Animal Model

Quantitative Trait Loci Influence Renal Disease Progression in a Mouse Model of Alport Syndrome

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Alport syndrome is a human hereditary glomerulonephritis which results in end-stage renal failure (ESRF) in most cases. It is caused by mutations in any one of the collagen $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, or $\alpha 5(\text{IV})$ chain genes (*COL4A3*-*COL4A5*). Patients carrying identical mutations can exhibit very different disease courses, suggesting that other genes or the environment influence disease progression. We previously generated a knockout mouse model of Alport syndrome by mutating *Col4a3*. Here, we show that genetic background strongly influences the timing of onset of disease and rate of progression to ESRF in these mice. On the 129X1/SvJ background, *Col4a3* $-/-$ mice reached ESRF at ~66 days of age, while on the C57BL/6J background, the mean age at ESRF was 194 days of age. This suggests the existence of modifier genes that influence disease progression. A detailed histopathological analysis revealed that glomerular basement membrane lesions typical of Alport syndrome were significantly more frequent in homozygotes on the 129X1/SvJ background than on the C57BL/6J background as early as two weeks of age, suggesting that modifier genes act by influencing glomerular basement membrane structure. Additional data indicated that differential physiological responses to basement membrane splitting also underlie the differences in disease progression. We attempted to map the modifier genes as quantitative trait loci (QTLs) using age at ESRF as the quantitative trait. Genome scans were performed on mice at the two extremes in a cohort of mutant F1 $\times$ C57BL/6J backcross mice. Analysis with Map Manager QT revealed QTLs linked to markers on chromosomes 9 and 16. A more detailed understanding of how these QTLs act could lead to new approaches for therapy in diverse renal diseases. (*Am J Pathol* 2002, 160:721-730)

Alport syndrome is a hereditary glomerulonephritis usually accompanied by sensorineural deafness and other defects.¹⁻³ The disease is caused by mutations in any one of the genes encoding the $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains of type IV collagen (*COL4A3*, *COL4A4*, and *COL4A5*, respectively).^{4,5} Because the *COL4A5* gene is X-linked,⁶ most Alport syndrome patients are males. The nephropathy is characterized clinically in the majority of cases by early onset hematuria, later onset proteinuria, and progression to end-stage renal failure (ESRF) as early as the second or as late as the fourth decade of life.⁷ Pathologically, the glomerular basement membrane (GBM) exhibits a characteristic thinning, thickening, and splitting.¹⁻³ This is consistent with the fact that the collagen IV chains affected by the mutations are normally found in the GBM, where they are thought to assemble into a specialized network that imparts a specialized structural stability to the GBM.⁴

When one of the $\alpha 3$ - $\alpha 5(\text{IV})$ chains is mutated, all three fail to accumulate in the GBM. This suggests the existence of a complex assembly process requiring all three chains that leads to formation of the specialized GBM collagen IV network.^{4,8,9} In Alport GBM, the missing collagen $\alpha 3$ - $\alpha 5(\text{IV})$ chains are replaced by the $\alpha 1$ and $\alpha 2(\text{IV})$ chains, which are normally found in the immature GBM.¹⁰ It has been hypothesized that the GBM network formed by the $\alpha 1$ and $\alpha 2(\text{IV})$ chains exhibits reduced structural stability or is more susceptible to proteolysis.¹¹

We and others have generated knockout mouse models of autosomal recessive Alport syndrome by mutating either the *Col4a3* gene^{12,13} or both the *Col4a3* and *Col4a4* genes.¹⁴ These mice all exhibit a delayed onset glomerulonephritis that progresses to ESRF. Importantly, the GBM lesions characteristic of the human disease are present, suggesting that the overt disease is rooted in the same GBM defects observed in human patients. At late stages of the disease, the mice exhibit glomerular pathology reminiscent of crescentic glomerulonephritis, as well

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as a robust tubulointerstitial component characteristic of progressive, chronic nephritis.

It has become apparent from studies of both human patients^{15–17} and animal models^{13,18–20} that individuals harboring identical mutations can exhibit different timing of disease onset and different rates of progression to ESRF. This phenotypic variation can likely be attributed to either differential environmental influences or variation in genetic background or both.

