A Human-Mouse Chimera of the $\alpha3\alpha4\alpha5(\text{IV})$ Collagen Protomer Rescues the Renal Phenotype in $\text{Col4a3}^{-/-}$ Alport Mice

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Collagen IV is a major structural component of basement membranes. In the glomerular basement membrane (GBM) of the kidney, the $\alpha3$, $\alpha4$, and $\alpha5(\text{IV})$ collagen chains form a distinct network that is essential for the long-term stability of the glomerular filtration barrier, and is absent in most patients affected with Alport syndrome, a progressive inherited nephropathy associated with mutation in *COL4A3*, *COL4A4*, or *COL4A5* genes. To investigate, *in vivo*, the regulation of the expression, assembly, and function of the $\alpha3\alpha4\alpha5(\text{IV})$ protomer, we have generated a yeast artificial chromosome transgenic line of mice carrying the human *COL4A3-COL4A4* locus. Transgenic mice expressed the human $\alpha3$ and $\alpha4(\text{IV})$ chains in a tissue-specific manner. In the kidney, when expressed onto a $\text{Col4a3}^{-/-}$ background, the human $\alpha3(\text{IV})$ chain restored the expression of and co-assembled with the mouse $\alpha4$ and $\alpha5(\text{IV})$ chains specifically at sites where the human $\alpha3(\text{IV})$ was expressed, demonstrating that the expression of all three chains is required for network assembly. The co-assembly of the human and mouse chains into a hybrid network in the GBM restores a functional GBM and rescues the Alport phenotype, providing further evidence that defective assembly of the $\alpha3\alpha4\alpha5(\text{IV})$ protomer, caused by mutations in any of the three chains, is the pathogenic mechanism responsible for the disease. This line of mice, humanized for the $\alpha3(\text{IV})$ collagen chain, will also provide a valuable model for studying the pathogenesis of Goodpasture syndrome, an autoimmune disease caused by

antibodies against this chain. (*Am J Pathol* 2003; 163:1633-1644)

The glomerular basement membrane (GBM), a specialized form of extracellular matrix that is positioned between layers of endothelial cells and epithelial visceral cells, is a key component of the glomerular filtration barrier in the kidney. The GBM is composed of type IV collagen networks that interact with cells through integrins and serve as a scaffold for binding other macromolecular constituents, including laminin, proteoglycan, and nidogen. Type IV collagen of normal human GBM exists as two distinct networks, composed of $(\alpha1)_2\alpha2(\text{IV})$ and $\alpha3\alpha4\alpha5(\text{IV})$ triple-protomers, respectively.^{1,2} The protomers are composed of three chains, each consisting of a 7S domain at the N-terminus, a long triple-helical domain and a noncollagenous (NC1) domain at the C-terminus. The protomers self-assemble into network by joining at the NC1 domains, forming dimers, and at the 7S domains, forming tetramers.^{3,4} The $(\alpha1)_2\alpha2(\text{IV})$ network is a component of all basement membranes (BMs), whereas the $\alpha3\alpha4\alpha5(\text{IV})$ network, extensively cross-linked by disulfide bonds,⁵ has a restricted distribution in human tissues, occurring in the kidney [GBM and some tubular basement membranes (TBMs)], lung, testis, cochlea, and eye.^{6,7}

Type IV collagens are directly involved in the pathogenesis of two human diseases: Goodpasture and Alport syndromes. Goodpasture syndrome is an autoimmune disease caused by autoantibodies against cryptic epitopes of the $\alpha3(\text{IV})$ NC1 domain,^{8,9} which bind to the glomerular and alveolar BMs, causing rapidly progressing glomerulonephritis associated with pulmonary hemorrhage. Alport syndrome, an inherited nephropathy characterized by irregular thinning, thickening, and splitting of the GBM and progressive renal failure, is associ-

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ated with mutations in any of the *COL4A3*, *COL4A4*, and *COL4A5* genes encoding the $\alpha 3$, $\alpha 4$ and $\alpha 5(\text{IV})$ chains. In the majority of Alport patients, the GBM shows the loss of the $\alpha 3.\alpha 4.\alpha 5(\text{IV})$ network and the persistence of the $\alpha 1.\alpha 2(\text{IV})$ network.¹⁰ The abnormal composition of networks predisposes the GBM to events, such as proteolysis, that cause progressive deterioration and loss of function.¹¹

Whereas mRNA expression of *COL4A3*, *COL4A4*, and *COL4A5* genes appears to be coordinated in dogs affected with Alport syndrome,¹² such a transcriptional regulation was not found in humans affected with Alport syndrome^{13,14} or in mouse models for Alport syndrome.^{15,16} Furthermore, the three $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains of native GBM co-exist within a triple-helical protomer,² and the NC1 domains of all three chains are required for the *in vitro* assembly of a hexamer complex, representative of the native network.¹ Together, these findings suggest that a mutation in one chain can prevent its association with the other two chains, thus disrupting the assembly of $\alpha 3.\alpha 4.\alpha 5$ triple-helical protomers and network.

To explore, *in vivo*, the mechanisms regulating the expression, assembly, and function of the $\alpha 3.\alpha 4.\alpha 5(\text{IV})$ protomer, we generated a yeast artificial chromosome (YAC) transgenic line of mice carrying the human *COL4A3*-*COL4A4* wild-type genes, either on a wild-type or on a *Col4a3*^{-/-} background. Transgenic mice were characterized with respect to renal pathology, type IV collagen genes transcription, and $\alpha(\text{IV})$ chains expression and network assembly. The results showed that the YAC rescues the Alport phenotype by assembly of a hybrid type IV collagen network containing human $\alpha 3$ and $\alpha 4(\text{IV})$ and mouse $\alpha 4$ and $\alpha 5(\text{IV})$ chains in the GBM, and provide new insights in understanding the pathogenesis of Alport syndrome, type IV collagen gene regulation, and $\alpha 3.\alpha 4.\alpha 5(\text{IV})$ network assembly.

