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Prospective collagen IV$^{\alpha 345}$ therapies for Alport syndrome

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Abstract

Purpose of review—In Alport syndrome, over 1,700 genetic variants in the *COL4A3*, *COL4A4*, and *COL4A5* genes cause the absence or malfunctioning of the collagen IV$^{\alpha 345}$ scaffold – an essential component of the glomerular basement membrane (GBM). Therapies are limited to treatment with ACE inhibitors to slow progression of the disease. Here, we review recent progress in therapy development to replace the scaffold or restore its function.

Recent findings—Multiple approaches emerged recently for development of therapies that target different stages of production and assembly of the collagen IV$^{\alpha 345}$ scaffold in the GBM. These approaches are based on 1) recent advances in technologies allowing to decipher pathogenic mechanisms that underlie scaffold assembly and dysfunction, 2) development of DNA editing tools for gene therapy, 3) RNA splicing interference, and 4) control of mRNA translation.

Summary—There is a growing confidence that these approaches will ultimately provide cure for Alport patients. Development of therapy will be accelerated by studies that provide a deeper understanding of mechanisms that underlie folding, assembly, and function of the collagen IV$^{\alpha 345}$ scaffold.

Keywords

gene editing; exon skipping; stop codon readthrough; chemical and pharmacological chaperones; protein replacement

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Conflicts of interest

None.

INTRODUCTION

In Alport syndrome, nearly two-thousand variants in the ***COL4A3, COL4A4*** and ***COL4A5* genes** cause a broad range of glomerulopathies affecting the function of the glomerular basement membrane (GBM) in millions of people worldwide (Fig. 1) [1••, 2••, 3, 4••]. The genes encode the assembly of the collagen IV$^{\alpha345}$ scaffold, the major GBM constituent, composed of α345 triple-helical protomers made of α3, α4 and α5 chains [4••, 5•] (Fig. 1B). The variants lead to a broad array of clinical manifestations, ranging from microscopic hematuria to end stage renal disease. Current therapy is limited to treatment with ACE inhibitors to slow progression [6]. Collagen IV$^{\alpha345}$ therapies aiming to repair the glomerular filter [7, 8] are in urgent need.

Development of an Alport cure hinges on answering the question of "**How the plethora of pathogenic variants, encoding defective α3, α4, and α5 chains, causes GBM dysfunction?"** Whereas certain variants result in absence of the collagen IV$^{\alpha345}$ in the GBM, others produce and deposit a scaffold with a defective structure (Fig. 1B). The answer requires basic knowledge of the pathobiology of collagen IV$^{\alpha345}$ in relation to its structure, assembly, stability, function, and dysfunction [9]. Here, we present recent breakthroughs that serve as groundwork for therapy development and summarize prospective therapies.

BREAKTHROUGHS IN THE PATHOBIOLOGY OF THE COLLAGEN IV^{A345} SCAFFOLD

A knock-in mouse model and the α345 hexamer as a focal point in GBM function

Several mouse models of Alport syndrome have been studied over the last 25 years. These include knockout mice for Col4a3, Col4a4 and Col4a5 [10–14], harboring variants that eliminate the α345 scaffold from the GBM function, as well as two models that incorporate defective scaffolds [15, 16]. However, the phenotypes in the latter mouse models were attributed to reduced amounts or proteolytic cleavage of α345. Collectively, these studies have verified the essentiality of the α345 scaffold for GBM function, but how the 1700 variants cause GBM dysfunction remains an enigma.