In our initial report of the *Col4a3* mutant phenotype, our data showed significant mouse to mouse variation in terms of the timing of onset of proteinuria and rate of progression to ESRF.¹³ Our original cohort of *Col4a3* $-/-$ mice had a mixed genetic background, suggesting the hypothesis that the variation could have been due to the activities of modifier genes, also known as quantitative trait loci (QTLs). Here, we used the power of mouse genetics and molecular biology to test this hypothesis and to attempt to localize QTLs affecting rate of progression of renal disease. Given that related human Alport patients carrying the same collagen IV gene mutation can exhibit variability in rate of disease progression, it is possible that QTLs identified in mice could have homologues in humans that also influence disease progression in Alport syndrome, as well as in other nephritides. Elucidation of such modifier pathways may provide novel targets for therapies aimed at slowing disease progression.

Materials and Methods

Mouse Breeding

Production of *Col4a3* mutant mice has been previously described.¹³ To generate incipient congenic 129X1/SvJ (129) and C57BL/6J (B6) *Col4a3* mutant mice, *Col4a3* $+/-$ males of mixed genetic background were initially crossed with inbred 129 and B6 females obtained from The Jackson Laboratory (Bar Harbor, ME). *Col4a3* $+/-$ males were then backcrossed for a total of six (129) or seven (B6) generations. Incipient congenic 129 or B6 *Col4a3* $-/-$ mice were generated by crossing heterozygotes. Incipient congenics were also outcrossed to generate the F1 hybrid mice. For generating most of the experimental N2 backcross mice used in the QTL analysis, B6 *Col4a3* $-/-$ males were mated to F1 hybrid *Col4a3* $+/-$ females.

Assays for Renal Function

Blood and urine were obtained at the time of sacrifice by cardiac and urinary bladder puncture of deeply anesthetized mice. Concentrations of blood creatinine and urea nitrogen and of urinary protein and creatinine were measured with a Cobas Mira Plus analyzer (Roche, Somerville, NJ). Urinary protein was normalized to creatinine concentration.

Histology

For conventional light microscopy, kidneys were fixed in 10% buffered formalin overnight, dehydrated through graded ethanols, and embedded in paraffin. Sections were cut and then stained with periodic acid-Schiff (PAS) reagent by standard techniques. For electron microscopy, small pieces of kidney cortex were fixed in 2% paraformaldehyde/2% glutaraldehyde in 0.15 mol/L sodium cacodylate buffer, pH = 7.2. After rinsing in buffer, the tissue was fixed in 1% osmium tetroxide, dehydrated, and embedded in plastic. Thin sections were cut with a diamond knife, stained in uranyl acetate and lead citrate, and viewed with a Zeiss transmission electron microscope (Thornwood, NY). To quantitate the extent of GBM splitting, capillary loops of several glomeruli from at least two mice of each genotype were photographed at $\times 4360$. The negatives were digitized, and images were enlarged and printed. Normal and split regions of the GBM were identified and measured with string and a ruler. The percentage of the GBM exhibiting splits was then determined. Reagents were obtained from Polysciences (Warrington, PA).

Immunohistochemistry

Freshly dissected kidneys were immersed in OCT compound and frozen in dry ice/ethanol cooled 2-methyl butane. Seven- μ m sections were cut on a cryostat and fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 10 minutes. After blocking in 10% goat serum, rabbit anti-human laminin $\alpha 2$ antiserum²¹ diluted in PBS with 1% bovine serum albumin was applied for 1 hour. After rinsing in PBS, Cy3-conjugated donkey anti-rabbit secondary antibody (Chemicon, Temecula, CA) was applied for 1 hour. Sections were rinsed in PBS, mounted in 90% glycerol/0.1X PBS/1 mg/ml *p*-phenylenediamine, and viewed with a Nikon Eclipse 800 fluorescence microscope (Tokyo, Japan). Images were captured with a Spot 2 cooled color digital camera (Diagnostic Instruments, Sterling Heights, MI) using Spot Software version 2.1. Reagents were obtained from Fisher Scientific (Pittsburgh, PA).