Materials and Methods

YAC Characterization and Manipulations

The YAC clone 870_B6, containing the human *COL4A3* and *COL4A4* genes located pairwise in a head to head manner, was previously isolated¹⁷ from the human CEPH library.¹⁸ Growth of yeast strain AB1380 containing YAC, agarose blocks containing yeast DNA, conventional Southern blotting and pulsed-field gel electrophoresis, isolation of YAC ends, mapping of YAC sequence-tagged sites and sequence analysis were performed as previously described.¹⁹ A 1041-bp fragment, located 42 kb downstream of *COL4A4* exon 48, was subcloned into a yeast truncation vector,²⁰ and used for truncating the YAC 870_B6 downstream of the 3' end of *COL4A4*, using a homologous recombination strategy in yeast as in Barton and colleagues.²¹ The targeting event in yeast was confirmed by pulsed-field gel electrophoresis, Southern blotting, and polymerase chain reaction (PCR) using *COL4A3* and *COL4A4* cDNA probes/exon primers.^{17,22}

Generation of Transgenic Mice and Genotyping

Purification of the YAC DNA and microinjection were performed as in Schedl and colleagues.²³ DNA was injected at a concentration of 5 ng/ μl into fertilized mouse oocytes isolated from F1 crosses of CBA/C57BL6 mice. Transgenic mice were identified by PCR using primers for *COL4A4* exon 48¹⁷ and by Southern blot using *COL4A3* and *COL4A4* cDNA overlapping probes. Mice carrying one (heterozygous) or two (homozygous) transgenic alleles were differentiated by real-time PCR, using 100 ng of genomic DNA, PCR primers 5'GTGTGTGTCTGAGCCCTAAT3' and 5'TGTGAATTCGCGATTCT3' (which amplify human *COL4A4* exon 48), and the Roche (Basel, Switzerland) LightCycler-fast start DNA Master SYBR green I kit (according to the manufacturer's instructions). Primers 5'GGGTTTCCTCTCTCTCT3' and 5'CTAGGGCAGTTAGTAAATGG3', amplifying mouse *Wt1* exon 10, were used as standards. *Col4a3*^{+/-} mice¹⁶ were purchased from the Jackson Laboratory (Bar Harbor, ME), and genotyping of the mouse *Col4a3* disrupted allele was performed by Southern blot analysis of *NcoI*-digested genomic DNA, as in Cosgrove and colleagues.¹⁶ All procedures using animals were conducted in accordance with national guidelines and institutional policies.

Fluorescence in Situ Hybridization Analysis

Metaphase spreads were prepared from heterozygous transgenic mice. Concanavalin A-stimulated lymphocytes were cultured at 37°C for 72 hours, with 5-bromodeoxyuridine added for the final 6 hours of culture (60 mg/ml of medium). The PAC clone 310_24 E (RPCI 1 library), covering *COL4A4* exons 43 to 48 and 50 kb downstream, was biotinylated by random priming with biotin 14-dCTP (Bioprime DNA labeling system; Invitrogen Carlsbad, CA). Hybridization to chromosome spreads and interphase nuclei was performed with standard protocols.²⁴ The hybridized probe was detected by means of fluorescence isothiocyanate-conjugated avidin. Chromosomes were counterstained and R-banded with propidium iodide diluted in anti-fade solution pH 11.0.²⁵

Urine and Blood Analyses

Samples of blood and urine were collected from deeply anesthetized mice before sacrifice. Total urinary protein was assayed with 1 μl of urine boiled in Laemmli sample buffer, electrophoretically separated on 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, using bovine serum albumin as a standard; protein was visualized with Coomassie blue. Hematuria was estimated using Multistix strips (Bayer Corporation). Serum creatinine and blood urea nitrogen were measured by the Jaffe method and by UV kinetic test respectively, using commercial kits.

Table 1. Properties of Anti-Collagen IV mAbs Used in This Study

mAb*	Isotype	Immunogen and reference	Chain and species specificity	IF [†]	IB [‡]	IP [§]
H31	Rat IgG2a	$\alpha 3(\text{IV})$ NC 1 peptide (26)	Murine, human $\alpha 3(\text{IV})$	+	+	
RH34	Rat IgG	Human r- $\alpha 3(\text{IV})$ NC1 (this work)	Human $\alpha 3(\text{IV})$	(D)	+	
Mab3	Mouse IgG1	NC1 hexamers from bovine GBM (27)	Murine, human $\alpha 3(\text{IV})$	+	+	+
H43	Rat IgG2b	$\alpha 4(\text{IV})$ NC1 peptide (26)	Murine, human $\alpha 4(\text{IV})$	(N)	+	
RH42	Rat IgG2a	Human r- $\alpha 4(\text{IV})$ NC1 (2)	Murine, human $\alpha 4(\text{IV})$	+	+	+
RH45	Rat IgG2a	Human r- $\alpha 4(\text{IV})$ NC1 (2)	Human $\alpha 4(\text{IV})$	(N)	+	+
mAb a11	Rat IgG2a	NC1 hexamers from rat GBM (this work)	Murine $\alpha 4(\text{IV})$	+		+
H53	Rat IgG	$\alpha 5(\text{IV})$ peptide (28)	Murine, human $\alpha 5(\text{IV})$	(N)		
mAb b14	Rat IgG2b	NC1 hexamers from rat GBM (this work)	Murine, human $\alpha 5(\text{IV})$	+		+
mAb 26-20	Mouse IgG1	NC1 hexamers from bovine testis BM (this work)	Murine, human $\alpha 3\alpha 4\alpha 5(\text{IV})$	(N)		+
mAb 15-47	Mouse IgG1	NC1 hexamer from bovine bladder BM (this work)	Murine, human $\alpha 1(\text{IV})$		+	+
mAb 5	Mouse IgG1	$\alpha 5(\text{IV})$ NC1 peptide (29)	Murine, human $\alpha 5(\text{IV})$		+	

* All mAbs are directed against epitopes on the NC1 domains, except for H53, which recognizes interruption III in the triple-helical domain of $\alpha 5(\text{IV})$.

[†] IF, immunofluorescence. Whereas most mAbs stained native (N) kidney sections, some mAbs required a brief pretreatment of the tissue with acid-urea to expose the cryptic epitopes (D).

[‡] IB, Immunoblotting.

[§] IP, Immunoprecipitation of native NC1 hexamers.

