Searching for a treatment for Alport syndrome using mouse models

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Abstract
Alport syndrome (AS) is a hereditary nephritis caused by mutations in COL4A3, COL4A4 or COL4A5 encoding the type IV collagen α3, α4, and α5 chains, which are major components of the glomerular basement membrane. About 20 years have passed since COL4A3, COL4A4, and COL4A5 were identified and the first Alport mouse model was developed using a knockout approach. The phenotype of Alport mice is similar to that of Alport patients, including characteristic thickening and splitting of the glomerular basement membrane. Alport mice have been widely used to study the pathogenesis of this syndrome and to develop effective therapies. In this review, the newer therapies for AS, such as pharmacological interventions, genetic approaches and stem cell therapies, are discussed. Although some stem cell therapies have been demonstrated to slow the renal disease progression in Alport mice, these therapies demand continual refinement as research advances. In terms of the pharmacological drugs, angiotensin-converting enzyme inhibitors have been shown to be effective in Alport mice. Novel therapies that can provide a better outcome or lead to a cure are still awaited.

Key words: Alport syndrome; Angiotensin-converting enzyme; Genetic; Hereditary nephritis; Pharmacological; Renal injury; Stem cell therapy

Core tip: There is currently no curative treatment for Alport syndrome, a progressive hereditary nephritis. However, many drugs have been demonstrated to slow the progression of renal injury in Alport mouse models. Alport mice treated with vasopeptidase inhibitors or angiotensin-converting enzyme inhibitors showed a more than two-fold longer survival than untreated Alport mice. A human clinical trial of an angiotensin-converting enzyme inhibitor is currently in progress. Genetic approaches have been used to elucidate the pathogenesis of this progressive renal disease. Stem cell therapies were also attempted, with some beneficial effects; however, they need to be improved before being tested in clinical trials.
disease: X-linked and autosomal. The X-linked type of AS is caused by mutations in COL4A3[4], while the autosomal type of AS is caused by mutations in COL4A3 or COL4A4[5,6]. COL4A3, COL4A4 and COL4A5 encode the type IV collagen α3, α4 and α5 chains, respectively. Since the type IV collagen α3, α4 and α5 chains are major structural components of the GBM, AS is a type IV collagen disease.

The purpose of this review is to summarize the current knowledge that has been obtained using mouse models of Alport syndrome.

### PATHOGENESIS

At the molecular level, there are only three triple-helical protomers, α1-α1, α2, α3-α4, α5 and α5-α5, α6, in type IV collagens[7]. The non-collagenous domain (NC1) at the carboxyl terminus of these protomers joins them to each other to make the superstructure of the GBM. The α1/α1, α2, α1/α2, α5/α6 and α3/α4/α5 heterohexamers were identified by digesting the NC1 hexamer from human glomeruli with bacterial collagenase[8]. Interestingly, the α3/α4/α5 heterohexamer consists of one α4-α4 homodimer and two α3-α5 heterodimers, while the α1/α1/α2 heterohexamer consists of two α1-α1 homodimers and one α2-α2 homodimer, and the α1/α2/α5/α6 heterohexamer consists of two α1-α5 heterodimers and one α2-α6 heterodimer[9]. The α3 (IV) and α4 (IV) chains have to accompany the α5 (IV) chain, and the α3/α4/α5 heterohexamer consists of compositions of (α3)2(α4)(α5). NC1 domains were also demonstrated to contain recognition sequences to form α1-α1, α2 (IV) and α3-α4, α5 (IV) networks[10].

There is a developmental switch from α1 and α2 (IV) chains to α3, α4 and α5 (IV) chains; the GBM from capillary loop stage contains α3, α4 and α5 (IV) chains, as well as α1 and α2 (IV) chains, while the GBM at the comma- and S-shaped stages contains only α1 and α2 (IV) chains[11,12]. In mature glomeruli, the GBM is mainly composed of α3, α4, and α5 (IV) chains. While only the distal tubular basement membranes (TBMs) are positive for the α3, α4 and α5 (IV) chains in humans, nearly the full range of TBMs in the mouse are positive for the α3, α4 and α5 (IV) chains[13].