Genome Scans

Genomic DNA was prepared from lungs by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. Microsatellite primer pairs (MapPairs) known or suspected to be polymorphic between 129 and B6 and spaced throughout the mouse autosomal genome were purchased from Research Genetics (Huntsville, AL). Polymorphic MapPairs were identified by polymerase chain reaction (PCR) on DNAs obtained from the inbred mice and from F1 hybrids. PCR reactions were performed in a volume of 20 μ l and contained 120 ng of genomic DNA, 10 pmol of each primer, 2.5 mmol/L $MgCl_2$, 150 μ mol/L dNTPs, 0.5 units *Taq* DNA Polymerase (Life Technologies, Rockville, MD), and 1X buffer supplied by the manufacturer. Reaction conditions were: 4 cycles of:

96°C for 2 minutes, 55°C for 30 seconds, 72°C for 30 seconds; 36 cycles of: 96°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; and 72°C for 7 minutes. PCR products were fractionated on 3% SFR agarose gels (Amresco, Solon, OH) in the presence of ethidium bromide. MapPairs which generated bands that differed significantly in size between the two inbred strains and that were spaced at a genetic distance of approximately 20 cM were used to perform genome-wide scans.²² Microsatellite map locations were obtained from the Mouse Genome Database on the Mouse Genome Informatics website maintained by The Jackson Laboratory. Whole genome scans were performed on DNAs obtained from a subset of the experimental N2 *Col4a3* $-/-$ backcross mice. This subset was composed of the five mice that reached ESRF soonest and the five mice that reached ESRF latest. In addition, microsatellite analysis of selected chromosomes was performed for 49 backcross mice.

Quantitative Trait Analyses

The results of the genome scans were organized and analyzed using the Map Manager QT program, version 3.0b29.²³ Age at ESRF was used as the continuous quantitative trait. An additive regression model with $P = 0.001$ was used to identify QTLs with the QT Links Report function. Logarithm of odds (LOD) scores were derived by dividing the likelihood ratio statistic by 4.6. For interval mapping, the critical threshold values for significance of linkage were determined by the QT Permutation Test function using 2000 permutations.^{22,24}

Results

Effect of Genetic Background on Progression of Renal Disease

In our initial studies of *Col4a3* $-/-$ mice, we reported significant heterogeneity in the timing of onset of impaired renal function and of progression to ESRF.¹³ Here we hypothesized that the basis for this heterogeneity was related to the mixed genetic background of the mutant mice, which was a variable, undefined mixture of C57BL/6J (B6), 129X1/SvJ (129), and 129S1/SvImJ strain genomes. To test this hypothesis, we backcrossed the mutation onto the B6 and 129 strains for seven and six generations, respectively. The resulting mice had genetic backgrounds that were theoretically >99% pure B6 and >98% pure 129. (These mice will hereafter be referred to as B6 *Col4a3* and 129 *Col4a3* mutants, respectively, though we note that 10 generations of backcrossing are considered necessary for formal congenic designation.²⁵) We then generated cohorts of B6 *Col4a3* $-/-$ and 129 *Col4a3* $-/-$ mice and determined the age at ESRF. B6 *Col4a3* $-/-$ mice survived much longer (mean age at ESRF 194 ± 24 days; $n = 28$) than the 129 *Col4a3* $-/-$ mice (mean age at ESRF 66 ± 6 days; $n = 16$).

Consistent with the observed effect of genetic background on progression to ESRF, there was a striking

Figure 1. Assays for renal function in 129 and B6 *Col4a3* $+/-$ and $-/-$ mice. Levels of blood urea nitrogen (BUN) and serum creatinine and the urinary protein:creatinine ratios were determined at various ages for mice of all four cohorts. Renal function declined much faster in 129 *vs.* B6 *Col4a3* $-/-$ mice, as denoted by the rapid rise in all three indicators.

influence of genetic background on renal function. Additional cohorts of B6 *Col4a3* and 129 *Col4a3* $-/-$ and $+/-$ control mice were sacrificed at various ages, and blood and urine were collected. The concentrations of blood urea nitrogen (BUN) and creatinine and the urinary protein:creatinine ratio were determined for each mouse as quantitative indicators of renal function. As shown in Figure 1, BUN and serum creatinine concentrations were elevated in 129 *Col4a3* $-/-$ mice at ~50 days of age, while concentrations in B6 *Col4a3* $-/-$ mice were normal until ~150 days of age. As also observed in humans, elevated urinary protein was an earlier indicator of renal malfunction; it was evident at ~35 days of age in the 129 *Col4a3* $-/-$ mice, and at ~100 days of age in the B6 *Col4a3* $-/-$ mice. Heterozygous control mice did not exhibit any consistent physiological abnormalities. Surprisingly, there was some variation among the B6 *Col4a3* $-/-$ mice in terms of both age of onset and degree of renal malfunction at various ages. This variation could have resulted from subtle environmental, developmental, or maternal factors. Nevertheless, these data clearly show that backcrossing to the parental strains to produce incipient congenics resulted in distinct, non-overlapping

phenotypes in terms of the timing of onset and rate of renal disease progression.