RNA Analysis, Reverse Transcriptase-PCR, and Real-Time PCR Analysis

Mouse kidneys were snap-frozen in liquid nitrogen. A human mature kidney not used for transplantation was also used for the study. Total RNA was extracted using the Rneasy kit (Qiagen, Hilden, Germany). First-strand cDNAs were generated using Superscript II (Invitrogen). Relative expression levels of type IV collagen genes were determined by real-time reverse transcriptase-PCR using the Roche LightCycler-fast start DNA Master SYBR green I kit, and different sets of primers. 1) Primers 5'-TGTTG-TACACAGTGCCTTAT-3' and 5'-GCCTTAGGTCCTG-GTAAT-3' amplify human *COL4A3* cDNA but not mouse *Col4a3*; 2) primers 5'-CCCTGTGGGCCAAGAGGTAA-3' and 5'-CATTGGCATCAGAGCTGGTG-3' match both mouse *Col4a3* and human *COL4A3* sequences and were shown to allow 100% PCR efficiency in both species; and 3) primers 5'-CACAGTCAGACGGACCAGGAG-3' and 5'-GCTGACATAGGGGCGGATCG-3' amplify human *COL4A4* cDNA but not mouse *Col4a4*. Individual samples were standardized by measuring the amount of mouse *Gapdh* RNA (primers 5'-ATTCAACGGCACAGTCAA-GG-3' and 5'-TGGATGCAGGGATGATGTTC-3') or human *GAPDH* RNA (primers 5'-ATTCCATGGCACCGT-CAAGGC-3' and 5'-TAGAGGCAGGGATGATGTTC-3'). Each sample was tested twice in each run, and the derived normalized values were the average of three runs.

Histology and Immunohistochemistry

Specimens used for histology were fixed in Dubosq-Brazil or formalin and dehydrated before embedding. Specimens used for immunofluorescence were snap-frozen in liquid nitrogen using CRYO-M-BED (Bright, Huntington, UK). Immunofluorescence labeling was performed as in Heidet and colleagues,¹⁴ except for Goodpasture antibodies, which required longer incubation (15 hours) with the cryostat sections under native conditions. For antibodies recognizing cryptic epitopes (as indicated in Table 1), the sections were pretreated with 6 mol/L of urea in 0.1 mol/L of glycine buffer, pH 3.5, for 10 minutes, then rinsed with distilled water. A Mouse to Mouse (M.O.M.) (Vector Laboratory, Burlingame, CA) immunodetection kit was used for localization of mouse primary antibodies. Tissue sections incubated with the appropriate preimmune serum or directly with the secondary antibodies served as control. Labeling was examined with a Leitz Orthoplan microscope equipped for light, fluorescence and phase contrast microscopy (Leica Microscopic Systems, Heezbrugg, Switzerland).

Antibodies

Affinity-purified antibodies recognizing human and mouse [$(\alpha 1)_2\alpha 2$] collagen IV protomers, raised against pepsin-digested human placenta type IV collagen, were obtained from Novotec (Lyon, France). Anti-type IV collagen mono-

clonal antibodies (mAbs) used in this work are described in Table 1. Several new mAbs were characterized for this study; their chain and species specificity was determined by comparing their pattern of reactivity in enzyme-linked immunosorbent assay, immunoblotting, and/or immunofluorescence staining to that of mAbs with known specificity. In particular, the availability of species-specific mAbs was critical for this work: mAbs RH34 and RH45 specifically recognized human $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ chains, respectively, whereas mAb a11 recognized the murine $\alpha 4(\text{IV})$. Human Goodpasture autoantibodies were affinity-purified from patient sera³⁰ and used at concentration of $\sim 2 \mu\text{g/ml}$. Antibodies directed against laminin chains $\alpha 1$, $\beta 1$, and $\gamma 1$, perlecan, and entactin were from Chemicon (Temecula, CA). Antibodies against fibronectin and agrin were from Novotec and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibodies directed against the $\alpha 2$ and the $\alpha 5$ chains of laminin were kind gifts from P. Yurchenko (Department of Pathology and Laboratory Medicine, Robert Wood Johnson Medical School, Piscataway, NJ) and J. Miner (Department of Medicine, Washington University School of Medicine, St. Louis, MO), respectively.

Electron Microscopy

Kidneys from 2-month-old mice were fixed in 2.5% glutaraldehyde/0.2 mol/L cacodylate buffer/phosphate-buffered saline (pH 7.4) for 24 hours at 4°C, postfixed in 2% osmium tetroxide for 1 hour at room temperature, and processed for electron microscopy as described.³¹

Analysis of Type IV Collagen from BMs by Immunoprecipitation and Immunoblotting

Basement membranes were prepared from homogenized mouse tissues, using the detergent extraction method, as described.³² The insoluble component, containing type IV collagen networks, was twice digested with bacterial collagenase, for 24 hours at 37°C, using 200 $\mu\text{g/g}$ of wet tissue. The identity of NC1 domains present in the solubilized fraction was determined by immunoblotting with chain-specific mAbs (as indicated in Table 1), after separation by SDS-PAGE in 6 to 22% polyacrylamide gradient gels and transfer onto Immobilon-P membranes (Millipore, Bedford, MA). Association of NC1 domains into NC1 hexamers was determined by analyzing the composition of fractions separated by affinity chromatography on immobilized anti-NC1 mAbs or by immunoprecipitation, as described.^{1,2,32}

Results

Generation of a Transgenic Mouse Line Carrying the Human COL4A3 and COL4A4 Genes, onto a Wild-Type (TG/Col4a3^{+/+}) or a Col4a3-Null Background (TG/Col4a3^{-/-})

The YAC 870.B6 was characterized by PCR, Southern blot, and pulsed-field gel electrophoresis restriction map-

ping, using COL4A3-COL4A4 cDNA and YAC end probes. The clone was shown to be 850 kb in length, to contain all of the COL4A3-COL4A4 exons and all human-specific EcoRI and PstI fragments. Pulsed-field gel electrophoresis analysis mapped the 3'-end of COL4A3 110 kb from the right (noncentromeric) arm of the YAC. This was subsequently confirmed by localization of the YAC end sequences in the human chromosome 2 working draft sequence (NT_005403). To facilitate DNA manipulation, the YAC 870.B6 was truncated 42 kb downstream of COL4A4 exon 48, by homologous recombination in yeast. The resulting clone was ~ 450 kb in length, contained both COL4A3 and COL4A4 genes, 42 kb downstream of COL4A4 and 110 kb downstream of COL4A3.

Injection of gel-purified YAC DNA into fertilized mouse oocytes yielded one transgenic mouse carrying human $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ collagen genes. To investigate the integrity of the integrated construct, EcoRI- and PstI-digested DNA from transgenic offspring was analyzed by Southern blot. Hybridization with overlapping human probes covering both COL4A3 and COL4A4 cDNAs showed the presence of all human restriction fragments (not shown). A total of 20 metaphase cells were analyzed by fluorescence *in situ* hybridization and in each of them, 100% of the cells showed a similar fluorescent signal on the subdistal part of a chromosome 16. No other significant hybridization signals were noted, indicating that the YAC had integrated into one site in the mouse genome. As multiple copies of a transgene usually integrate as a tandem array, individual copies of the YAC should be separated by 450 kb of DNA, a distance distinguishable by fluorescence *in situ* hybridization analysis on interphase chromatin. A single signal was detected, highly suggesting that a single copy of the transgene was present in this line of mice.