GBM in X-linked AS patients consists of only α1 and α2 (IV) chains because the developmental switch does not occur[14]. The loss of the α5 (IV) chain leads to the loss of all three chains (α3, α4 and α5 (IV) chains) in the GBM because of the defective assembly of triple-helical α3, α4, α5 (IV) protomers[15]. This abnormal GBM in X-linked AS patients is more susceptible to proteolysis by bacterial collagenase, cathepsin B, cathepsin G and Pseudomonas elastase than that in normal humans[16], because the collagenous domain of α1-α1, α2 (IV) protomers contains fewer disulfide cross-links than do α3-α4, α5 (IV) protomers[17].

Interestingly, AS patients with 5’ glycine mutations have a later onset of end-stage renal failure than those with 3’ glycine mutations, which is compatible with the fact that type IV collagen assembly starts from the NCI domain at the carboxyl terminus[12,13].

By generating two hybrid kidneys that contained wild endothelial cells and COL4A3 -/-/podocytes or COL4A3 -/-/endothelial cells and wild podocytes, type IV collagen α3, α4 and α5 chains proved to be originally produced specifically by podocytes in the kidney[18], thus suggesting that AS is podocyte-associated disease.

### MOUSE MODELS OF ALPORT SYNDROME

There were two COL4A3 knockout models reported in 1996. One model was generated by cloning a neomycin cassette into exon 48 of COL4A3[19]. The other model was generated by deleting three exons between exons 48 and 50 of COL4A3[20]. Both models aimed to disrupt exons in the NCI domain, and the resulting phenotypes resembled those of autosomal recessive AS in human. The COL4DELTA3-4 model, which has a large deletion between exon 2 of COL4A3 and exon 12 of COL4A4, was also reported[21]. This mouse model was found because of the observation that there was unexpected renal disease in a transgenic line, and this model had a more severe type of AS than the above COL4A3 knockout models, because the expression of COL4A3 and COL4A4 mRNAs were not detected due to a lack of the intergenic region of COL4A3-COL4A4. A new COL4A4 mouse model, which has a splice site mutation and skips exon 30 of Col4a4, was also recently reported[22]. Since this mutation does not cause a frame shift, this mouse model retains a mutant α4 (IV) chain in the GBM and represents a good new AS model.

Regarding the X-linked type, a COL4A5 knockout model was generated by making a nonsense mutation in exon 1 of COL4A5, and this has made the analysis of female carriers easier[23]. These five mouse models are summarized in Table 1.

| Gene          | Mutation | Ref. |
|---------------|----------|------|
| ARAS          |          |      |
| COL4A3       | exon 48  | [15] |
| COL4A3       | exon 48-50 | [16] |
| COL4A3-COL4A4| COL4A3 exon 2-COL4A4 exon 12 | [17] |
| COL4A4       | exon 30  | [18] |
| XLAS         |          |      |
| COL4A5       | exon 1   | [19] |

ARAS: Autosomal recessive Alport syndrome; XLAS: X-linked Alport syndrome.
ACE: Angiotensin converting enzyme; ARB: Angiotensin-II receptor blocker; CCR1: Chemokine (CC motif) receptor 1; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A; TNF: Tumor necrosis factor.

C57BL/6J background.[28] A linkage analysis of quantitative trait loci identified three markers on chromosome 9 and one marker on chromosome 16 that were suggested to be modifier genes. In this regard, it is important to use appropriate control littermates for all experiments. Although the 129 genetic background is good enough to assess the efficacy of new therapies in AS, the C57 genetic background might be better for assessing the long-term effects of new therapies.

The big difference between COL4A3-/- mice and COL4A5-/- mice is the presence of the α5 (IV) chain in the GBM of COL4A3-/- mice.[21] Of note, the expression level of the α5 (IV) chain is more prominent in mice with a C57 genetic background than in those with a 129 genetic background.[20] To assess the efficacy of regeneration therapy in COL4A3-/- mice, it is recommended that the α3 and α4 (IV) chains, not the α5 (IV) chain, should be used.