Because all mice carried the same targeted *Col4a3* mutation and were housed under the same conditions, we conclude that the genetic backgrounds differentially influenced the rate of progression to ESRF, perhaps by modifying the animals' physiological responses to the *Col4a3* mutation. In addition, the heterogeneity we observed in the original *Col4a3* $-/-$ mice on the mixed genetic background¹³ likely resulted from random segregation of B6 and 129 alleles of modifier genes unlinked to *Col4a3*.

Effect of Genetic Background on Histopathology

We next attempted to correlate the striking differences in the rate of progression to ESRF on the two genetic backgrounds with differences in renal histopathology. One possibility is that lesions appear in the mutant kidneys of both strains at the same age, but progression to ESRF is much faster in 129 *Col4a3* $-/-$ mice. Alternatively, lesions in B6 *Col4a3* $-/-$ mice may not appear until much later than they do in 129 *Col4a3* $-/-$ mice. To distinguish between these possibilities, we examined PAS-stained sections from 129 and B6 *Col4a3* $-/-$ kidneys at several ages. At ESRF, 129 and B6 *Col4a3* $-/-$ kidneys appeared very similar (Figure 2, E and data not shown), showing extensive damage to glomeruli, including a thickened GBM and fibrocellular material in Bowman's space. In addition, there was extensive tubulointerstitial fibrosis and inflammation. These lesions are consistent with what we previously reported for near end-stage mutant kidneys on the mixed genetic background.¹³

Comparison of sections from younger mutants revealed that at ~3 weeks of age (Figure 2, A and B), no significant pathology was evident on either genetic background. However, at ~6.5 weeks of age (Figure 2, C and D), there were obvious glomerular and tubulointerstitial lesions in the 129 *Col4a3* $-/-$ kidneys, while little pathology was observed in the B6 *Col4a3* $-/-$ kidneys. Even at ~12 weeks of age (Figure 2, E and F), when all 129 *Col4a3* $-/-$ mice had reached ESRF, the B6 *Col4a3* $-/-$ mice exhibited only limited pathology. Analysis of older B6 *Col4a3* $-/-$ kidneys (data not shown) indicated that the pathology slowly worsened over a 13 to 16 week period as the kidney progressed to ESRF. Thus, in the 129 *Col4a3* $-/-$ mice the rapid progression to ESRF was reflected by the rapid appearance and advancement of lesions, while in the B6 *Col4a3* $-/-$ mice, the slow progression to ESRF was reflected by the later onset and slower advancement of histopathological lesions.

Because ultrastructural GBM splits are one of the hallmarks of Alport syndrome, we hypothesized that GBM splits would be detected earlier in the 129 than in the B6 *Col4a3* $-/-$ kidneys, and that this would explain in part the physiological and histopathological differences between the two strains. We therefore used electron microscopy to examine the GBMs in kidneys from 2 and 3 week

old 129 and B6 *Col4a3* $-/-$ and $+/-$ mice (Figure 3 and data not shown). At 2 weeks (Figure 3, A–C), we found that GBM splits were present in both the 129 and the B6 *Col4a3* $-/-$ kidneys. Importantly, by quantitating the percentage of the GBM exhibiting splits, we found that there was significantly more splitting in the 129 than in the B6 *Col4a3* $-/-$ GBM (Figure 4). The frequency of splitting observed in the B6 *Col4a3* $-/-$ GBMs was not significantly different from that observed in either the B6 or 129 *Col4a3* $+/-$ controls (Figure 4), suggesting that this splitting primarily represents portions of the GBM that are still in the process of maturing.^{26,27} At ~3 weeks of age (Figure 3, D–F), no splitting was detected in the heterozygotes, but splitting was detected in both the 129 and the B6 *Col4a3* $-/-$ GBMs. However, the percentage of splitting in the 129 *Col4a3* $-/-$ GBMs was reduced from the level observed at 2 weeks and was no longer significantly different from the level observed in the B6 *Col4a3* $-/-$ GBMs (Figure 4). We therefore conclude that the high level of GBM splitting observed specifically in 129 *Col4a3* $-/-$ GBMs at 2 weeks of age may be responsible for the rapid progression to ESRF, despite partial recovery from splitting at 3 weeks of age. This recovery is intriguing and may result from the activity of whatever developmental processes assure the proper fusion of the developing GBM.

