Feasibility of Repairing Glomerular Basement Membrane Defects in Alport Syndrome

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ABSTRACT
Alport syndrome is a hereditary glomerular disease that leads to kidney failure. It is caused by mutations affecting one of three chains of the collagen α3α4α5(IV) heterotrimer, which forms the major collagen IV network of the glomerular basement membrane (GBM). In the absence of the α3α4α5(IV) network, the α1α1α2(IV) network substitutes, but it is insufficient to maintain normal kidney function. Inhibition of angiotensin-converting enzyme slows progression to kidney failure in patients with Alport syndrome but is not a cure. Restoration of the normal collagen α3α4α5(IV) network in the GBM, by either cell- or gene-based therapy, is an attractive and logical approach toward a cure, but whether or not the abnormal GBM can be repaired once it has formed and is functioning is unknown. Using a mouse model of Alport syndrome and an inducible transgene system, we found that secretion of α3α4α5(IV) heterotrimers by podocytes into a preformed, abnormal, filtering Alport GBM is effective at restoring the missing collagen IV network, slowing kidney disease progression, and extending life span. This proof-of-principle study demonstrates the plasticity of the mature GBM and validates the pursuit of therapeutic approaches aimed at normalizing the GBM to prolong kidney function.

The glomerular basement membrane (GBM) is the extracellular matrix component of the glomerular filtration barrier that lies between podocytes and endothelial cells. It is composed primarily of collagen α3α4α5(IV), laminin-521 (α5β2γ1), nidogen, and agrin.1 Alport syndrome is a hereditary nephritis caused by mutation of any one of the three GBM collagen IV chain genes (COL4A3, COL4A4, or COL4A5).2 The major molecular features usually observed in Alport GBM are (1) the absence of the collagen α3α4α5(IV) network, which is made by podocytes,3,4 and (2) compensation by increased levels of the α1α1α2(IV) network, which is normally a minor GBM component found on the subendothelial aspect.5 Compensation by α1α1α2(IV) keeps the GBM intact and functioning for years, but eventually GBM splitting and thickening imparts a basket-weave appearance.2 A progression of hematuria, proteinuria, and glomerulosclerosis leads to ESRD, usually by adolescence or young adulthood. Angiotensin-converting enzyme (ACE) inhibition slows progression to proteinuria and ESRD,6 but there is no cure for Alport syndrome.

Mouse and dog Alport syndrome models, which demonstrate GBM lesions and a disease progression similar to that in human Alport syndrome,7,8 have been instrumental for gaining insights into disease pathogenesis and for testing potential therapies. The efficacy of ACE inhibition in human patients was predicted by the ameliorative effects of treating Alport dogs and mice with ACE inhibitors.9,10 The ease with which Alport mice can be manipulated has made them the preferred choice for preclinical investigations since the first models were generated by mutating Col4a3.11,12

Several approaches have been used in attempts to cure Alport syndrome in mice, or to at least slow progression of the disease and lengthen lifespan. These include efforts to prevent the development of GBM lesions and glomerulosclerosis13–15; to attenuate the glomerular and tubulointerstitial inflammation that inevitably occurs16–18; and to transplant or infuse various types of stem cells, with or without irradiation, with the goal of replacing mutant podocytes with exogenous cells capable of expressing the missing collagen α3α4α5(IV).19–22 Despite claims of success at replacing podocytes and/or restoring GBM composition,19–21 these results stirred controversy23 and have not been convincingly replicated. The recent finding that injection of amniotic fluid stem cells is beneficial in Alport mice without any

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effect on GBM composition or glomerular cell identity suggests that exogenous cells do not need to home to glomeruli, cross the GBM, and replace or fuse with mutant podocytes (if such events are even possible) to be ameliorative. Nevertheless, should breakthroughs occur such that a large percentage of podocytes can be replaced or genetically altered to repair the mutation, perhaps via a renal perfusion approach developed for gene therapy, it is important to know whether or not the missing collagen α3α4α5(IV) network can be incorporated into an existing defective Alport GBM and restore function.

Previously a transgenic approach was used to restore the missing collagen α3 (IV) chain in Col4a3−/− mice using regulatory elements of the human COL4A3 gene to drive expression in a developmentally correct fashion. In contrast, here we used a very different transgenic approach to express COL4A3 on the Col4a3−/− background via a doxycycline-inducible system. A cDNA encoding full-length COL4A3 was placed under the control of the (TetO)7/CMV promoter (Figure 1A). Eight (TetO)7/CMV-Col4a3 (T-Col4a3) transgenic lines were generated, and each was bred to the Rosa26-reverse tetracycline transactivator (rtTA) line, induced with doxycycline during gestation, and tested for expression in multiple tissues with an anti-COL4A3 antibody. Several lines showed novel expression in intestine and spleen that was not present in controls (data not shown), and one line was deemed the best expressor and was used for subsequent studies.

