Matrix Pathobiology

Sequential Expression of Type IV Collagen Networks: Testis as a Model and Relevance to Spermatogenesis

Scott J. Harvey,* Julie Perry,* Keqin Zheng,*
Dilys Chen,* Yoshikazu Sado,[†]
Barbara Jefferson,[‡] Yoshifumi Ninomiya,[§]
Robert Jacobs,[‡] Billy G. Hudson,[¶] and
Paul S. Thorner*^{||}

From the Division of Pathology,* Hospital for Sick Children, Toronto, Canada; the Division of Immunology,[†] Shigei Medical Research Institute, Okayama, Japan; the Department of Pathobiology,[‡] University of Guelph, Guelph, Canada; the Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences,[§] Okayama, Japan; the Department of Medicine,[¶] Vanderbilt University, Nashville, Tennessee; and the Department of Laboratory Medicine and Pathobiology,^{||} University of Toronto, Toronto, Canada

The six α chains of type IV collagen are organized into three networks: $\alpha 1/\alpha 2$, $\alpha 3/\alpha 4/\alpha 5$, and $\alpha 1/\alpha 2/\alpha 5/\alpha 6$. A shift from the $\alpha 1/\alpha 2$ to the $\alpha 3/\alpha 4/\alpha 5$ network occurs in the developing glomerular basement membrane, but how the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network fits into this sequence is less clear, because the three networks do not colocalize. Here, we studied the seminiferous tubule basement membrane of normal canine testis where all three networks do colocalize: the $\alpha 1/\alpha 2$ network is expressed from birth, the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network by 5–6 weeks of age, and the $\alpha 3/\alpha 4/\alpha 5$ network by 2 months of age. A canine model of Alport syndrome allowed study of the absence of $\alpha 3/\alpha 4/\alpha 5$ and $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ networks in testis. In Alport dogs, the seminiferous tubule basement membrane was thinner than in controls. Spermatogenesis began at the same time as with normal dogs; however, the number of mature sperm was significantly reduced in Alport dogs. Thus, it would appear that $\alpha 3/\alpha 4/\alpha 5$ and $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ networks are not essential for onset of spermatogenesis, but long-term function may be compromised by the loss of one or both networks. This situation is analogous to the glomerular basement membrane in Alport syndrome. In conclusion, testis can serve as a model system to study the sequence of type IV collagen network expression. (*Am J Pathol* 2006; 168:1587–1597; DOI: 10.2353/ajpath.2006.050816)

Type IV collagen is assembled from a family of six distinct chains (reviewed in Refs. 1 and 2) designated $\alpha 1$ to $\alpha 6$, which are encoded by six different genes, *COL4A1* to *COL4A6*, respectively. The six type IV collagen chains self-assemble into three basic protomers with the composition $\alpha 1_2\alpha 2$, $\alpha 5_2\alpha 6$, and $\alpha 3\alpha 4\alpha 5$. These protomers are organized into three distinct networks, $\alpha 1/\alpha 2$, $\alpha 3/\alpha 4/\alpha 5$, and $\alpha 1/\alpha 2/\alpha 5/\alpha 6$.^{2–6} Whereas basement membranes of all organs contain the $\alpha 1/\alpha 2$ network, the other two networks are expressed in selected sites only, in a manner that is remarkably consistent across mammals. For example, in human, murine, bovine, and canine kidney, the $\alpha 3/\alpha 4/\alpha 5$ network is present in the mature glomerular basement membrane (GBM), and the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ is present in Bowman's capsule and around smooth muscle cells.^{7–11} This site-specific expression presumably reflects function, but there are few experimental data to support this concept.