Next, we compared GBM ultrastructure in the B6 and 129 *Col4a3* $-/-$ kidneys at ~6 weeks of age (Figure 3, G–I). GBM splitting was evident in *Col4a3* $-/-$ mice of both genetic backgrounds, but not in the control. This is an age at which B6 *Col4a3* $-/-$ kidneys exhibited little if any histopathology, while the 129 *Col4a3* $-/-$ kidneys exhibited significant histopathology (Figure 2, C and D). The fact that GBM splitting was present in B6 *Col4a3* $-/-$ kidneys in the absence of significant pathology suggests that differences in secondary physiological responses to the primary GBM damage likely influence the rate of disease progression.

Effect of Genetic Background on GBM Composition

Recently, we and others showed that aberrant deposition of the laminin $\alpha 2$ chain in the GBM is an abnormality consistently observed in Alport syndrome.^{28,29} Normally, the GBM contains laminin-11, a trimer containing the $\alpha 5$, $\beta 2$, and $\gamma 1$ chains.^{30,31} The additional presence in Alport GBM of the laminin $\alpha 2$ chain, which is normally restricted to the mesangial matrix,³¹ together with the abnormal collagen IV chain repertoire, may alter the filtration properties of the GBM or perturb podocyte homeostasis. The mechanism of aberrant laminin chain deposition and its pathological consequences are not understood. Nevertheless, Cosgrove and colleagues²⁹ reported that crossing the mouse *Col4a3* mutation onto an integrin $\alpha 1$ -null background significantly slowed progression to ESRF, and this was associated with reduced accumulation of laminin $\alpha 2$ in the GBM. We therefore examined by indirect immunofluorescence whether there was differential accu-

Figure 2. Comparative histology of 129 and B6 *Col4a3*^{-/-} kidneys. By PAS staining, no pathology was evident at 22 days of age on either background. Significant pathologies, including glomerular crescents (**asterisks**), cellular infiltrates (**white arrows**), tubular casts (tc), and interstitial fibrosis (**black arrows**), were evident in the 129 *Col4a3*^{-/-} kidney at 47 days of age and later, but only limited pathology was observed in the B6 *Col4a3*^{-/-} kidney as late as 82 days of age. g, glomerulus. Scale bar, 50 μ m.

mulation of laminin α 2 in the 129 vs. B6 *Col4a3*^{-/-} GBM. Indeed, at both 2 and 3 weeks of age, 129 *Col4a3*^{-/-} GBMs exhibited significantly more laminin α 2 antibody immunoreactivity than did the B6 *Col4a3*^{-/-} GBM (Fig-

ure 5). We therefore conclude that differential accumulation of this normally mesangial laminin chain in the 129 and B6 *Col4a3*^{-/-} GBMs may partially explain the striking differences in rates of progression to ESRF.

Figure 3. Comparative ultrastructural analysis of 129 and B6 *Col4a3*^{-/-} glomerular basement membranes (GBMs). At 14 days of age, widespread splitting was observed in the 129 *Col4a3*^{-/-} GBM, while less splitting was observed in the B6 *Col4a3*^{-/-} and in the *Col4a3*^{+/-} GBMs. At 22 and 44 days of age, significant splitting was observed in both the 129 and B6 *Col4a3*^{-/-} GBMs, but not in the *Col4a3*^{+/-} GBM. **Arrows** indicate regions of GBM splitting. Scale bar, 2.3 μ m.

Mapping of Quantitative Trait Loci

Data presented so far suggest that there are genetic modifiers which influence the timing of onset of GBM lesions and the physiological responses to them. We wished to characterize these modifier genes further. To determine whether the 129 or B6 alleles of these genes exhibit simple dominance, we performed an outcross to generate *Col4a3*^{-/-} mice on the F1 hybrid (B6x129) background. These mice reached ESRF at an intermediate age of 113 ± 16 days (Figure 6), suggesting that the alleles involved in determining rate of progression to ESRF could be semidominant. Alternatively, there could exist dominant B6 and 129 alleles of multiple genes that

together, in the context of the hybrid background, result in the intermediate age at ESRF.