Transgenic mice were intercrossed, and mice carrying one (TG^{+/-}) or two (TG^{+/+}) copies of the transgenic allele were distinguished by real-time PCR. To generate mice carrying the human transgene on a Col4a3^{-/-} background, TG^{+/-} mice were crossed with Col4a3^{+/-} mice. Offspring heterozygous for both the transgene and the Col4a3 disrupted allele (TG^{+/-}Col4a3^{+/-}) were subsequently intercrossed.

Expression of Human COL4A3 and COL4A4 Transcripts in Transgenic Mouse Kidneys

Real-time PCR showed a level of transcription of human COL4A3 and COL4A4 in TG^{+/-}Col4a3^{+/+} mouse kidney ($n = 3$) that was 9.5% (range, 7.3 to 12.1%) and 10.2% (range, 8.6 to 11.3%), respectively, of the level of transcription of the genes observed in human kidney. In TG^{+/+}Col4a3^{+/+} ($n = 2$) mice, the level of transcription rose to 17.5% (range, 16.4 to 18.5%) for COL4A3 and 19.7% (range, 18.7 to 20.8%) for COL4A4. Using primers amplifying equally human and mouse cDNAs, the overall expression of $\alpha 3(\text{IV})$ transcripts in TG^{+/+}Col4a3^{+/+} mice was found to be 124% (range, 111 to 137%) of that of wild-type (Col4a3^{+/+}) mouse kidney. The human genes were expressed at comparable levels onto a Col4a3-null

Figure 1. SDS-PAGE analysis of proteinuria. **Lanes 1 to 4:** Bovine serum albumin: 0.5, 1, 5, and 10 µg; **lane 5:** 8-week-old *Col4a3*^{-/-} mouse; **lane 6:** 8-week-old *Col4a3*^{+/+} (wild-type control) littermate; **lane 7:** 8-week-old TG/*Col4a3*^{-/-} littermate. Heavy albuminuria was observed in *Col4a3*^{-/-} mice, but not in TG/*Col4a3*^{-/-}.

background: TG^{+/-}*Col4a3*^{-/-} mice (*n* = 2) expressed COL4A3 at 9.5% (range, 8.2 to 10.8%) and COL4A4 at 13.1% (range, 12.1 to 14.1%) of the levels found in the human kidney, whereas the respective levels in TG^{+/+}*Col4a3*^{-/-} mice (*n* = 6) rose to 21% (range, 16 to 25.7%) and 22.9% (range, 21 to 28.6%). By comparison to the expression of *Col4a3* in wild-type (*Col4a3*^{+/+}) mice, expression of COL4A3 in transgenic mice was 11.5% (range, 11 to 12%) in TG^{+/-}*Col4a3*^{-/-} mice and 18.2% (range, 16.1 to 20.4%) in TG^{+/+}*Col4a3*^{-/-} mice.

Rescue of the Renal Function and Morphology in *Col4a3*^{-/-} Mice by the Human COL4A3-COL4A4 Transgene

Clinical Phenotype

Animals carrying the transgene onto a wild-type background (TG/*Col4a3*^{+/+}) were fertile and displayed normal survival. They showed no hematuria/proteinuria with a follow-up of 6 months, and their renal function was normal by comparison with nontransgenic littermates (not shown). In contrast, the transgene had a dramatic effect on the *Col4a3*^{-/-} mice, which display an Alport-like phenotype. As previously described,¹⁶ *Col4a3*^{-/-} mice lacking the transgene (*n* = 16) presented with microhematuria as early as 10 days of age, while proteinuria appeared approximately at 30 days of life and persisted until end-stage renal disease. They began to lose weight at ~2 months of age, became lethargic, and died at 65.6 ± 2.2 days with severe renal failure; blood urea nitrogen and creatinine levels were 109.5 ± 26.7 mmol/L and 120.6 ± 24.5 µmol/L, respectively. By contrast, both TG^{+/-}*Col4a3*^{-/-} and TG^{+/+}*Col4a3*^{-/-} mice were clinically undistinguishable from wild-type mice or mice hemizygous for *Col4a3*. Urine analysis from 2-month-old TG^{+/-}*Col4a3*^{-/-} mice showed no hematuria or proteinuria (Figure 1). Their renal function was tested at 30 days (*n* = 7), 60 days (*n* = 2), 90 days (*n* = 2), 150 days (*n* = 5), and 180 days (*n* = 3). Blood urea nitrogen and creatinine levels were comparable to those of wild-type mice (8.7 ± 0.9 mmol/L and 29 ± 3.1 µmol/L, respectively).

Renal Morphology

Animals carrying the transgene onto a wild-type background display normal kidney histology (not shown). As previously reported,¹⁶ kidneys of *Col4a3*^{-/-} mice at the time of death showed severe abnormalities affecting both glomeruli and tubulointerstitium (Figure 2a). Histology of the kidney of TG^{+/-}*Col4a3*^{-/-} and TG^{+/+}*Col4a3*^{-/-} littermates (*n* = 4) resembled that of wild-type mice, (Figure 2b). By electron microscopy, compared with wild-type mice (Figure 2c), extensive alteration of the GBM typical of Alport syndrome was observed in *Col4a3*^{-/-} mice (Figure 2d). Effacement of epithelial foot processes and formation of microvilli were commonly associated. Irregular thickening of the proximal and distal tubular basement membrane (TBM) and interstitial fibrosis were also seen. In contrast, most GBM in TG^{+/-}*Col4a3*^{-/-} littermate had a normal thickness and structure (Figure 2e), except for focal thickening and splitting of rare GBM segments. Podocyte foot processes were preserved and TBMs were normal.