The molecular composition of many specialized basement membranes is not static. Rather, there are changes in the expression of specific isoforms of type IV collagen during normal development. This phenomenon has been observed for rat, mouse, dog, and human GBMs,^{10,12,13} mouse and dog inner ear,^{14,15} and mouse and human lens capsule.¹⁶ In all these sites, the $\alpha 3/\alpha 4/\alpha 5$ network is expressed at a later stage in normal development than the $\alpha 1/\alpha 2$ network, which is ubiquitous in site and time for basement membranes. To fit the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network into this sequence requires a temporal study on a basement membrane that contains all six isoforms. Only rare basement membranes such as the seminiferous tubule basement membrane (STBM), epithelial basement mem-

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Address reprint requests to Paul Thorner, M.D., Ph.D., Division of Pathology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario M5G 1X8, Canada. E-mail: thorner@sickkids.on.ca.

brane of the epididymis, and lens capsule of the eye contain all three networks.^{16–18}

Further insight into the role of specific type IV collagen networks has come from studies on Alport syndrome, a hereditary disorder of type IV collagen characterized by progressive nephropathy, ocular abnormalities, and high tone sensorineural deafness.^{19–22} In this disease, mutations in any of the *COL4A3*, *COL4A4*, or *COL4A5* genes result in a GBM with abnormal morphology and composition.^{23–25} The GBM usually contains only the $\alpha 1$ and $\alpha 2$ chains and lacks the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains.^{7,8,26,27} The existence of the $\alpha 3/\alpha 4/\alpha 5$ network provides a plausible explanation for the absence of these chains in the GBM in Alport syndrome, in that all three chains are required for the assembly of this network.^{3,6} Studies from a canine model of X-linked Alport syndrome have shown the $\alpha 3/\alpha 4/\alpha 5$ network is not required for normal GBM formation but is required for long-term maintenance of GBM structure and function.¹⁰ In this “natural knockout” model, there is a nonsense mutation in the *COL4A5* gene,²⁸ resulting in no $\alpha 5$ chain being produced and, in turn, loss of the $\alpha 3/\alpha 4/\alpha 5$ and $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ networks.^{10,11}

With the above considerations in mind, the goal of the present study was to delineate the sequential expression of all three type IV collagen networks using the testis as a model system, in particular the STBM. We chose to study canine testis for three reasons: 1) the tissue is relatively easy to access, 2) only some mammalian STBM (including canine) have been reported to express all three collagen networks, and 3) the availability of a canine model of Alport syndrome allows one to examine testis in which specific type IV collagen networks can be predicted to be absent. In turn, this may provide clues to the functions of these networks.

Materials and Methods

Tissue Samples

Testes were available from normal dogs and dogs affected with X-linked hereditary nephritis. A total of 17 normal dogs and 27 affected dogs (paired littermates) were examined. Testis from one to four normal and one to four affected dogs (mode = 2) were examined at 1, 5, 6, 8, 12, 16, 20, 24, 28, 32, and 36 weeks. In addition, testes were available (formalin-fixed material only) from a 33-month-old Alport dog that had received a renal transplant at 3 months of age and six normal dogs, 2–6 years old. Samples from four normal human testes (patient ages 12, 12, 14, and 17 years) and one cryptorchid testis (patient age 16 years) were available for immunohistochemistry analyses. These samples were obtained from the remaining tissue after diagnostic workup, during surgical procedures performed solely for clinical reasons. Use of these tissues was granted by the Research Ethics Board of the Hospital for Sick Children.

Immunohistochemistry

Expression of the $\alpha 1/\alpha 2$, $\alpha 3/\alpha 4/\alpha 5$, and $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ networks was studied in the testis using normal dogs and dogs with X-linked Alport syndrome and normal human testis. Tissues were snap-frozen in OCT, and 5- μ m sections were stained using monoclonal rat antibodies to each of the six α -chains of human type IV collagen (1:100 dilution). The specificity of these antibodies has been established previously on human and canine tissue.^{7,10,29} Sections were pretreated for 10 minutes with a 100 mmol/L acid-KCl solution (pH 1.5) to expose epitopes then blocked with 1.5% rabbit serum (Vector Laboratories, Burlingame, CA). Sections were incubated with the primary antibodies for 90 minutes, followed by a biotinylated rabbit anti-rat antibody (1:200 dilution) for 60 minutes (Vector) and then a peroxidase-conjugated avidin-biotin complex for 30 minutes (Vector). Diaminobenzidine was used as a chromogen, and sections were counterstained with hematoxylin.