To further investigate how the genetic modifiers influence age at ESRF, and to determine whether it might be worthwhile to attempt to map such modifiers, we backcrossed F1 hybrids to parental B6 *Col4a3*^{+/-} mice to generate a cohort of 51 *Col4a3*^{-/-} N2 backcross mice. The mean age at ESRF for these N2 mice was 136 ± 27 days (Figure 6), and there was significant variability: the youngest age of ESRF was 81 days, and the oldest was 193 days, with a continuous range of ages in between. These backcross mice were, on average, homozygous B6 at 50% of loci and heterozygous 129/B6 at the remain-

Figure 4. Quantitation of GBM splitting in 129 and B6 *Col4a3*^{-/-} and +/- mice. At 14 days of age, the 129 *Col4a3*^{-/-} GBMs exhibited significantly more splitting than either the B6 *Col4a3*^{-/-} or the heterozygous control GBMs. At 22 days of age, however, both the 129 and B6 *Col4a3*^{-/-} GBMs exhibited splitting, but were not significantly different from each other. Error bars indicate S.E.M.

ing loci, but each mouse was heterozygous at a different set of loci due to meiotic recombination. Because the 129 *Col4a3*^{-/-} mice progressed more rapidly to ESRF (Figure 6), we proposed the simple hypothesis that at a particular modifier locus influencing the rate of renal disease progression, the backcross mice that progressed to ESRF earliest would harbor both 129 and B6 alleles, while those that progressed to ESRF latest would be homozygous B6.

To test this hypothesis and to attempt to map the modifier loci, we performed genome scans on the indi-

Figure 6. Mean age at ESRF for *Col4a3*^{-/-} mice on different genetic backgrounds. The actual values ($\pm$ SD) were: 129, 66 ± 6 days ($n = 16$); F1, 113 ± 16 days ($n = 26$); N2, 136 ± 27 days ($n = 51$); B6, 194 ± 24 days, ($n = 28$). The F1 and N2 cohorts exhibited a normal distribution.

vidual backcross mice at the two extremes of age at ESRF. Microsatellite markers were evaluated at approximately 20 cM intervals along each chromosome. We sought chromosomal segments exhibiting both excess 129 allele sharing in the five mice that reached ESRF earliest and excess B6 allele sharing in the five mice that reached ESRF latest. Because age at ESRF is a quantitative trait, the genome scan results were analyzed using Map Manager QT software.^{23,32} Three markers on chromosome 9 and one marker on chromosome 16 were identified as exhibiting significant linkage to QTLs that influence age at ESRF.

Figure 5. Immunohistochemical analysis of laminin $\alpha 2$ deposition in 129 and B6 *Col4a3*^{-/-} and +/- glomeruli. Laminin $\alpha 2$ was detected in the GBM at both 14 and 22 days in the 129 *Col4a3*^{-/-} kidney (arrows in A and D), but was only observed in the mesangium in the B6 *Col4a3*^{-/-} and control kidneys. This early deposition of $\alpha 2$ in the GBM may be related to the rapid onset and progression of disease. Scale bar, 25 μ m.

Figure 7. LOD scores for linkage of the markers tested on chromosomes 9 and 16 to QTLs influencing age at ESRF. The analysis incorporated data from 49 backcross mice. Schematic diagrams of the two chromosomes show the regions of linkage. As determined by the permutation test, critical threshold values for highly significant linkage were LOD = 3.26 for chromosome 9 and 3.1 for chromosome 16.

Because of the significance of QTL linkage to markers on chromosome 9 (LOD = 7.1) and 16 (LOD = 3.0), we performed additional analyses in other backcross mice. We genotyped 49 of the 51 backcross mice on chromosomes 9 and 16, and included an increased density of markers for chromosome 9. When QTL analysis was performed with these new data, the linkage remained highly significant on both chromosomes (Figure 7). Interval mapping using critical values obtained from the permutation test (2000 permutations, additive regression model)^{22,24} produced similar results (Figure 7). We conclude that the region of chromosome 9 between D9Mit4 (29 cM) and D9Mit12 (55 cM) and the region of chromosome 16 near D16Mit153 (56.8 cM) contain QTLs influencing the rate of progression of renal disease in *Col4a3* $-/-$ mice.