Expression of Collagen IV Chains and Networks in the Renal Basement Membranes of Transgenic Mice

Immunofluorescence Analysis of Collagen IV Chains

Expression of murine and human collagen IV chains in the kidney BMs of transgenic mice was evaluated by indirect immunofluorescence using chain- and species-specific mAbs. Using antibodies that recognize both human and mouse type IV collagen chains, TG/*Col4a3*^{+/+} mouse kidneys display the same pattern as previously described in control mouse kidneys:^{16,33} the α3, α4, and α5(IV) collagen chains were found consistently co-expressed in the GBM, as well as in the proximal and distal tubules BMs, and α5(IV) was also present in the Bowman capsule BM. Using monoclonal antibodies that recognize the human but not the murine α3 and α4(IV) chains (RH34 and RH45, respectively), a faint staining was observed in the GBM and focally in segments of distal TBM, at the vascular pole of the glomerulus, reminiscent of the human pattern of expression of these chains (Figure 3).⁷

In TG/*Col4a3*^{-/-} mice, the GBM staining for the human chains was more intense than in TG/*Col4a3*^{+/+} mice, albeit less intense than in the human GBM. Again, the α3 and α4(IV) chains were expressed in a human-like pattern (Figure 4). Interestingly, the mouse α4 and α5(IV) chains (recognized by mAbs a11 and H53, respectively), absent [except focally in the Bowman capsule for α5(IV) chain] in the kidneys of *Col4a3*^{-/-} mice, were re-expressed along with the human α3(IV) and α4(IV) in the GBM and small segments of the TBM in TG/*Col4a3*^{-/-} mice. The GBM of the TG/*Col4a3*^{-/-} mice was also clearly labeled with mAb 26-20, an antibody to native α3/α4/α5(IV) NC1 containing hexamers, demonstrating a co-assembly of the human and mouse chains in hexamers. In contrast to the strong GBM expression of the α1 and α2(IV) chains in Alport mice, a normal glomerular distribution of these chains was ob-

Figure 2. Comparative histology of *Col4a3*^{-/-} (a) and TG/*Col4a3*^{-/-} littermate (b) mice at 8 weeks of age. Severe lesions, including glomerular sclerosis, glomerular crescents, tubular dilatations, and interstitial fibrosis were evident in the *Col4a3*^{-/-} mouse kidneys, whereas the renal morphology was preserved in the TG/*Col4a3*^{-/-} mice (trichrome light green stain). c to e: Electron microscopy of 8-week-old mice: *Col4a3*^{+/+} mouse (c); *Col4a3*^{-/-} mouse (d); and TG/*Col4a3*^{-/-} littermate (e). In the *Col4a3*^{+/+} mouse, the GBM was globally regular, except for some very focal thickening (arrow). Foot processes were normal. In the *Col4a3*^{-/-} mouse, an irregular thickening of the GBM with focal loss of foot processes was observed. In the TG/*Col4a3*^{-/-} mouse, the normal structure of the GBM and the foot processes were preserved, albeit very focal thickening of the GBM was observed (arrow). Original magnifications: $\times 200$ (a); $\times 400$ (b); $\times 4350$ (c); $\times 4800$ (d); $\times 4500$ (e).

served in TG/*Col4a3*^{+/+} (not shown) and TG/*Col4a3*^{-/-} mice (Figure 4).

Immunoblotting Analysis of Collagen IV Chains

Expression of the collagen IV chains in the murine kidneys was further analyzed by determining the composition of NC1 hexamers solubilized from mouse kidney BMs by collagenase digestion. NC1 hexamers were re-

solved into their constituent monomers and dimers by SDS-PAGE, then immunoblotted with mAbs specific for $\alpha 1$ - $\alpha 5$ (IV) chains (Figure 5). The results paralleled those found by immunofluorescence staining. The $\alpha 1$ - $\alpha 5$ (IV) chains were expressed in the renal BMs of wild-type control, TG/*Col4a3*^{+/+} and TG/*Col4a3*^{-/-} mice, but the $\alpha 3$ (IV) and $\alpha 4$ (IV) chains were absent in the *Col4a3*^{-/-} mice. Using human-specific mAbs RH34 and RH45, the presence of human $\alpha 3$ (IV) and $\alpha 4$ (IV) chains was clearly

Figure 3. Immunohistological analysis of human *Col4a3*^{+/+} mouse (middle) and TG/*Col4a3*^{+/+} mouse (right) kidneys using antibodies recognizing human (Hu) $\alpha 3$ and $\alpha 4$ (IV) chains. Antibodies strongly stained the human GBM and distal tubule BM, but did not label wild-type *Col4a3*^{+/+} control mouse BMs. A discrete GBM and focal TBM labeling was observed in TG/*Col4a3*^{+/+} mice.

detected only in transgenic mice, more strongly in TG/*Col4a3*^{-/-} mice, and weaker but clearly discernable in TG/*Col4a3*^{+/+} mice. For comparison, digests of renal BMs from control mice did not react with either RH34 or RH45, whereas a human GBM digest reacted strongly. Importantly, all NC1 domains were present predominantly in cross-linked form (as NC1 dimers), indicating

Figure 4. Immunohistological analysis of *Col4a3*^{+/+} mouse (left), *Col4a3*^{-/-} mouse (middle), and TG/*Col4a3*^{-/-} mouse (right) kidneys using antibodies recognizing human (Hu) and mouse (Ms) $\alpha 1$, $\alpha 2$ (IV), human and mouse $\alpha 3$ (IV), human and mouse $\alpha 4$ (IV), human $\alpha 3$ (IV), human $\alpha 4$ (IV), mouse $\alpha 4$ (IV), mouse $\alpha 5$ (IV), and human and mouse $\alpha 3/\alpha 4/\alpha 5$ (IV) containing native hexamers. The human $\alpha 3$ - $\alpha 4$ (IV) were expressed in the TG/*Col4a3*^{-/-} mouse GBM, but not in TBM. The mouse $\alpha 4$ (IV) and $\alpha 5$ (IV) chains were expressed in GBM and TBM in *Col4a3*^{+/+} mice, but were absent in all renal BM [except focally in the Bowman capsule for $\alpha 5$ (IV)] in *Col4a3*^{-/-} mice, and were expressed, along with human $\alpha 3$ and $\alpha 4$ (IV), in the GBM in TG/*Col4a3*^{-/-} mice.

Figure 5. Western blot analysis of collagen IV chain expression in the kidneys of *Col4a3*^{+/+} and *Col4a3*^{-/-} mice, in the presence or absence of the human transgene (TG). Collagenase-solubilized NC1 hexamers from murine renal BMs were resolved by SDS-PAGE into component NC1 monomers (M) and dimers (D), transferred to PVDF membranes, and immunoblotted with chain-specific mAbs recognizing both human and murine NC1 domains ($\alpha 1$ to $\alpha 5$), as well as with mAbs specific for human $\alpha 3$ and $\alpha 4$ (IV) NC1 domains (Hu $\alpha 3$, Hu $\alpha 4$). Human GBM hexamers were used as positive control.

that the human $\alpha 3$ (IV) and $\alpha 4$ (IV) collagen chains, like all other chains, assemble into cross-linked networks in the renal BMs *in vivo*. For all chains, including the human transgenes, the relative proportion of NC1 monomers to NC1 dimers was lower in mouse renal BMs than in human GBM, suggesting more efficient cross-linking of collagen IV protomers into networks in the murine BMs.