Electron Microscopy and Morphometry

Samples of testis from dogs 0.3 to 9 months of age (14 affected and 9 normal littermates) were fixed in 4% paraformaldehyde-1% glutaraldehyde, postfixed in 2% osmium tetroxide, and embedded in Epon-Araldite. Sections were cut at 50-nm thick and stained with uranyl acetate and lead citrate. Random electron photomicrographs (JEM1230; Jeol, Tokyo, Japan) of STBMs were captured electronically at $\times 50,000$ magnification and transferred to a morphometric analysis program (Scion Image, Frederick, MD). A blinded observer examined five images per sample, and the thickness of the STBM was measured at three different segments for each image. Relationships between age and thickness of the STBM were examined by least squares linear regression (Sigmaplot, Systat Software Inc., Point Richmond, CA). Median values of STBM thickness of affected and normal dogs were compared using the Mann-Whitney test.

Spermatogenesis

Spermatogenesis was assessed by light microscopic examination of Epon-embedded sections of testis, in which there was a clear separation of mature sperm. Three affected dogs (ages 7.5, 8, and 9 months) and two normal littermates (ages 7.5 and 8 months) were examined. Two normal dogs ages 3 and 4 years were examined as a control for the transplanted affected dog. To ensure that our findings were representative of the tissue as a whole, histological studies were performed using three samples of testis from each dog. The number of mature sperm in each tubular cross-section was counted using a $\times 20$ objective. At this magnification, an entire cross-section could be viewed. For longitudinal sections, nonoverlapping fields were examined at this same magnification. Fields without a tubular lumen were not counted. An average of 31 fields (range, 11 to 81) were counted per dog. Results were expressed as the number of mature

Figure 1. Transverse section of seminiferous tubules. **A:** The outermost cellular layer is composed of Sertoli cells and spermatogonia. Toward the lumen, spermatocytes and spermatozoa are seen at various stages of development. **B:** The STBM is seen underlying the seminiferous epithelium (SE), in this case, a spermatogonium. Underlying the STBM is an extracellular matrix (ECM) rich in collagen fibrils, and beneath this, one or more layers of peritubular myoid cells (MYO), each bounded by a discontinuous basement membrane (BM). These cells are separated from the interstitium by a second layer of ECM. **A,** toluidine blue $\times 200$; **B,** electron micrograph $\times 50,000$.

sperm per luminal cross-section. Each dog was treated as its own experiment with results analyzed by analysis of variance. Because the data were not normally distributed, the Kruskal-Wallis one-way analysis of variance on ranks was used. The differences in sperm counts between the dogs were determined to be significant ($P < 0.001$). Pairwise comparisons were done by a posthoc test of significance (Dunn's methods).

Results

Distribution of the $\alpha 1$ to $\alpha 6$ Chains of Type IV Collagen in Normal Canine Testis

STBM

The appearance of the seminiferous tubule and surrounding structures is provided for reference in Figure 1. By 2 months of age, testes from normal dogs showed positive staining of the STBM for all six type IV collagen chains (Figure 2). Before this time, the $\alpha 1$ and $\alpha 2$ chains were consistently present. The $\alpha 5$ and $\alpha 6$ chains first appeared around 5 weeks, with the $\alpha 3$ and $\alpha 4$ chains appearing by 2 months of age. As previously reported for murine testis,³⁰ a double layer of basement membrane was apparent. The first layer, corresponding to the STBM, was located immediately beneath the seminiferous epithelium and stained in a discrete linear fashion. The second less regular layer of staining was localized among the peritubular myoid cells and corresponded to the discontinuous basement membrane that sheathes these cells as viewed by electron microscopy (Figure 1).

Interstitial

Also present was a population of cells in the region between tubules, which showed pericellular staining for the $\alpha 1$, $\alpha 2$, $\alpha 5$, and $\alpha 6$ chains but never for the $\alpha 3$