### PHARMACOLOGICAL INTERVENTIONS

A vasopeptidase inhibitor, AVE7688, extended the lifespan of COL4A3-/- mice dramatically, and it is the most effective drug against COL4A3-/- mice identified so far.[22] The various drugs that have shown efficacy in treating COL4A3-/- mice are summarized in Table 2.

An angiotensin-converting enzyme (ACE) inhibitor, Ramipril, was demonstrated to be effective for treating COL4A3-/- mice.[23] Notably, early initiation of ACE inhibitor treatment was associated with a longer survival time, and this indicated that the ACE inhibitor had a renoprotective effect in the COL4A3-/- mice, regardless of its impact on the blood pressure.

Moreover, Gross et al.[24] compared the antifibrotic effects between an ACE inhibitor and an angiotensin receptor blocker (ARB), which was also known to be an angiotensin receptor 1 antagonist. Although both drugs prolonged the survival of COL4A3-/- mice, the ACE inhibitor was much more effective than the ARB. Treatment with an ACE inhibitor reduced the transforming growth factor-beta 1 (TGFB1) and connective tissue growth factor (CTGF) levels more effectively than did treatment with an ARB, which might explain the different effects between ACE inhibitors and ARBs, because TGFB1 was demonstrated to be associated with renal disease progression in COL4A3-/- mice.[25]

A 3-hydroxy-3-methylglutaryl-coenzyme (HMG-CoA) reductase inhibitor, which was originally used for the treatment of hypercholesterolemia, showed an antifibrotic effect in COL4A3-/- mice, because it prolonged the survival by inhibiting the activation of fibroblast markers.[26] Interestingly, late initiation of treatment with the HMG-CoA reductase inhibitor at week 7 prolonged the survival of the mice from 71.3 to 90.5 d, while late initiation of ACE inhibitor treatment did not.[25]

A chemokine receptor 1 antagonist, BX471, prolonged the survival of COL4A3-/- mice by preventing interstitial macrophage recruitment.[27] That study showed the involvement of chemokines in the renal fibrosis of COL4A3-/- mice. However, Ccl2 blockade did not prolong the survival of COL4A3-/- mice even though it reduced the number of renal macrophages.[20]

A tumor necrosis factor alpha antagonist prolonged the survival of COL4A3-/- mice by decreasing podocyte apoptosis.[20] Aliskiren, a direct renin inhibitor, prolonged the survival of COL4A3-/- mice by 18% by downregulating both TGFB1 and CTGF in the kidney.[30] The combination of paricalcitol with an ACE inhibitor led to longer survival than the combination of calcitriol with the ACE inhibitor, which indicated that the different analogs of the active form of vitamin D exert different effects.[31]

A matrix metalloproteinase (MMP) -2, -3, and -9 inhibitor cocktail prolonged the survival of COL4A3-/- mice if it was administered before the onset of proteinuria.[30] In contrast, late administration of the inhibitor cocktail after the onset of proteinuria aggravated the renal disease of COL4A3-/- mice, which was associated with increased interstitial fibrosis. This dual effect might explain why MMPs played a pathogenic role in the early stage, although they played a protective role in the late stage of disease in COL4A3-/- mice.[32] MMP-12, also known as macrophage metalloelastase, was upregulated in the podocytes of Alport mice, and a MMP inhibitor, MMI270, which blocks MMP-2, -3, -9, -12 and -14, prolonged the survival of COL4A3-/- mice from eight to 10 wk, while treatment with a MMP inhibitor that blocked MMP-2, -3 and -9 did not.[33] The authors of that study also showed that a CC chemokine receptor 2 antagonist, propagermanium, also prolonged the survival of COL4A3-/- mice from eight to 11 wk.

At present, an ACE inhibitor has been reported to be the most effective treatment in humans.[14] A vasopeptidase inhibitor might be considered as the next candidate, since this drug led to the longest survival in COL4A3-/- mice (Table 2).