Discussion

Many questions regarding the progression of renal disease in human Alport syndrome still remain. Mutation of any one of the genes encoding the $\alpha 3$, $\alpha 4$, or $\alpha 5$ chains of type IV collagen is clearly the initiating event. However, the mechanism whereby this leads to the characteristic GBM thinning, thickening, and splitting is a mystery. Furthermore, how these GBM lesions lead to the complex changes in kidney architecture and physiology that are associated with progressive renal disease is also unknown.

The production of mouse models and the identification of naturally occurring dog models for Alport syndrome have permitted in depth physiological, molecular, and histological studies of disease initiation and progression^{12–14,18–20,28,29,33–37}. Here, we have used mouse genetics and molecular biology to explore roles for genes distinct from those encoding type IV collagen in the pathogenesis of Alport syndrome. Our motivation was the finding in our mouse model that genetic background has a striking influence on the rate of renal disease progression: 129 *Col4a3* $-/-$ mice reached ESRF at ~ 2 months of age, while B6 *Col4a3* $-/-$ mice routinely lived to

greater than 6 months of age. This is consistent with the fact that in humans with Alport syndrome, individuals with identical mutations can exhibit very different rates of progression to ESRF.^{15–17}

Quantitation of the extent of GBM splitting at 2 weeks of age in our mice showed that the 129 *Col4a3* $-/-$ GBM was more severely affected than was the B6 *Col4a3* $-/-$ GBM, which was not significantly different from controls. The simplest interpretation is that the QTLs act by influencing the integrity of the GBM in the absence of the normal collagen IV chains. However, the fact that significant GBM splitting was evident in the B6 *Col4a3* $-/-$ mice at ~ 6 weeks of age, in the absence of significant pathology or proteinuria, suggests that the observed differences in disease progression are also based on differential physiological responses to GBM splitting. Alternatively, the earlier splitting in the 129 *Col4a3* $-/-$ GBM could be responsible for inducing the rapid and robust physiological response, which is exemplified by the increased deposition of ectopic laminin $\alpha 2$ in the GBM at 2 to 3 weeks of age and the aggressive fibrosis and inflammation observed in the 129 *Col4a3* $-/-$ kidneys at young ages (< 7 weeks). The laminin $\alpha 2$ deposition in the young 129 *Col4a3* $-/-$ GBM may lead to immediate disruptions in podocyte homeostasis, early onset proteinuria, and subsequent glomerular and tubular damage. Together, these would be expected to lead to ESRF. A similar but slower process appears to occur in the B6 *Col4a3* $-/-$ mice.

We identified QTLs on chromosomes 9 and 16 as being involved in modifying this disease process. The regions of significant linkage to these QTLs contain hundreds of genes. We are currently attempting to narrow these intervals by isolating and testing additional recombinants. In addition, we are generating modified B6 *Col4a3* $-/-$ strains harboring 129 derived chromosomal segments from the regions linked to the QTLs, to determine whether these can influence rate of progression to ESRF in the absence of other 129 alleles. Once the intervals have been narrowed, the QTLs can be positionally cloned or known candidate genes in the region can be tested. With similar methods, we hope to determine whether the two chromosomal regions act independently to modify kidney disease. For example, one QTL may influence the stability of the molecularly aberrant GBM, perhaps in an age-dependent fashion, while the other QTL may influence physiological responses to glomerular damage. If true, then the former QTL might be relevant only to Alport syndrome, while the latter would be expected to modify the progression of other glomerulopathies as well.

Another genetic kidney disease which exhibits significant variation is polycystic kidney disease. Several groups have mapped QTLs that modify disease severity in mouse models of polycystic kidney disease.^{38–41} As with the QTLs we mapped, there are likely to be homologous genes in humans which are similarly involved in modifying severity of disease. Identifying and mapping QTLs are important steps in attempting to gain a better understanding of how modifier genes influence disease progression. With inevitable breakthroughs in gene ex-

pression profiling and an increased understanding of whole genomes on the horizon, it is expected that these QTLs can someday be understood at a molecular level. This knowledge will lead to novel therapies aimed at slowing progression of renal disease.

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