Immunoaffinity Fractionation Analysis of Collagen IV Networks

Separation of the NC1 hexamers using chain-specific mAbs provided further information about the network organization of murine α (IV) collagen chains in wild-type

Figure 6. Affinity fractionation of murine NC1 hexamers from *Col4a3*^{+/+} mice (**top**) and TG/*Col4a3*^{-/-} mice (**bottom**), using mAb 26-20 [which bound hexamers containing $\alpha3/\alpha4/\alpha5$ (IV) NC1 domains] and mAb 15-47 [which bound hexamers containing $\alpha1$ (IV) NC1 domain]. The composition of the NC1 hexamer fractions bound or not bound to the immobilized mAbs was analyzed by Western blot with chain-specific mAbs recognizing both human and murine NC1 domains ($\alpha1$ to $\alpha5$), as well as with mAbs specific for human $\alpha3$ and $\alpha4$ (IV) NC1 domains (Hu $\alpha3$, Hu $\alpha4$).

(*Col4a3*^{+/+}) and in TG^{+/+}/*Col4a3*^{-/-} mice. Using renal BMs from wild-type mice, mAb 26-20, recognizing native hexamers containing $\alpha3/\alpha4/\alpha5$, bound hexamers composed of $\alpha1$ - $\alpha5$ (IV) NC1 domains (Figure 6). This indicates that the murine $\alpha3$ and $\alpha4$ (IV) chains exist not only in association with $\alpha5$ (IV) chain, but also with the $\alpha1$ and $\alpha2$ (IV) chains. Hexamers not bound to mAb 26-20 were composed of $\alpha1$, $\alpha2$, and $\alpha5$ (IV) NC1 domains, likely representing a mixture of $\alpha1\alpha2$ (IV) hexamers and $\alpha5$ -containing hexamers. Co-assembly of murine $\alpha1$ - $\alpha5$ (IV) chains was also demonstrated using mAb 15-47 (to $\alpha1$ -containing hexamers), which bound all $\alpha1$ and $\alpha2$ (IV) NC1 domains along with a large proportion of $\alpha3$ - $\alpha5$ (IV) NC1 domains. A small amount of hexamers was not bound to mAb 15-47 and contained only the $\alpha3$, $\alpha4$, and $\alpha5$ (IV) NC1 domains, indicating the existence of $\alpha3.\alpha4.\alpha5$ (IV) hexamers. These results, together with the localization by immunofluorescence staining, indicate that murine $\alpha3$ - $\alpha5$ (IV) chains are co-deposited into both the GBM and the TBMs of wild-type mouse kidneys and

Figure 7. Immunohistological analysis of kidneys from *Col4a3*^{+/+} mice (**left**), *Col4a3*^{-/-} mice (**middle**), and TG/*Col4a3*^{-/-} mice (**right**) using antibodies recognizing mouse $\alpha2$ ($\alpha2$ LN), $\beta1$ ($\beta1$ LN), and $\gamma1$ ($\gamma1$ LN) laminin chains, perlecan, and fibronectin (FN). These proteins are mainly expressed in the mesangium of control *Col4a3*^{+/+} mice, but strongly stained in the GBM of *Col4a3*^{-/-} mice. In TG/*Col4a3*^{-/-} mice, the expression is comparable to control *Col4a3*^{+/+} mice.

occur both in an $\alpha3.\alpha4.\alpha5$ (IV) network (likely similar to that recently described in the human GBM), as well as in a distinct $\alpha1$ - $\alpha5$ (IV) network. The $\alpha1$ - $\alpha5$ (IV) network must be composed of dissimilar protomers, most likely of $\alpha3\alpha4\alpha5$ (IV) protomers interacting with ($\alpha1$)₂ $\alpha2$ (IV) protomers via their respective NC1 domains. The relative distributions of the murine $\alpha3.\alpha4.\alpha5$ (IV) and $\alpha1$ - $\alpha5$ (IV) networks between the GBM and TBMs could not be ascertained from the present study because total renal BM were used for the biochemical analysis.

Analysis of the kidney BMs from the TG^{+/+}/*Col4a3*^{-/-} mice with mAb 26-20 and mAb 15-47 yielded similar results (Figure 6). Both mAbs bound the human $\alpha3$ and $\alpha4$ (IV) NC1 domains along with murine $\alpha1$, $\alpha2$, and $\alpha5$ (IV) NC1 domains, indicating that human $\alpha3$ and $\alpha4$ (IV) chains co-assemble with the murine chains into a hybrid $\alpha1$ - $\alpha5$ (IV) collagen network. Co-assembly of the $\alpha3$, $\alpha4$, and $\alpha5$ (IV) chains into the same protomers and networks was further confirmed by co-precipitation of $\alpha3$ - $\alpha5$ NC1 domains by other chain-specific mAbs: Mab3 to $\alpha3$ (IV), RH42 to $\alpha4$ (IV), mAb b14 to $\alpha5$ (IV), and mAb a11 to mouse $\alpha4$ (IV) (data not shown). Based on the findings of immunofluorescence staining, the hybrid $\alpha1$ - $\alpha5$ (IV) network occurs in the kidneys of TG/*Col4a3*^{-/-} mice in the GBM and focally in distal TBMs.

Expression of Other Extracellular Matrix Proteins in the Kidney of TG/*Col4a3*^{-/-} Mice

The expression of laminin chains, heparan sulfate proteoglycan, nidogen, and fibronectin was studied in *Col4a3*^{-/-}, TG^{+/+}/*Col4a3*^{-/-}, and wild-type mice, at 2 months of age (Figure 7). None of the changes in the distribution of noncollagenous extracellular matrix pro-

Figure 8. Western blot analysis of collagen IV chain expression in the extrarenal BMs of *Col4a3*^{+/+} and TG/*Col4a3*^{-/-} mice. Collagenase-solubilized NC1 hexamers from murine lung and bladder BMs were resolved by SDS-PAGE into component NC1 monomers (M) and dimers (D), transferred to PVDF membranes, and were immunoblotted with chain-specific mAbs recognizing both human and murine NC1 domains ($\alpha 1$ to $\alpha 5$), as well as mAbs specific for human $\alpha 3$ and $\alpha 4$ (IV) NC1 domains (Hu $\alpha 3$, Hu $\alpha 4$).

teins observed in *Col4a3*^{-/-} mice were detected in TG/*Col4a3*^{-/-} animals. In short, fibronectin; laminin $\alpha 2$, $\beta 1$, and $\gamma 1$ chains; and perlecan, which normally have a restricted mesangial distribution in the glomerulus, were strongly expressed in the GBM in *Col4a3*^{-/-} mice, as previously reported.^{16,34,35} In contrast, in the TG/*Col4a3*^{-/-} animals, normal mesangial expression of these antigens was observed. No significant changes in the distribution of proteins normally expressed in the GBM (laminin $\alpha 5$ chain, entactin, and agrin) were detected in either *Col4a3*^{-/-} or TG/*Col4a3*^{-/-} mice, compared to controls.