### GENETIC APPROACHES

TGFB1 is involved in the progression of renal disease in COL4A3-/- mice.[30] TGFB1 was found to be sig-

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**Table 2  The efficacy of pharmacological drugs in COL4A3-/- mice**

| Drug                          | Survival (d) | Efficacy |
|-------------------------------|--------------|----------|
| Vasopeptidase inhibitor[29]   | 172          | (+++)    |
| ACE inhibitor[29]             | 150          | (+++)    |
| ARB[26]                       | 98           | (+)      |
| HMG-CoA reductase inhibitor[26]| 91           | (+)      |
| CCR1 inhibitor[22]            | 86           | (+)      |
| TNF-alpha antagonist[26]      | 81           | (+)      |
| Renin inhibitor[28]           | 78           | (*)      |
| Vitamin D analog[20]          | 75           | (*)      |
| Untreated (129SvJ background) | 71           |          |
significantly upregulated after the onset of proteinuria. TGF-β1 and integrin α1β1 were found to affect distinct pathways in the pathogenesis of COL4A3 -/- mice. While TGF-β1 inhibition prevented the thickening of the GBM, the deletion of integrin α1β1 diminished the foot process effacement of podocytes. Treatment with a combination of these approaches prolonged the survival of Alport mice. Recently, the same group showed that integrin q1 deletion in COL4A3 -/- mice decreased the mesangial invasion into the capillary loops of glomeruli. Integrin α2 deletion in COL4A3 -/- mice prolonged the survival by 20% on a C57Bl6 background.

The deletion of discoidin domain receptor 1 (DDR1) in COL4A3 -/- mice prolonged the survival from 64.3 to 94.2 days. Since DDR1 is expressed in podocytes, these results again showed the importance of podocyte involvement in the pathogenesis of AS.

Uterine sensitization-associated gene-1 (USAG-1) deletion in COL4A3 -/- mice improved the renal phenotype and improved the survival. This result was compatible with the finding that recombiant human bone morphogenetic protein-7 (BMP-7) had a protective effect in COL4A3 -/- mice, because USAG-1 is known to counteract BMP-7 and is normally expressed in the distal tubules of the kidney. Interestingly, they found that USAG-1 was also expressed in the macula densa, and showed the possibility of crosstalk between the macula densa and extraglomerular mesangial cells.

Although MMPs had been thought to be involved in the damage to the GBM in COL4A3 -/- mice, MMP-9 deletion did not affect the progression of renal disease in these mice. Three MMPs, MMP-2, -3, and -9, were genetically ablated in COL4A3 -/- mice, and compensatory upregulation was shown among these MMPs. Therefore, broad-spectrum MMP inhibition is likely required for any effects associated with the MMPs.

A mouse line which had a yeast artificial chromosome including COL4A3 and COL4A4 was generated, and this transgene could rescue the phenotype of COL4A3 -/- mice. Although the expression level of the COL4A3 and COL4A4 transgenes were about 20% of the levels of COL4A3 and COL4A4 in a wild type mouse, the human α3 and α4 (IV) chains could assemble with the mouse α5 (IV) chain. This finding is very interesting, because the amino acid sequence homology of the α3 and α4 (IV) chains between the human and mouse, which are 79% and 78%, respectively, still allows for the formation of triple-helical α3α4α5 (IV) protomers.

The expression of an inducible human/mouse chimeric COL4A3 transgene after birth prolonged the lifespan of COL4A3 -/- mice by expressing α3, α4 and α5 (IV) chains in the GBM. Notably, expression of the inducible transgene after three weeks of age could still rescue the phenotype of COL4A3 -/- mice, and the α3α4α5 (IV) protomers could integrate into the damaged GBM that was comprised by mainly α1α1α2 network.