Three different rtTA lines were used to drive expression of T-Col4a3 in Col4a3−/− mice. The extent and level of expression were assayed using antibodies to COL4A3 (Figure 1) and to the α3α4α5 (IV) hexamer (Figure 2), staining for which indicates α3α4α5(IV) heterotrimer assembly and network formation. The Nphs2 (podocin)−/−rtTA driver was tried first, but it drove expression of T-Col4a3 and of the (TetO)7/CMV-histone 2B-GFP fusion transgene (T-H2BGF) in only a subset of podocytes (not shown). We therefore generated a new podocyte driver using the 4.1 kb Nphs1 (nephrin) promoter trade-named rtTA-3G. Microinjection of the Nphs1-rtTA-3G transgene (hereafter called NEFTA) (Figure 1B) yielded 36 transgenic founders. Expression in a subset was assayed by breeding to T-H2BGFP and to a (TetO7)/CMV-human laminin α5 (T-hLAMA5) transgene and inducing with doxycycline. Several good expressors were identified by nuclear green fluorescent protein fluorescence in podocyte nuclei and continuous staining for human laminin α5 in the GBM (C). Immunostaining for Wilms tumor 1 identifies podocyte nuclei, all of which are GFP-positive (green/yellow in the merge in E). Kidney sections of 23-week-old Col4a3−/−; NEFTA; T-Col4a3 (G), and Col4a3−/−; Rosa26; rtTA; T-Col4a3 (H) mice fed doxycycline from birth were stained for COL4A3. Original magnifications: C, ×200; D and E, ×600; F–H, ×400. g, glomerulus.

Figure 1. NEFTA and Rosa26-rtTA drive expression of T-Col4a3 and deposition of transgene-derived protein into the GBM. (A) The (TetO)7/CMV-Col4a3 (T-Col4a3) transgene is induced by a doxycycline (Dox)-bound rtTA. (B) To achieve rtTA expression in podocytes, the nephrin (Nphs1) promoter was placed upstream of the rtTA-3G cDNA and the SV40 large T-antigen poly A signal (SV40pA) to generate the NEFTA transgene. (C–E) Kidney sections from a 10-day-old NEFTA; T-HISGFP; T-hLAMA5 mouse induced with doxycycline prenatally shows green fluorescent protein (GFP) fluorescence in podocyte nuclei and continuous staining for human laminin α5 in the GBM (C). Immunostaining for Wilms tumor 1 identifies podocyte nuclei, all of which are GFP-positive (green/yellow in the merge in E). (F–H) Kidney sections of 23-week-old Col4a3−/−; NEFTA; T-Col4a3 (G), and Col4a3−/−; Rosa26; rtTA; T-Col4a3 (H) mice fed doxycycline from birth were stained for COL4A3. Original magnifications: C, ×200; D and E, ×600; F–H, ×400. g, glomerulus.
which drove assembly of a collagen $\alpha_3\alpha_4\alpha_5$(IV) network (Figure 2Ac); with Rosa26-rtTA as driver, the GBM and many tubular basement membranes contained COL4A3, although GBM staining was weak compared with tubular basement membranes (Figure 1H), indicating that the Rosa26 promoter is probably weak in podocytes. For this reason, we focused on the Col4a3$^{-/-}$; NEFTA;T-Col4a3 mice for functional analyses.

As alluded to above, the data in Figure 2A show that the COL4A3 encoded by the T-Col4a3 transgene is capable of assembling with COL4A4 and COL4A5 to form $\alpha_3\alpha_4\alpha_5$(IV) heterotrimers, and that expression only from podocytes is sufficient; this finding agrees with data showing that podocytes, but not endothelial cells, make collagen $\alpha_3\alpha_4\alpha_5$(IV). Many GBM segments of Col4a3$^{-/-}$; NEFTA;T-Col4a3 mice induced at birth were ultrastructurally normal even at 23 weeks of age compared with Col4a3$^{-/-}$ littermates (Figure 2Db,c), and the mice were still alive with either no or low levels of albuminuria long after their Col4a3$^{-/-}$ littermates reached ESRD (Figure 3 and data not shown).