Expression of Collagen IV Chains and Networks in Extra-Renal BMs

To determine whether regulatory elements in the human transgene can control their tissue-specific expression in mice, we compared transgenic mice and wild-type controls with regard to the collagen IV chain composition of extra-renal BMs. Consistent with previous immunohistochemical localization studies^{36,37} in control wild-type mice, the $\alpha 3$ (IV) and $\alpha 4$ (IV) chains were expressed in lung BMs (along with $\alpha 5$ (IV) chain), but not in the bladder BMs. The same expression pattern was found in TG/*col4a3*^{-/-} mice; further analysis of their lung BMs with human-specific mAbs confirmed the presence of human $\alpha 3$ and $\alpha 4$ (IV) chains (Figure 8). Together with the find-

Figure 9. Top: Indirect immunofluorescence staining with Goodpasture autoantibodies of native (A–E) and acid urea-treated (F) kidney sections. The sections were from *Col4a3*^{-/-} mouse (A), human (B), TG/*Col4a3*^{-/-} mouse (C), and *Col4a3*^{+/+} mouse (D–F). Under native conditions, antibodies bound in a linear manner to human and TG/*Col4a3*^{-/-} mouse GBM but not to *Col4a3*^{+/+} mouse GBM. In *Col4a3*^{+/+} mice, only podocytes were clearly labeled under native conditions, whereas GBM and TBM were stained after acid urea treatment. Bottom: The reactivities with Goodpasture antibodies of native (solid bars) and dissociated (open bars) NC1 hexamers from human GBM and mouse kidneys were compared by enzyme-linked immunosorbent assay. Under native conditions, the Goodpasture epitopes were much less accessible in the murine than in the human NC1 hexamers, consistent with the results of immunofluorescence staining.

ings in renal BMs, these results indicate that expression of the *COL4A3-COL4A4* transgene is tissue-restricted and occurs only in BMs that normally express the $\alpha 3$ (IV) and $\alpha 4$ (IV) collagen chains.

Human Goodpasture Autoantibodies Bind to the GBM of *Col4a3*^{-/-} Transgenic Mice

Because the human $\alpha 3$ (IV) collagen chain is the Goodpasture (GP) autoantigen,³⁸ the TG/*Col4a3*^{-/-} mouse may be uniquely suited to study *in vivo* the interaction

between human GP autoantibodies and the GP autoantigen. To explore the feasibility of these studies, we compared the binding of GP autoantibodies to human, *Col4a3*^{+/+}, *Col4a3*^{-/-}, and *Tg/Col4a3*^{-/-} mouse GBM by indirect immunofluorescence (Figure 9, top). Under native conditions, GP antibodies stained human GBM and *Tg/Col4a3*^{-/-} mouse GBM, but not the wild-type nor the *Col4a3*^{-/-} mouse GBM. Surprisingly, only podocytes were labeled in wild-type mice, suggesting that the GP epitopes are completely inaccessible to autoantibodies in the native murine GBM and TBM networks. Indeed, GP antibodies produced the expected GBM and TBM pattern of $\alpha 3(\text{IV})$ expression after the wild-type mouse kidney sections were treated with acid urea to expose the cryptic epitopes. Furthermore, by enzyme-linked immunosorbent assay (Figure 9, bottom), native NC1 hexamers from human GBM and mouse kidneys exhibited ~34% and ~6.5% of the GP immunoreactivity of the respective dissociated hexamers. This indicates that GP epitopes are sequestered more tightly in mouse than in human native NC1 hexamers, and only human hexamers are capable of reacting with GP antibodies under native conditions. Because GP antibodies stain the GBM of the *Tg/Col4a3*^{-/-} mice under native conditions, the hybrid human-mouse $\alpha 3.\alpha 4.\alpha 5(\text{IV})$ network is more similar to its human than to its wild-type counterpart with regard to the accessibility of GP epitopes.

Discussion

The GBM is an essential component of the glomerular capillary wall, which is responsible for plasma ultrafiltration during primary urine formation. The mature GBM mainly contains an $\alpha 3.\alpha 4.\alpha 5(\text{IV})$ collagen network that is required for the long-term maintenance of the glomerular filtration function¹¹ but which is absent in the majority of the patients affected with Alport syndrome.¹⁰ Several lines of evidence suggest that the absence of the whole $\alpha 3.\alpha 4.\alpha 5(\text{IV})$ network in most Alport patients, even though only one chain is mutated, is because of a post-translational co-regulation of the expression of these three chains, which either prevents the assembly of the $\alpha 3.\alpha 4.\alpha 5$ protomers, or produces defective protomers that are degraded or unable to assemble into supramolecular network.^{1,2,14–16}

Alport syndrome progresses slowly but inexorably toward end-stage renal failure, requiring dialysis or transplantation. Any therapeutic approach aimed at correcting the underlying defect in Alport patients by restoring the mutated chain is highly desirable. The feasibility of gene therapy in Alport syndrome in the future is suggested by recent data showing expression of a recombinant $\alpha 5(\text{IV})$ within the GBM chain after *in vivo* kidney perfusion in a pig model.³⁹ However, neither *in vitro* nor *in vivo* studies have so far addressed the question of whether the re-expression of the mutated chain in its wild-type form will correct the defect in the assembly of collagen IV protomers and networks in the GBM. In addition, very little is known regarding the level of type IV collagen that is required for making a functional BM.