STEM CELL THERAPIES

There have been two reports that showed the efficacy of wild-type bone marrow transplantation (BMT) against the renal injury in COL4A3 -/- mice. Prodrumidi et al. reported that the blood urea nitrogen (BUN) and serum creatinine (Cr) levels were significantly improved in COL4A3 -/- mice that received wild-type (WT) bone marrow compared to those that received COL4A3 knockout (KO) mouse bone marrow (Table 3). The renal histopathology showed significant improvement of the glomerular injury and tubulointerstitial fibrosis in the WT to KO transplanted mice than in the KO to KO transplanted mice. Moreover, the α3 (IV) chain could be detected partially by immunofluorescence, but not in a Western blot analysis. Sugimoto et al. reported similar results (Table 3). They also showed that the BUN, Cr, and renal histopathology were significantly improved in the COL4A3 -/- mice that received 21-wk WT bone marrow than did the mice that received KO mouse bone marrow. An immunofluorescence study showed patchy staining of the α3 (IV) chain in the GBM of WT to KO transplanted mice. These two reports shared a common finding that BMT after irradiation from WT to COL4A3 -/- mice dramatically improved the renal injury even though the expression level of the α3 (IV) chain was very low. Neither group examined the survival after BMT as an absolute evaluation marker, so it is unclear whether the BMT could prolong the survival of the mice.

We also reported the results of BMT after irradiation in COL4A3 -/- mice. In contrast to the previous two reports, the BUN, Cr, renal histopathology and survival were significantly improved in both WT to KO and KO to KO mice compared to the untreated KO mice, but there were no significant differences between the WT to KO and KO to KO mice (Table 3). The de novo expression of the α3 (IV) chain could not be detected in the WT to KO mice by immunofluorescence and Western blot analyses. However, wild type COL4A3 mRNA could be identified in the WT to KO, not in the KO to KO, mice by reverse transcription polymerase chain reaction. In fact, fewer than 1% of the podocytes were donor-derived when BMT was performed in a mouse model of mesangial sclerosis. Since KO bone marrow had similar effects as WT bone marrow in the COL4A3 -/- mice, the effect of irradiation itself was examined at sublethal doses. Surprisingly, a sublethal dose of irradiation without subsequent BMT improved the survival of COL4A3 -/- mice. This suggests that the renal injury of COL4A3 -/- mice was improved by the irradiation, not by the BMT. The mechanism by which irradiation improved the survival remains to be clarified, since radiation exposure induces numerous effects.

Another group reported that multipotent mesenchymal stromal cells (MSCs) could not prolong the survival of COL4A3 -/- mice although they improved the interstitial fibrosis by producing vascular endothelial growth factors.
factor⁴⁹. MSCs in the kidney that transdifferentiated into renal cells could not be identified.

However, wild-type bone marrow cells were also shown to prolong the survival of unirradiated COL4A3 -/- mice³². Surprisingly, wild-type blood transfusion, as well as the injection of undifferentiated mouse embryonic stem cells, improved the renal function of unirradiated COL4A3 -/- mice, with the appearance of the de novo expression of the α3 (IV) chain in the GBM. Although these data confirmed that cell-based therapies could be effective, there was a large discrepancy between the expression patterns of the α3 and α5 (IV) chains: the expression of the α3 (IV) chain was patchy, while that of the α5 (IV) chain was linear. There might be an unknown association between the small amount of de novo α3 (IV) chains and the renal improvement of COL4A3 -/- mice that received WT bone marrow. Of interest, a single injection of amniotic fluid stem cells was recently shown to prolong the survival of COL4A5 -/- mice without de novo expression of α5 (IV) chains⁹³.

### CONCLUSION

At present, there is no treatment available that can cure AS, and symptomatic renal protective therapies are currently the mainstay of treatment for AS. During the search for a treatment in Alport mice, ACE inhibitors were found to be the most promising therapeutic drugs as first-line therapy. This is a good example of the benefits of mouse studies, because this has led to a double-blind, randomized, placebo-controlled, multicenter EARLY PRO-TECT Alport trial⁴². BMT therapy is also promising, but is still controversial, given the fact that BMT itself is invasive⁴³. Other therapeutic agents that have been proven effective in AS mouse models should be considered as the next options for clinical trials in patients with AS.

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