With proof that the transgene-encoded COL4A3 is functional and expressed as anticipated, we next induced its expression at weaning and continuously thereafter by feeding doxycycline to Col4a3$^{-/-}$;NEFTA;T-Col4a3 mice starting at 3 weeks, when all glomeruli have GBMs with abnormal composition (i.e., containing collagen $\alpha_1\alpha_1\alpha_2$(IV)) that are functioning in glomerular ultrafiltration. After induction, we collected urine every three to four weeks to monitor the integrity of the filtration barrier. SDS-PAGE analysis of urine showed that Alport mice with transgene induction beginning at 3 weeks (or earlier) remained nonalbuminuric for months, even at ages when their littermates without transgene expression had easily detectable urinary albumin (Figure 3). That albuminuria is a sign of progressive disease in Alport syndrome was reflected by the fact that mice with the higher levels of urinary albumin died of ESRD by 4–8 months of age, while most of their Col4a3$^{-/-}$ littermates expressing the Col4a3 transgene (beginning prenatally, at birth, or at 3 weeks) remained alive, either with no or low levels of urinary albumin (Figure 3). The survival data for these mice showed that no Alport mice without transgene expression survived beyond 32 weeks, whereas all transgene-expressing Alport mice survived beyond 32 weeks.
survived longer. As of this writing, two such mice induced with doxycycline at 3 weeks are still alive, though with some albuminuria at 15 and 16 months of age, respectively.

Immunostaining showed that the transgenic "rescued" Col4a3−/− mice did have a GBM with a collagen α3α4α5(IV) network, but some segments appeared thickened and had significant accumulation of collagen α1 and α2(IV) chains, although less than observed in non-transgenic Alport mice (Figure 2, A and B). Ultrastructural analysis showed some segmental GBM thickening in the rescued Alport mice, but in many cases podocyte foot process architecture was maintained (Figure 2D). Consistent with this, light microscopy showed that glomerulosclerosis and tubular protein cast formation were attenuated in rescued Alport mice, in agreement with the lack or low level of urinary albumin and longer lifespan compared with Alport mice not expressing the transgene (Figures 2C and 3).

These results show that once formed and functioning in filtration, the Alport GBM’s composition can still be changed and partially normalized by incorporation of the missing collagen α3α4α5(IV) network. We also found segmental GBM splitting and thickening even after postnatal collagen α3α4α5(IV) network assembly, yet kidney function decline was attenuated and lifespan was lengthened. Interestingly, scanning electron microscopy showed that decellularized Col4a3−/− GBM had a rough, blebby surface compared with the smooth surface of control GBM (Figure 3, D and E); the P21 rescued GBM exhibited some blebs, consistent with the transmission electron microscopy (Figure 2D), but an overall smooth surface (Figure 3F) that we speculate was conferred by incorporation of the collagen α3α4α5(IV) network. Together, these data suggest that a split, thickened GBM can be compatible with long-term kidney function, despite the ultrastructural abnormalities. This study validates the utility of therapeutic approaches aimed at normalizing the composition of the Alport GBM. Importantly, our results suggest that even imperfect molecular and structural repair of the GBM can significantly delay the onset of proteinuria and extend the time to ESRD.

**CONCISE METHODS**

**New Genetically-Altered Mice and Doxycycline Treatment**

All animal experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Washington University Animal Studies Committee.

The T-Col4a3 transgene contained the (TetO)7/CMV regulatory element (a gift from Jeffrey Whitsett, University of Cincinnati) driving a chimeric human/mouse Col4a3 cDNA, followed by the BGH polyadenylation signal. The full-length Col4a3 coding
sequence was constructed before the 5' end of
the mouse cDNA had become available, so a
human cDNA fragment was used to encode
the first 133 amino acids; it was fused to a
mouse cDNA encoding the remaining 1536
amino acids. T-CoHa3 transgenic mice were
identified by PCR using a pair of primers (5'-
ATCAAGGTTTCCAGGGACAAGGAT-
CAC-3' and 5'-CCCTGGAGGCCCCTCAGA-
GCCGGGCTCTTCC-3') that amplify a 247-bp
fragment of the cDNA and a 661-bp fragment
from endogenous Col4a3.

The NEFTA transgene contained the 4.1-kb
mouse nephrin promoter30 (a gift from Dr.
Susan Quagggin, Northwestern University)
driving rtTA-3G31 cDNA (Clontech), fol-
lowed by an SV40 polyadenylation signal.
NEFTA mice were identified by PCR using
a forward primer from the Nphs1 promoter
(5'–GAGACGGAGATGATGCACGGCCTC-
GGTCC-3') and a reverse primer from rtTA
(5'–ACATTTGCTCTTGAGCTCAGA-
CATGCGT–3') to generate an approximately
400-bp amplicon.

Transgenes were purified away from plas-
mid vector sequences and microinjected into
the pronucleus of B6CBAF2/J single-celled
embryos. Breeding of the transgenics to
generate the transgene-rescued Col4a3–/–
mice and their nonrescued and control litter-
mates resulted in a mixed genetic back-
ground containing C57BL/6J, CBA/J, and
129S1/SvImJ strain contributions. Trans-
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DISCLOSURES
None.

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