In the present study, we generated a transgenic line of mice carrying the human *COL4A3-COL4A4* genomic locus. We used YAC because 1) type IV collagen genes are large;^{17,22} 2) these genes are alternatively spliced;^{40,41} and 3) the transcriptional regulation of these genes is complex and implies regulatory elements located in introns.^{42,43} We used the human genes to distinguish between the transgenic and natively expressed chains by using species-specific antibodies. The expression of transgenic type IV collagen was studied both onto a wild-type and a *Col4a3*^{-/-} mouse background. The level of transcription of the human genes in mice carrying two copies of the transgene on either background was found to be ~20% of the level of transcription observed in the human kidney or in the wild-type (*Col4a3*^{+/+}) mouse kidney. This low level of expression might be because of differences between *COL4A3-COL4A4* and *Col4a3-Col4a4* loci, and mouse-specific regulatory factors may act less efficiently on the human transgene. Alternatively, *cis*-elements required for full expression of the genes might not be included in our 450-kb YAC. Finally, although YAC transgenesis is thought to overcome, in part, position effects related to the integration site, we cannot rule out the hypothesis of a low level of expression caused by a positional effect. The study of the overall level of transcription $\alpha 3(\text{IV})$ in transgenic mice onto a wild-type and a *Col4a3*-null background suggests that the level of transcription of *Col4a3* gene does not modify the level of transcription of the transgene, and vice versa. In this model, up-regulation of the glomerular expression of the $\alpha 3$ and $\alpha 4(\text{IV})$ does not seem to be deleterious.

In the transgenic mice, the human $\alpha 3$ and $\alpha 4(\text{IV})$ chains were found to be expressed in lung and kidney but not in bladder BMs, suggesting that the genomic sequences included in our construct are able to direct the correct tissue-specific expression of the genes. Moreover, the human $\alpha 3$ and $\alpha 4(\text{IV})$ chains were expressed only in the GBM and distal TBM of transgenic mice. This distribution, similar to the expression pattern in human kidneys, contrasts with that observed in wild-type mice, in which $\alpha 3$ and $\alpha 4(\text{IV})$ are present in all TBMs as well as in the GBM.³³ These results indicate that the differences between the human and the rodent pattern of expression of type IV collagen chains in the kidney are probably transcriptional and because of *cis*-regulatory elements carried by the transgene, rather than to factors acting in trans that could have been differentially expressed in the two species.

Whereas the mouse $\alpha 4(\text{IV})$ and $\alpha 5(\text{IV})$ chains were consistently absent in the GBM and TBM of *Col4a3*^{-/-} mice, they were re-expressed in *Tg/Col4a3*^{-/-} mice, along with the human $\alpha 3$ and $\alpha 4(\text{IV})$ chains, specifically in the GBM. We show that the human chains co-assembled with the mouse $\alpha 4$ and $\alpha 5(\text{IV})$ chains, into a hybrid collagen IV network in the mouse GBM. Thus, correction of $\alpha 3(\text{IV})$ expression by the human transgene rescues the expression of the mouse $\alpha 4(\text{IV})$ and $\alpha 5(\text{IV})$, specifically at sites where the human $\alpha 3(\text{IV})$ was expressed, emphasizing the need of all three chains for making an appropriate network and a functional GBM. A similar correction of the

glomerular type IV collagen networks may be expected, ultimately, from gene therapy.

Despite a relatively low level of expression, the assembly of human $\alpha 3$ and $\alpha 4$ (IV) chains in an hybrid collagen network in the GBM was able to rescue the Alport phenotype in *Col4a3*^{-/-} mice. Furthermore, despite the absence of $\alpha 3.\alpha 4.\alpha 5$ (IV) collagen, the ultrastructural aspect of the proximal TBM in TG/*Col4a3*^{-/-} mice was normal, suggesting that the TBM expression of the $\alpha 3.\alpha 4.\alpha 5$ (IV) network in mouse is not critical and that severe tubular changes observed in *Col4a3*^{-/-} mice are not directly linked to the TBM defect, but could be the consequence of persistent proteinuria. This observation is of importance because severe tubulointerstitial lesions also develop in Alport syndrome patients lacking the $\alpha 3\alpha 4\alpha 5$ (IV) chains in the distal tubules. Glomerular gene delivery correcting the GBM collagen IV network could prevent the progressive tubulointerstitial damage in Alport syndrome kidneys.

In *Col4a3*^{-/-} mice, deposition within the GBM of BM-associated proteins that are normally expressed in the mesangial matrix might play a role in the progression of the disease.^{16,35} It was shown recently that mRNAs encoding these extracellular proteins were significantly elevated in the podocytes, suggesting that a change in gene expression in these cells results in elevated synthesis and deposition of extracellular matrix in the GBM of *Col4a3*^{-/-} mice.⁴⁴ In the TG/*Col4a3*^{-/-} mice that we report here, the pattern of expression of $\alpha 2$ and $\beta 1$ laminin, heparan sulfate proteoglycans, and fibronectin was normal. Altogether, these data suggest that even a relatively low level of expression of the missing $\alpha 3$ (IV) collagen chain is sufficient for restoring the structural and functional integrity of the GBM and for proper phenotypic differentiation of podocyte cells abutting the GBM. Whether such a low expression will be sufficient for long-term stability of the GBM remains to be determined by a longer follow-up. Indeed, despite the absence of renal symptoms, the normal appearance of the GBM by light microscopy, the normal distribution of other matrix proteins and the normal architecture of the podocyte foot processes, ultrastructural GBM lesions were focally observed in TG/*Col4a3*^{-/-} mice. It is likely that the focal defects are because of the relatively low levels of $\alpha 3.\alpha 4.\alpha 5$ (IV) expression, and may suggest a risk for late progression of the renal disease. Indeed, the character of the lesions is reminiscent of that seen in some individuals carrying heterozygous *COL4A3* or *COL4A4* mutations. Those patients mostly present with benign hematuria and thin GBM, but can also develop nephropathies of variable severity with focal splitting of the GBM.²²

Finally, the mouse model we report here may provide an ideal model for studying the pathogenesis of Goodpasture (GP) syndrome, an autoimmune disease caused by antibodies against the $\alpha 3$ (IV) collagen chain. The two major GP epitopes, mapped to two regions of the human $\alpha 3$ (IV) NC1 domain,⁴⁵ are conformational and normally sequestered within the $\alpha 3$ (IV)-containing hexamers of the native GBM,³⁰ but how pathogenic antibodies gain access to these cryptic epitopes is not known. Binding of human GP antibodies to the native GBM of TG/*Col4a3*^{-/-}

(but not wild-type) mice suggests that the quaternary organization of the GP epitopes in the hybrid human-mouse GBM network resembles that found in the human GBM network, and that transgenic mice may be susceptible to passive transfer of anti-GBM nephritis by human GP antibodies. The line of mice we report here may lead, along with transgenic lines humanized for MHC molecules and/or human immunoglobulins,^{46,47} to the development of new mouse models that very closely resemble the human GP disease at the molecular level. This may provide new means to decipher early pathogenic events implicated in the human GP syndrome, and to test specific therapies.

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