Our recent studies have focused on how the 176 known variants in NC1 domains cause GBM dysfunction (Fig. 1B). These domains have been extensively studied and found to function as key recognition modules in the assembly of the α345 scaffold [17, 18]. Two variants are distinct and encode an 8-residue appendage, called Zurich (Z-) variant, attached to the C-terminus of the α3NC1 domain, and a 74-residue appendage, attached to the α5NC1 domain (Fig. 1B). We developed a knock-in mouse model harboring the Z-appendage [4••]. This variant incorporated into the α345 scaffold, cause GBM ultrastructural abnormalities and proteinuria (Fig. 2A, middle panel), phenocopying a wide spectrum of glomerular phenotypes in human AS. The Z-appendage pathogenicity, in both homozygous and heterozygous mice, pinpointed the α345 hexamer as a critical structure of the collagen IV scaffold that governs morphology and ultrastructure of the GBM, features that enabled permselective ultrafiltration.

Z-mouse model provides a unique strategy to acquire seminal information about the pathobiology of the collagen IV$^{\alpha345}$ scaffold at the molecular, cellular, and tissue levels. In particular, the model provides a trackable strategy to discover functions of the α345 hexamer at specific sites and pathogenic mechanisms that are relevant to other hypomorph variants in the NC1 domains. Moreover, this model provides a strategy for testing potential pharmacological chaperones and gene editing therapies.

Single-chain NC1 trimer technology and crystal structure of α345 hexamer

We developed a groundbreaking technology for the synthesis of recombinant single-chain NC1 trimers to explore how the "Zurich" and other variants causes GBM dysfunction [20••, 21••]. Importantly, this technology provided the pivotal advance to solve the crystal structure of the α345 hexamer [21••], a key assembly and connection module within the collagen IV$^{\alpha345}$ scaffold. The hexamer structure (Fig. 2B) revealed a ring of twelve chloride ions at the trimer-trimer interface, analogous to the collagen IV$^{\alpha121}$ scaffold [20••, 22••]. The surface of the α345 hexamer is marked by multiple pores and crevices that are potentially accessible to small molecules. Over 170 variants occur within the α3, α4 and α5 NC1 domains, including the Zurich variant of α3NC1, a C-terminal 8-amino acid appendage (Fig. 1B). Most variants are truncating and missense, both of which can cause total loss of hexamer in the GBM and renal dysfunction. Whereas, the Z-appendage, inserted at the apex of the hexamer, allowed the incorporation of the variant into the GBM causing renal dysfunction. The pathogenicity of the Z-appendage (*vide supra*) revealed that the α345 hexamer is a critical domain for GBM structure and function.

Furthermore, our single-chain trimer technology, coupled with the α345 hexamer structure, provides a platform to decipher how the multiple variants in the hexamer cause GBM dysfunction. The Z-appendage and various missense variants (hypomorphs) can alter the hexamer conformation. These sites, along with the pores and crevices at the hexamer surface are prospective targets for pharmacological chaperone therapy to restore native function.

Miniprotomer recombinant technology and assembly of the α345 hexamer

We explored the α345 hexamer as a step to decipher mechanisms that underlie the assembly of the collagen IV$^{\alpha345}$ scaffold. The α3, α4 and α5 NC1 monomers are encoded with structural determinants that govern both chain selection in protomer assembly and in protomer oligomerization into scaffold (Fig. 3A). Chloride ions signal protomer assembly and stabilize the α345 hexamer - an essential step in the oligomerization of protomers [23••]. Bromide ions function as cofactors in the formation of sulfilimine crosslinks, which reinforce the stability of the α345 hexamer [24••] (Figs. 2B, 3A). Furthermore, the loop-crevice-loop (LCL) bioactive sites on the hexamer surface exhibit conformational plasticity, supporting hexamer functions that include cell signaling and organizing macromolecular complexes in the assembly of the GBM, as summarized in Fig. 2B. We validated these *in vitro* results by expressing a mini version of the full-length α345 protomer in cell culture [23••], which undergoes the same assembly mechanism triggered by chloride ions (Fig. 3B). The miniprotomers technology is also a step towards production of recombinant full-length protomers suitable for protein replacement therapy [9].

