A mouse *Col4a4* mutation causing Alport glomerulosclerosis with abnormal collagen α3α4α5(IV) trimers

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A spontaneous mutation termed bilateral wasting kidneys (*bwk*) was identified in a colony of NONcNZO recombinant inbred mice. These mice exhibit a rapid increase of urinary albumin at an early age associated with glomerulosclerosis, interstitial nephritis, and tubular atrophy. The mutation was mapped to a location on chromosome 1 containing the *Col4a3* and *Col4a4* genes, for which mutations in the human orthologs cause the hereditary nephritis Alport syndrome. DNA sequencing identified a G-to-A mutation in the conserved GT splice donor of *Col4a4* intron 30, resulting in skipping of exon 30 but maintaining the mRNA reading frame. Protein analyses showed that mutant collagen α3α4α5(IV) trimers were secreted and incorporated into the glomerular basement membrane (GBM), but levels were low, and GBM lesions typical of Alport syndrome were observed. Moving the mutation into the more renal damage–prone DBA/2J and 129S1/SvImJ backgrounds revealed differences in albuminuria and its rate of increase, suggesting an interaction between the *Col4a4* mutation and modifier genes. This novel mouse model of Alport syndrome is the only one shown to accumulate abnormal collagen α3α4α5(IV) in the GBM, as also found in a subset of Alport patients. These mice will be valuable for testing potential therapies, for understanding abnormal collagen IV structure and assembly, and for gaining better insights into the mechanisms leading to Alport syndrome, and to the variability in the age of onset and associated phenotypes.

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Alport syndrome is a human hereditary glomerulonephritis that, in most cases, results in end-stage renal disease (ESRD).[1] Other clinical symptoms include high-tone sensorineural deafness and ocular defects affecting the lens and the fundus. Alport syndrome is the most common inherited glomerular disease leading to renal failure and is caused by mutations in any one of the genes encoding the α3, α4, or α5 chains of type IV collagen (*COL4A3*, *COL4A4*, and *COL4A5*, respectively).[2] α3α4α5(IV) heterotrimers are secreted by podocytes[3,4] and polymerize to form the major collagen IV network of the glomerular basement membrane (GBM).[5] *COL4A5* is located on the X chromosome (Chr) and is mutated in X-linked Alport syndrome, whereas mutations in *COL4A3* and *COL4A4*, which are located on human Chr 2, lead to autosomal-recessive Alport syndrome and, in rare cases, to autosomal-dominant Alport syndrome.[6]

All collagen IV α-chains have a large, central, triple helical collagenous domain with multiple interruptions, an NH2-terminal noncollagenous domain termed 7S, and a COOH-terminal noncollagenous domain termed NC1. The NC1 domain promotes both heterotrimerization of α-chains to form a protomer and association of two protomers to form a macromolecule containing six NC1 domains. These six NC1 domains constitute a collagenase-resistant structure called a hexamer. Hexamer formation, together with interactions among 7S domains from four different protomers, contribute to collagen IV network polymerization.[5]

As the GBM functions normally in Alport patients for several to many years, it is possible that blocking the events that trigger initiation of overt disease might arrest Alport syndrome in its prepathogenic state. Therefore, understanding disease initiation and progression and the factors that are involved in these processes could lead to therapeutic interventions.[7] Animal models can have a critical role in achieving this understanding.[8]

In the past two decades, knockout (KO) mice for *Col4a3*,[9,10] *Col4a4*,[11] *Col4a5*,[12] and both *Col4a3* and *Col4a4* together[13] have been generated and characterized. Studying these mice has led to an understanding of several important aspects of the disease and its progression, such as the α5/α6 switch, in which

the disease in *Col4a3* KO mice can be partially delayed by ectopic deposition of collagen α5α5α6(IV) protomers in the GBM; this feature is highly dependent on genetic background.[8] Another important finding is the presence of modifier genes that influence the GBM ultrastructure and mean age at renal failure in *Col4a3* KO mice.[14]

In 2002, a spontaneous mutant was identified in a colony of severely obese NONcNZO4/Lt (RCS-4) recombinant congenic mice.[15] The mutant was identified because of its leanness. Subsequent phenotyping showed chronic nephritis, and the mutation was named bilateral wasting kidneys (*bwk*). We now report mapping the *bwk* mutation to Chr 1 and identify *Col4a4* as the mutated gene. Immunohistochemical analyses revealed this mutant to be the first mouse model of Alport syndrome with detectable collagen α3α4α5(IV) in the GBM. However, because the collagen IV is abnormal, the typical GBM lesions observed in Alport syndrome are present, consistent with the observed progression to ESRD.