and $\alpha 4$ chains (Figure 2). Staining for the $\alpha 5$ and $\alpha 6$ chains was barely detectable at 11 days and was stronger by 5 weeks of age, whereas the $\alpha 1$ and $\alpha 2$ chains were clearly expressed at all times examined. The interstitial cells examined by electron microscopy had the features of Leydig cells that possessed a basement membrane that ranged from complete to incomplete in a single layer or sometimes duplicated. Capillaries in the interstitial region were positive at all time points for the $\alpha 1$ and $\alpha 2$ chains only. Smooth muscle in arterioles showed strong staining for both the $\alpha 5$ and $\alpha 6$ chains at all times examined (Figure 2), before the appearance of these chains in STBM. Immunostaining with antibody B66, which was raised against the bovine $\alpha 6$ chain, labeled the basement membranes of arteriolar smooth muscle cells, interstitial cells, and the STBM. In contrast, antibody M69, which was raised against the murine $\alpha 6$ chain, labeled only the arterioles and the interstitial cells, suggesting that the epitope recognized by this antibody might be hidden within the $\alpha 6$ -containing network of the STBM (data not shown).

Rete Testis

The seminiferous tubules converge into a series of ducts (the vasa recta) as they approach the mediastinum testis along the posterior aspect of the gland. These end in an anastomosing network of tubules (the rete testis), which are continuous with the epididymis. The temporal pattern of type IV collagen expression in basement membranes of the rete testis differed from that of the STBM (Figure 3). In 11-day-old normal dogs, the basement membranes of the rete testis stained intensely for all six isoforms of type IV collagen, and this pattern continued into adulthood. Thus, the expression of the $\alpha 3$ to $\alpha 6$ chains in the rete testis precedes their appearance in the STBM by at least 1 month.

Figure 2. Distribution of the $\alpha 1$ – $\alpha 6$ chains of type IV collagen in normal canine testis. Each chain is shown in a panel of three time points in development: 11 days, 1.5 months, and 3 months of age. The $\alpha 1$ and $\alpha 2$ chains were present in all basement membranes (STBM, interstitial cells, capillaries, and smooth muscle cells) at all time points. The $\alpha 3$ and $\alpha 4$ chains were present only in the STBM and not before 2 months. The $\alpha 5$ and $\alpha 6$ chains appeared in the STBM of normal dogs around 5 weeks of age, in contrast to basement membranes around smooth muscle cells that showed much stronger staining for both the $\alpha 5$ and $\alpha 6$ chains at this time. Interstitial cells were also positive at this time. H&E counterstain, $\times 400$.

Figure 3. Distribution of the $\alpha 1$ – $\alpha 6$ chains in the normal canine rete testis. In 11-day-old normal dogs, the basement membranes of the rete testis stain intensely for all six isoforms of type IV collagen, and this pattern continues into adulthood. H&E counterstain, $\times 200$.

Distribution of the $\alpha 1$ to $\alpha 6$ Chains of Type IV Collagen in Human Testis

The same sequence could not be delineated to the same degree in human testis, due to the few samples available. Nevertheless, there was a shift in collagen network expression in the normal human testis. All six chains were expressed in the STBM in a sample from a normal 14-year-old subject, before there was spermatogenesis (Figure 4), and in a testis from a normal 17-year-old. Staining for the $\alpha 1$ and $\alpha 2$ chains was considerably stronger than the other four chains. In contrast, in one normal 12-year-old, only the $\alpha 1$ and $\alpha 2$ chains were expressed in the STBM, whereas in a second normal 12-year-old, the $\alpha 5$ and $\alpha 6$ chains were weakly expressed, in addition to strong expression of the $\alpha 1$ and $\alpha 2$ chains (data not shown). In the cryptorchid testis, there was strong expression of the $\alpha 1$ and $\alpha 2$ chains in the STBM and weak expression of the $\alpha 5$ and $\alpha 6$ chains (data not shown). No expression of the $\alpha 3$ and $\alpha 4$ was noted. No spermatogonia were observed in any of the seminiferous tubules.

Distribution of the $\alpha 1$ to $\alpha 6$ Collagen Chains in Canine Alport Testis

The STBM and the basement membrane of interstitial cells and vascular smooth muscle cells contained the $\alpha 1$ and $\alpha 2$ chains at all ages examined (Figure 5). However, the $\alpha 3$ to $\alpha 6$ chains were not detected at any time in any basement membranes. In general, the STBM of Alport dogs appeared thinner than age-matched normal dogs

as judged by the intensity and distribution of staining for the $\alpha 1$ and $\alpha 2$ chains.