PROSPECTIVE THERAPEUTIC APPROACHES

Premature termination codon readthrough therapy

Truncated α3, α4, and α5 chains lacking an intact NC1 domain due to a premature termination codon (PTC) cannot assemble into heterotrimers and lead to the absence of the α345 scaffold in the GBM. Achieving full-length protein expression via readthrough truncating nonsense mutations is a potential therapy for AS. Small molecule-based PTC readthrough (PTC-RT) therapy has been well studied in other genetic diseases.

Recently, 49 individual nonsense *COL4A5* mutations found in AS patients were screened for PTC-RT in cell culture experiments [25••]. 11 mutations were found susceptible to PTC-RT induced by G418, which is known to have high readthrough activity in a class of aminoglycoside derivatives. These results suggest that PTC-RT therapy is a feasible approach for some patients with AS.

Exon skipping therapy

The *COL4A5* gene consists of 51 exons with 44 of these exons belonging to the collagenous domain (exons 3–46). Among these 44 exons, 35 exons have nucleotide numbers that are a multiple of 3, which is a requirement not to have a frame shift upon exon skipping. When patients have truncating mutations in one of these exons, exon skipping can shift the truncation to a non-truncating mutation, that is, in-frame deletion mutations that can delay the development of ESRD in AS.

Recently, truncating variants in exon 21 of the *COL4A5* gene were targeted by exon skipping, which enabled trimer formation, leading to clinical and pathological improvements including deposition of the α5 chain into the glomerular and tubular basement membrane [26••]. In addition, the survival period was clearly prolonged in the antisense oligonucleotide treated mice group. This data suggests that exon skipping may represent a promising therapeutic approach for treating severe AS cases.

Gene therapy

Alport syndrome caused by a pathogenic variant in one or more of three genes: *COL4A3*, *COL4A4*, or *COL4A5*. Progress in development of gene therapy for Alport syndrome is currently limited to early testing. Nevertheless, several key achievements are foundational and demonstrate potential for this approach.

An artificial chromosome transgenic line of mice carrying the human COL4A3-COL4A4 locus was generated [27•]. In the kidney, when expressed onto a Col4a3$^{(-/-)}$ background, the human α3 chain restored the expression of and co-assembled with the mouse α4 and α5 chains. The co-assembly of the human and mouse chains into a hybrid network in the GBM restored a functional GBM and rescued the Alport phenotype.

Lin et al. [28] demonstrated effective restoration of the missing α345 collagen IV network in a mouse model of Alport syndrome using an inducible transgene system. This proof-of-principle study demonstrated the plasticity of the mature GBM and validated the pursuit of therapeutic approaches aimed at normalizing the GBM to prolong kidney function.

Another proof-of-concept study used podocyte-lineage cells from two AS patients to test whether gene therapy can correct mutations in the *COL4A3* and *COL4A5* genes. Researchers used the CRISPR/Cas9 system to target the faulty genes in kidney cells isolated from urine [29••]. They achieved reversion of variants greater than 40% with undesired insertions/deletions lower than 15%.

Although still in its infancy, gene therapy may one day be part of clinical trials assessing its potential in Alport syndrome patients.

Pharmacological chaperones

The assembly of the α345 scaffold requires hexamer formation of the α3, α4, and α5 NC1 domains, suggesting that mutations within NC1 in Alport syndrome may disrupt this assembly [18]. The atomic structure of the α345 hexamer revealed multiple pores, crevices, and inner cavities [21••] (Fig. 2B), which may not be functionally important but are potential targets for small molecules (SMOLs) that can affect folding, assembly and stability of the quaternary structure without detrimental effect on the function. Trimerization of α345 collagen IV protomer is a culprit for multiple variants causing AS. Omachi et al. [30•] developed a system to identify α345 collagen IV protomer trimerization in cells. They demonstrated that chemical chaperones rescue the trimer formation ability of clinically reported α5 variants.

