acad Natl Sci USA* 91:3989–3993, 1994
 17. JANSEN B, THORNER P, BAUMAL R, VALLI V, MAXIE MG, SINGH A: Samoyed hereditary glomerulopathy (SHG): Evolution of splitting of glomerular capillary basement membranes. *Am J Pathol* 125:536–545, 1986
 18. JANSEN B, TRYPHONAS L, WONG J, THORNER P, MAXIE MG, VALLI VE, BAUMAL R, BASRUR P: Mode of inheritance of Samoyed hereditary glomerulopathy: An animal model of hereditary nephritis in humans. *J Lab Clin Med* 107:551–555, 1986
 19. JANSEN B, VALLI VE, THORNER P, BAUMAL R, LUMSDEN JH: Samoyed hereditary glomerulopathy (SHG): Serial clinical and laboratory (urine, serum biochemistry and hematology) studies. *Can J Vet Res* 51:387–393, 1987
 20. THORNER PS, ZHENG K, KALLURI R, JACOBS R, HUDSON BG: Coordinate gene expression of the $\alpha 3$, $\alpha 4$ and $\alpha 5$ chains of collagen type IV: Evidence from a canine model of X-linked nephritis with a COL4A5 gene mutation. *J Biol Chem* 271:13821–13828, 1996
 21. SADO Y, KAGAWA M, KISHIRO Y, SUGIHARA K, NAITO I, SEYER JM, SUGIMOTO M, OOHASHI T, NINOMIYA Y: Establishment by the rat lymph node method of epitope-defined monoclonal antibodies recognizing the six different α chains of human type IV collagen. *Histochem Cell Biol* 104:267–275, 1995
 22. MINER JH, SANES JR: Collagen IV $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains in rodent basal laminae: Sequence, distribution, association with laminins, and developmental switches. *J Cell Biol* 127:879–891, 1994
 23. ENDERS GC, KAHSAI TZ, LIAN G, FUNABIKI K, KILLEN PD, HUDSON BG: Developmental changes in seminiferous tubule extracellular matrix components of the mouse testis: $\alpha 3(\text{IV})$ collagen chain expressed at the initiation of spermatogenesis. *Biol Reprod* 53:1489–1499, 1995
 24. GATTONE VH, SCHIEREN G, KILLEN PD: Aberrant expression of extracellular matrix in infantile-type polycystic kidney disease in cpk/cpk mice. *J Am Soc Nephrol* 6:694, 1995
 25. MINER JH, SANES JR: Molecular and functional defects in kidneys of mice lacking collagen $\alpha 3(\text{IV})$: Implications for Alport syndrome. *J Cell Biol* 135:1403–1413, 1996
 26. PROCKOP DJ, KIVIRIKKO KI: Collagens: Molecular biology, diseases and potentials for therapy. *Annu Rev Biochem* 64:403–434, 1995
 27. KASHTAN CE, KIM Y: Distribution of the $\alpha 1$ and $\alpha 2$ chains of collagen type IV and of collagens V and VI in Alport syndrome. *Kidney Int* 42:115–126, 1992
 28. DESJARDINS M, BENDAYAN M: Ontogenesis of glomerular basement membrane: Structural and functional properties. *J Cell Biol* 113:689–700, 1991
 29. MARTIN J, DAVIES M, THOMAS G, LOVETT DH: Human mesangial cells secrete a GBM-degrading neutral proteinase and a specific inhibitor. *Kidney Int* 36:790–801, 1989
 30. LOVETT DH, JOHNSON RJ, MARTI HP, MARTIN J, DAVIES M, COUSER WG: Structural characterization of the mesangial cell type IV collagenase and enhanced expression in a model of immune complex-mediated glomerulonephritis. *Am J Pathol* 141:85–98, 1992
 31. KAUSHAL GP, WALKER PD, SHAH SV: An old enzyme with a new function: Purification and characterization of a distinct matrix-degrading metalloproteinase in rat kidney cortex and its identification as meprin. *J Cell Biol* 126:1319–1327, 1994
 32. KNOWLDEN J, MARTIN J, DAVIES M, WILLIAMS JD: Metalloproteinase generation by human glomerular epithelial cells. *Kidney Int* 47:1682–1689, 1995