Electron Microscopy and Morphometry of the Canine STBM

All seminiferous tubules were bounded by a continuous basement membrane (Figure 6). The thickness of the STBM decreased with age in both normal and Alport dogs. In Alport dogs, the STBM was consistently thinner compared to normal age-matched controls, but at no time point was a multilaminar appearance noted. In both normal and Alport dogs, the thickness of the STBM decreased to nearly 40% of its original width between 5 weeks and 8 months of age. For any given time point, the width of the STBM in Alport dogs was $\sim 70\%$ that of age-matched normal dogs. Pooling the data into groups less than and greater than 4 months of age showed the STBM thickness to be significantly less in affected dogs under 4 months of age compared to normal dogs ($P = 0.04$). At ages greater than 4 months, there was no significant difference between normal and affected dogs.

Spermatogenesis in Normal and Alport Dogs

Spermatogenesis was first evident in normal and Alport dogs at ~ 6 months of age (data not shown), consistent with the findings of others in this species.³¹ In normal canine testis, numerous spermatozoa could be seen in the lumen of the tubules (Figure 7A). In Alport canine

Figure 4. Distribution of the $\alpha 1$ – $\alpha 6$ chains of type IV collagen in normal human testis. In this biopsy from a normal 14-year-old, all six chains were expressed in the STBM. The pattern of staining was similar to that seen in normal canine testis. In another sample from a normal 12-year-old, only the $\alpha 1$ and $\alpha 2$ chains were present (data not shown). H&E counterstain, $\times 400$.

testis, spermatogenesis was present at markedly reduced levels compared to age-matched normal controls (Figure 7B). Spermatogenesis continued to be reduced

compared to normal dogs up to 9 months of age, which was the last time point examined. In this model, animals are terminated in advance of end-stage renal failure at 10

Figure 5. Distribution of the $\alpha 1$ – $\alpha 6$ chains in affected canine testis. The $\alpha 1$ and $\alpha 2$ chains were present in all basement membranes (STBM, capillaries, and interstitial cells) at all times examined (3 months of age is illustrated). The STBM appeared thinner in Alport dogs compared to normal. The $\alpha 3$, $\alpha 4$, $\alpha 5$, and $\alpha 6$ chains were never present in the basement membranes around seminiferous tubules, interstitial cells, or vascular smooth muscle. H&E counterstain, $\times 400$.

Figure 6. Electron microscopy and morphometry of canine STBM. Top: seminiferous tubules of normal and Alport dogs were surrounded by a complete basement membrane. The thickness of the STBM appears decreased in Alport dogs compared to normal age-matched controls (data for 3 months of age are illustrated, magnification $\times 50,000$). At no time point was a multilaminar appearance noted. Bottom: Graph of STBM thickness versus age in normal and Alport dogs. Data points and error bars represent the mean $\pm$ SD from 9 normal and 14 affected dogs. In both normal and Alport dogs, the thickness of the STBM decreased to nearly 40% of its original width between 5 weeks and 8 months of age. For any given time point, the width of the STBM in Alport dogs was $\sim 70\%$ that of age-matched normal dogs. The STBM thickness was significantly less in affected dogs under 4 months of age compared to normal dogs ($P = 0.04$).

months of age.³² The average number of sperm per tubular cross-section in normal canine testis at this age was significantly ($P < 0.05$) greater (31.2 ± 18.0 (mean $\pm$ SD)) compared to that in affected dogs (1.2 ± 2.8 (mean $\pm$ SD)). Because renal failure can delay puberty and impair spermatogenesis,³³ the reduction in spermatogenesis in Alport dogs may simply have been a reflection of deteriorating renal function. In an attempt to address this, one male Alport dog, that had received a renal transplant and consistently maintained normal renal function, underwent bilateral orchidectomy at age 33 months. Examination of Epon sections from the testes showed a marked reduction in spermatogenesis (Figure 7D) compared to normal controls ages 2 to 6 years (Figure 7C). The number of sperm per tubular cross-section in normal canine testis at this age was significantly ($P < 0.05$) greater (52.8 ± 17.2 (mean $\pm$ SD)) compared to the testis of the renal-transplanted affected dog (6.3 ± 9.9 (mean $\pm$ SD)). There was no significant difference be-

tween sperm counts in younger versus older normal dogs or between younger versus older affected dogs.