Pharmacological chaperones (pharmacoperones) initially defined as molecules correcting misfolding caused by pathogenic variants have recently been extended with molecules stabilizing final structure of a protein as they are also able to facilitate folding of aberrant variants and in addition slow down degradation (turnover rate) of the folded proteins [31•, 32]. The best class, also referred to as “second-generation” pharmacoperones, targets binding pockets that do not affect the function of the protein [31•]. This new class of molecules can potentially correct and stabilize the α345 hexamer, without affecting its function, and can be developed into a new therapy for Alport patients. Our single-chain trimer technology provides a strategy for high-throughput screening to identify potential drugs for Alport syndrome.

Protein replacement therapy

Protein replacement therapy remains an attractive and unexplored opportunity for Alport patients. Delivery of full-length recombinant laminin molecules to the GBM [33•] set up a possibility that a full-length or mini-α345 protomer can be delivered therapeutically to the glomerulus, where it can oligomerize forming the α345 scaffold in the GBM. Recent advancements in producing the α345 NC1 single-chain trimer [21••] and a miniature version of α345 collagen IV protomer [23••], named miniprotomer (Fig. 3B), provide tools to begin development of protein replacement therapy. Both trimers and miniprotomers may harbor sufficient activities to have a therapeutic effect. Further steps will include development and testing of full-length α345 protomers.

CONCLUSION

The discoveries of the α3, α4 and α5 chains of collagen IV set the foundation for unraveling the mystery of Alport syndrome and development of therapy [34•, 35•, 36•, 37•, 38•, 39•, 40•]. Over the preceding 30 years, sequences of cognate genes COL4A3, COL4A4 and COL4A5 were determined and shown to harbor nearly two thousand pathogenetic variants that cause GBM dysfunction [1••, 2••, 3, 4••]. In parallel, advances were made in understanding the structure and assembly of chains into a complex collagen IV$^{\alpha345}$ scaffold, as summarized in [5•, 39•, 41•]. Collectively, this knowledge has provided fundamental underpinnings for prospective therapeutic approaches that are summarized in Fig. 4.

Attractive approaches include protein replacement and pharmacological chaperones because the GBM is directly accessible to protein delivery *via* the bloodstream. Recent advances in protein design set up a possibility that a full-length or mini-α345 protomer can be delivered therapeutically to the glomerulus, where it can oligomerize forming the collagen IV$^{\alpha345}$ scaffold in the GBM. Moreover, because a significant number of hypomorph variants occurs within the α345 hexamer, the multiple pores, crevices, and cavities of the hexamer surface can be potential targets for pharmacological chaperones that correct misfolding or stabilize the protein from proteolytic degradation.

Yet only a paucity of information is known about how the chains assemble into a collagen IV$^{\alpha345}$ scaffold in the context of the complex supramolecular structure of the GBM. Moreover, how the scaffold functions at the molecular and cellular levels, and how pathogenetic variants cause GBM dysfunction remain obscure. This lack of knowledge impedes the development of precision therapies aimed at restoring/repairing function of the scaffold.

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• of special interest

•• of outstanding interest

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KEY POINTS

- Cure for Alport patients is to restore production and function of the collagen IV$^{\alpha 345}$ scaffold in the GBM (collagen IV therapy)
- Collagen IV therapies are possible at various stages of production and assembly of scaffold
- A new mouse model of Alport syndrome with an aberrant scaffold incorporated into the GBM is valuable for studying pathobiology and developing therapies
- Development of protein-based therapies hinge on a deep understanding of the pathobiology of the scaffold in GBM