RESULTS

Origin of the *bwk* mutation

The *bwk* mutant phenotype, determined by breeding to be autosomal recessive, was observed in the NONcNZO4/Lt (RCS-4) recombinant congenic strain at generation N2F10. Mutants on this inbred genetic background began to appear leaner than NONcNZO4/Lt (RCS-4) mice 1–2 weeks after weaning and died between 8 and 10 weeks of age. At autopsy, the kidneys of affected mice appeared pale and pitted. Because of inbreeding depression, mice of this mutant subline were crossed one generation with NON/ShiLtJ (NON) mice; offspring of this cross were then crossed with N2F12 *bwk/bwk* cousins. Since then, the *bwk* mutation has been maintained homozygous on this inbred genetic background by sibling or cousin matings.

Characterization of *bwk* mutant mice

The *bwk* mutation causes ESRD associated with glomerulosclerosis, synechiae (adhesions of the glomerular tuft to Bowman's capsule), glomerular crescents, tubular protein casts, tubular atrophy, and tubulointerstitial nephritis as major histopathologic components (Figure 1 and data not shown). The expanded interstitium, which appears to include inflammatory cells, eventually replaces or impinges on many of the tubules (Figure 1). To determine whether the inflammatory phenotype might be caused by T or B cells, we crossed *bwk* mice with B6.129S7-*Rag1*^{tm1Mom}/J (*Rag1* KO) mice. The presence of a similar kidney phenotype in *bwk/bwk; Rag1*^{tm1Mom}/*Rag1*^{tm1Mom} mice (data not shown) showed that the inflammation was not due to exclusively T or B cells, which are absent in B6.129S7-*Rag1*^{tm1Mom}/J mice.[16] B6.129S7-*Rag1*^{tm1Mom}/J mice still have functional cells of the innate immune system; hence, the inflammatory cells in the double KO kidneys must be of 'innate immunity' type, as opposed to 'adaptive (T and B cells) immunity' type.

Consistent with the observed histopathology (Figure 1), *bwk/bwk* mice rapidly developed high albuminuria. A time course of albumin-to-creatinine ratios (ACR) showed an already slightly elevated ACR at 4 weeks compared with controls, which increased in the course of only 6 weeks to 3400 mg/g in both males and females (Figure 2, top panels).

Mapping of *bwk* to a region on Chr 1

An initial genome-wide scan of DNA markers in the progeny of a backcross between CAST/EiJ (CAST) and *bwk* allowed us to map the mutation to a 16-Mb region on Chr 1 between *D1Mit46* and *D1Mit488*. Subsequent fine mapping in an intercross with CAST narrowed the region to a 4.2-Mb interval (Figure 3a) containing 29 genes, including *Col4a3* and *Col4a4*. Because of their known roles in kidney disease in both mice and humans (Alport syndrome), these genes were deemed to be excellent candidates for harboring the *bwk* mutation.

A complementation test excludes *Col4a3* as a candidate gene

To directly test whether *bwk* is a mutation in *Col4a3*, we performed a complementation test in which *Col4a3*+/− females were mated to *bwk/bwk* males. This cross produced two distinct groups of mice: those with a *Col4a3* KO allele and a *bwk* allele ($n=15$), and those with one obligate *Col4a3* wild-type (WT) allele and a *bwk* allele ($n=5$). As *bwk* is recessive, mice in the first group were expected to develop albuminuria only if *bwk* resides in the *Col4a3* gene, whereas those in the second group were expected not to show any albuminuria. Neither group showed either elevated albuminuria or renal damage at any time point (data not shown), indicating that *bwk* could not be a mutation in *Col4a3*. This left *Col4a4* as the prime candidate gene.

Identification of a mutation in *Col4a4* and its effects on *Col4a4* mRNA and protein

Sequencing of *Col4a4* exons, intron splice sites, and the 3′-untranslated region revealed a G-to-A point mutation in the first base following the 3′-end of exon 30 (i.e., the first base of intron 30; Figure 3b). This mutation, which was not detected in any other strains, affects the GT consensus splice donor of intron 30 and should therefore prevent proper splicing of the mRNA.

To investigate the effect of the mutation on *Col4a4* mRNA, we performed reverse-transcription PCR using primers from exons 28 and 32 on whole kidney RNA. The primers (Figure 3c) generated the expected product from WT RNA but only a shorter product from *bwk* mutant RNA (Figure 3d). Sequencing of the short PCR product showed a direct splice from exon 29 to exon 31, indicating a clean skip of exon 30 (Figure 3c and e). Quantitative real-time reverse-transcription PCR using primers from *Col4a4* exons 3′ of exon 30 showed an average 55% reduction ($P<0.0006$ by Student's two-tailed *t*-test) in the level of transcript in mutant versus WT whole kidney RNA samples. We therefore suggest that the alternative splicing was not 100% efficient and that a large subset of mutant transcripts retained intron 30 and were degraded due to nonsense-mediated mRNA decay.[17]