Discussion

The results on normal canine STBM indicate that expression of the six chains of type IV collagen networks is separated distinctly in time, with the $\alpha 1$ and $\alpha 2$ chains strongly expressed at 11 days (the earliest time point examined), the $\alpha 5$ and $\alpha 6$ chains by 1.5 months, and the $\alpha 3$ and $\alpha 4$ chains by 2 months of age. Assuming the three basic protomers in STBM have the same composition (ie, $\alpha 1_2\alpha 2$, $\alpha 5_2\alpha 6$, and $\alpha 3\alpha 4\alpha 5$) and undergo network assembly in the same manner as in other tissues,^{5,6} then when interpreted at the network level, our results indicate the sequence of expression is the $\alpha 1/\alpha 2$ network appearing first, followed by the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network, and lastly the $\alpha 3/\alpha 4/\alpha 5$ network.

Figure 7. Spermatogenesis is reduced in Alport dogs. The histology of the testis in normal (**A** and **C**) and affected (**B** and **D**) dogs is presented in representative sections at 8 months (**A** and **B**), 4 years (**C**), and 3 years (**D**) of age. Spermatogenesis is first observed around 6 months of age in both normal and Alport dogs (data not shown). Spermatogenesis was present at reduced levels in Alport dogs compared to age-matched normal dogs as evident from the decreased number of spermatozoa in the tubular lumen. The dog illustrated in **D** underwent a renal transplant at 3 months of age and maintained normal renal function thereafter. Bilateral orchidectomy was performed at age 33 months (toluidine blue counterstain, $\times 200$). The reduction of sperm counts in affected dogs was statistically significant in both age groups ($P < 0.05$).

This developmental shift is consistent with earlier results on the expression of type IV collagen in the developing testis in mice^{18,30} and rats,³⁴ in which the $\alpha 1$ and $\alpha 2$ chains appear before the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains. Curiously, the $\alpha 6$ chain is apparently not expressed in mouse testis,¹⁸ and the distribution of this chain was not evaluated in the other studies on mouse and rat, so no conclusions could be made on the timing of developmental shifts involving the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network. On the other hand, the epithelial basement membrane in murine epididymis was found to undergo the same sequence of chain expression as canine STBM.¹⁸ Bovine STBM contains all six isoforms,¹⁷ but no sequential studies have been performed. There is only one study on expression of type IV collagen chains in human testis, based on adult specimens, and concluding that the STBM contains only the $\alpha 1$ and $\alpha 2$ chains.³⁵ In contrast, we found that human STBM contains all six α chains, as do canine and bovine testis. In fact, despite the limited number of specimens available to us, there is the suggestion that human testis undergoes the same developmental shift as canine

testis in the expression of type IV collagen networks in STBM and that the shift takes place around the time of puberty. The basis for our detecting more (ie, all six) α chains in our study compared to the other is not entirely clear. The primary antibodies in both studies are from the same source, but there are several clones available for each chain, which show different degrees of reactivity in tissue, and it is unknown if exactly the same clones were used in both studies. Our immunostaining technique would favor higher sensitivity with longer incubation times for the primary and secondary antibodies and the avidin-biotin complex.

Not all basement membranes in the canine testis have the same developmental time course or network composition as the STBM. At the earliest time point studied (11 days), all three networks were already expressed in the rete testis, preceding STBM by several weeks. A similar result has been noted for murine testis.¹⁸ Such differences may reflect the different embryologic origins of these tissues.^{36,37} Leydig cells have the same basement membrane composition as smooth muscle cells. How-

ever, Leydig cells undergo a shift in network expression from $\alpha 1/\alpha 2$ to $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ around 1.5 months of age as does the STBM, whereas vascular smooth muscle cells contain the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network from the earliest time point examined (11 days). We have found the same to be true for smooth muscle in canine bladder and in vessels of the kidney that already contain the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ as early as 40 days gestation, ~20 days before birth (data not shown). Hence, if expression of the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network follows the $\alpha 1/\alpha 2$ network in smooth muscle cells, this must occur earlier than has so far been examined.