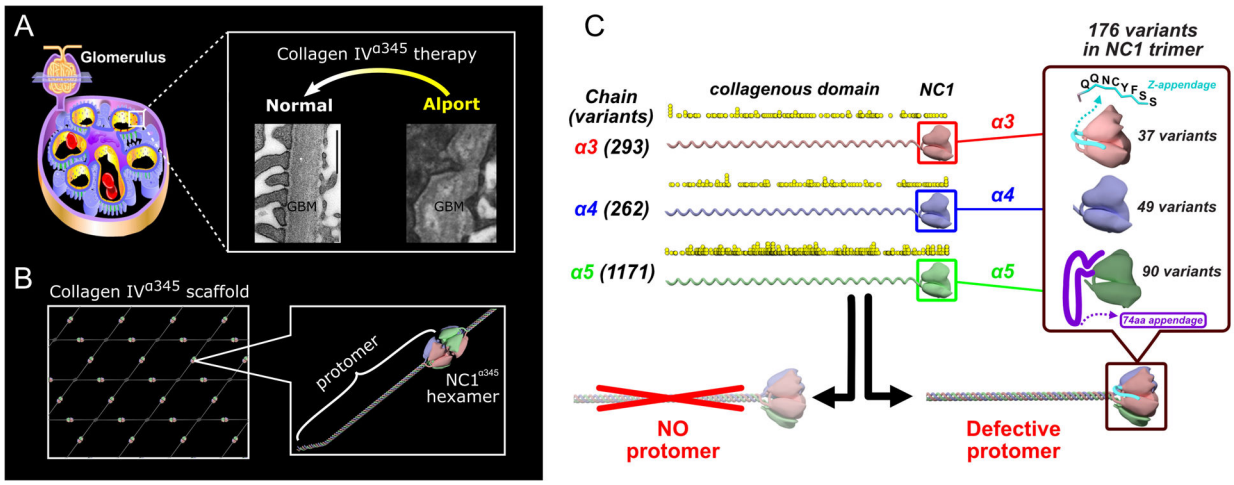

Figure 1. Alport pathology in kidney.

A. Alport syndrome is a glomerulopathy characterized by irregular thickening and splitting of glomerular basement membrane (GBM). Electron microscopy images of human GBM are from [7] for Alport and from [8] for normal. Collagen IV$^{\alpha345}$ therapies aim to restore the morphology and function of GBM. **B.** Schematic representation of the collagen IV$^{\alpha345}$ scaffold assembled from the protomers where NC1 domain hexamerization plays a crucial role. **C.** Alport variants (previously published in [4••]). The number and location of over 1700 genetic Alport-associated variants (indicated by yellow dots) in the α3, α4, and α5 chains of collagen IV. Pathogenic variants cause either loss of the α345 protomers in the GBM or assembly of defective α345 protomers that can incorporate into the GBM, causing a broad spectrum of GBM phenotypes. Significant number of Alport-associated variants occur in the NC1 domain of α3, α4, and α5 chains (right insert).