Other sites that undergo developmental shifts in type IV collagen network appear to follow the same sequence. In the glomerulus, the $\alpha 1/\alpha 2$ network is expressed from the earliest stages.^{10,12,13} The $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network appears in developing glomeruli in the late S phase, just before there are defined capillary loops.¹⁰ The appearance of the $\alpha 3/\alpha 4/\alpha 5$ network coincides with the time when capillary loops become apparent,^{10,12,13} and the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network comes to reside in Bowman's capsule. In lens capsule^{16,38} the developmental shifts in networks are less well defined. Prenatal murine and human lens capsules contain the $\alpha 1$, $\alpha 2$, $\alpha 5$, and $\alpha 6$ chains, with the $\alpha 3$ and $\alpha 4$ chains appearing only postnatally.¹⁶ Hence, expression of the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network precedes that of the $\alpha 3/\alpha 4/\alpha 5$ network, but there is no stage identified with the $\alpha 1/\alpha 2$ network only. Since the $\alpha 1/\alpha 2$ network is believed to be ubiquitous, the developmental shift involving the $\alpha 5$ and $\alpha 6$ chains likely occurs before the earliest time point yet examined. In the murine and canine inner ear, only the $\alpha 1/\alpha 2$ network is present at birth, with the $\alpha 3/\alpha 4/\alpha 5$ network not detected until around 2 weeks in mice¹⁴ and 3 to 4 weeks in dogs.¹⁵ The $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network is not expressed in this site except in vascular smooth muscle.

During the early stages of testicular development, only Sertoli cells rest on the STBM, and germ cells are located along the luminal aspect of the seminiferous tubule. Shortly after birth, murine germ cells migrate to the periphery where they contact the STBM.³⁹ Based on immunohistochemical studies,^{18,30} it can be concluded that murine STBM contains only the $\alpha 1$ and $\alpha 2$ chains before germ cells reach the tubule wall. Similarly in dogs, spermatogonia first contact the STBM by 11 days of age (data not shown) before any of the $\alpha 3$ to $\alpha 6$ chains are expressed. Hence, migration of germ cells to the STBM might provide a signal to the Sertoli cell to start manufacturing these chains. However, the cryptorchid human testis in our study was totally lacking in germ cells but still showed the $\alpha 5$ and $\alpha 6$ chains in the STBM. It is unknown whether germ cells were at one time present in this testis or not. Further studies are warranted before any definite conclusions can be drawn.

In X-linked Alport syndrome, there is progressive renal disease, and the GBM usually contains only the $\alpha 1/\alpha 2$ network.^{7,8,26} In the canine model for Alport syndrome used in this study, the ultrastructure and function of the GBM are initially normal, although the $\alpha 3/\alpha 4/\alpha 5$ network is absent.¹⁰ Deterioration of the GBM begins later in the course of the disease leading to the conclusion that the $\alpha 3/\alpha 4/\alpha 5$ network is necessary for long-term maintenance of function.

The situation with the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ is much less clear. In X-linked Alport syndrome, the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ network is usually absent from the kidney and skin^{7,8,29} and smooth muscle of the bladder, arterioles, and the bronchial tree in canine X-linked Alport syndrome,^{10,11} without clinical or pathological consequences.¹¹ The absence of these networks was previously speculated to originate at the translational level based on the finding that there were reduced levels of mRNA for the $\alpha 3$ and $\alpha 4$ chains in the kidney in the canine Alport model.⁴⁰ More recent studies of this model have indicated that the absence is caused by a failure at the protein assembly level, because the mRNA levels for these two chains are normal at birth in the kidney, and normal in the testis throughout the life span of the affected dogs.⁴¹

The present study allows us to speculate on the role the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ and/or $\alpha 3/\alpha 4/\alpha 5$ networks might play in the STBM. In normal mouse testis, the appearance of $\alpha 3/\alpha 4/\alpha 5$ network coincides with the onset of spermatogenesis, prompting speculation that expression of this network might be required for normal function.³⁰ In the normal human testis, all three networks were present before the onset of spermatogenesis. In normal dogs, spermatogenesis began at ~6 months of age, which is 4 months after the appearance of the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ and $\alpha 3/\alpha 4/\alpha 5$ networks. However, in Alport dogs, which lack these networks, the onset of spermatogenesis occurred at the same time as normal dogs. Hence, comparable to the GBM in the kidney, the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ and $\alpha 3/\alpha 4/\alpha 5$ networks in the testis are not necessary for initial development and function. In both normal and Alport dogs, the thickness of the STBM decreased to nearly 40% of its original width between 5 weeks and 8 months of age. Thinning of the normal STBM has been reported in bovine⁴² and in humans testis⁴³ and takes place before, or coincident with, the onset of spermatogenesis. This suggests that thinning of the STBM may be required for normal function. However, the STBM of Alport dogs was thinner than age-matched normal dogs at all ages. This finding has not been investigated in human patients with Alport syndrome, but paradoxically, diffuse thinning of the GBM is occasionally seen in human Alport patients¹⁹ but not in Alport dogs.

The question then arises as to whether the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ and/or $\alpha 3/\alpha 4/\alpha 5$ networks are important for long-term maintenance of function, as is the case for the $\alpha 3/\alpha 4/\alpha 5$ network in the GBM. If one uses fertility as a long-term measurement of testicular function, the Alport dog in this study that received a renal transplant is now over 3 years old and has produced offspring (R. Jacobs, unpublished observations). Similarly, in *Col4a3* knockout mouse models of autosomal recessive Alport syndrome, the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains are absent from the STBM,^{9,44} yet mating of homozygous mutant to wild-type mice yields litters of normal size.⁹ There is no evidence in the literature that suggests fertility is reduced in human males with X-linked Alport syndrome; however, this has never been formally investigated. Sperm counts have not been reported in this condition. Only one study has examined the testis by routine light microscopy in a 15-year-old patient with

Alport syndrome.⁴⁵ The testis showed a pre-pubertal histology, inconsistent with the patient's age; however, it is difficult to infer any role from loss of type IV collagen networks in this testis, because a delay in pubertal maturation can occur with chronic renal failure.³³ Additional studies on murine and human testis in Alport syndrome would help to resolve this question.

Our results indicate that spermatogenesis is impaired in Alport dogs. From age 6 months onward, spermatozoa were consistently present at significantly lower levels in Alport dogs compared to normal age-matched littermates ($P < 0.05$). One explanation could be that spermatogenesis is impaired on the basis of chronic renal failure. However, the testis in the transplant dog continued to show reduced levels of spermatogenesis, which were significantly lower than age-matched controls ($P < 0.05$). This dog always maintained normal renal function, and the only drug used was low dose (1.25 mg/kg/day) cyclosporine A, which was discontinued 24 days before orchidectomy. This drug has been associated with impairment of spermatogenesis in rats but only at doses 20 mg/kg/day or higher^{46–48} and with a return to normal spermatogenesis 2 weeks after stopping the drug.⁴⁸ We hypothesize that the loss of the $\alpha 3$ to $\alpha 6$ chains from the STBM might impair spermatogenesis, perhaps by disturbing cell-matrix interactions or by changing the permeability of this structure thereby altering the local microenvironment in the germinal epithelium.

In conclusion, we have shown that, during testicular development, there is sequential expression of the three networks of type IV collagen, beginning with the ubiquitous $\alpha 1/\alpha 2$ network, followed by the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ and $\alpha 3/\alpha 4/\alpha 5$ networks. Although the actual networks involved may vary from site to site (testis, GBM, inner ear, lens capsule), their order of expression is consistent. In a canine model of Alport syndrome, the absence of the $\alpha 1/\alpha 2/\alpha 5/\alpha 6$ and the $\alpha 3/\alpha 4/\alpha 5$ networks from the STBM leads to thinning of this structure. These two networks do not appear to be required for normal development and function but may be required for long-term maintenance and function, a concept that parallels the role of these type IV collagen networks might have in the kidney and inner ear. The advantage of using STBM to study basement membranes is that all three collagen networks colocalize, and the sequence of expression is more clearly separated than in any other organ. A study of normal and Alport testis could therefore provide a model system to unravel the mechanisms involved in these collagen network shifts, investigate the roles of specific networks, and determine the physicochemical manner in which these networks interact within a basement membrane.

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