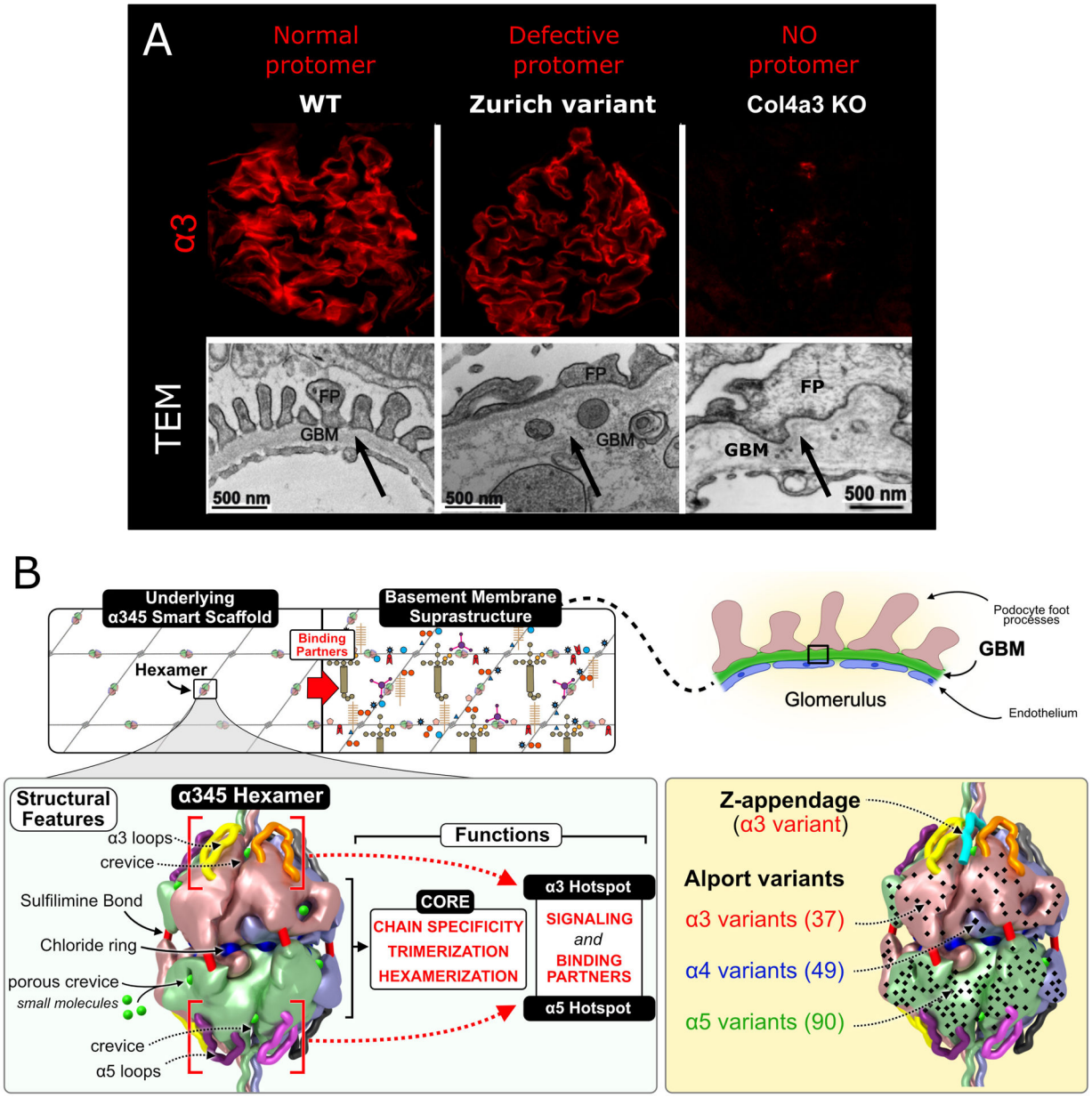

Figure 2. Pathobiology of the collagen IV$^{\alpha345}$ scaffold.

A. Mouse models for Alport syndrome. Two types of Alport animal models based on presence or absence of the collagen IV$^{\alpha345}$ scaffold in the GBM represented. Top panel demonstrates immunofluorescent staining for alpha 3 chain of collagen IV (previously published in [4••]). In the glomeruli of Col4a3 KO mouse the collagen IV$^{\alpha345}$ scaffold is absent while in Zurich variant mouse defective collagen IV$^{\alpha345}$ scaffold is incorporated in the GBM and staining intensity is similar to that of the wild type control. Lower panel shows defects in the GBM of both models observed by electron microscopy. EM image of Col4a3 KO GBM (lower right) is previously published in [19]. **B**. The α345 hexamer is a focal point of GBM function and is altered in Alport syndrome (modified from [4••]). The collagen IV$^{\alpha345}$ scaffold is populated with several classes of macromolecules - laminins,

nidogens and proteoglycans, forming the glomerular basement membrane suprastructure. The α345 hexamer harbors a number of features involved in pathogenesis of Alport syndrome. Multiple Alport-associated variants occur within α3, α4, and α5 NC1 domains (black dots) including the Zurich variant of α3 NC1. The overall concept is presented in three companion papers in *J Biol Chem*. [4••, 21••, 23••].

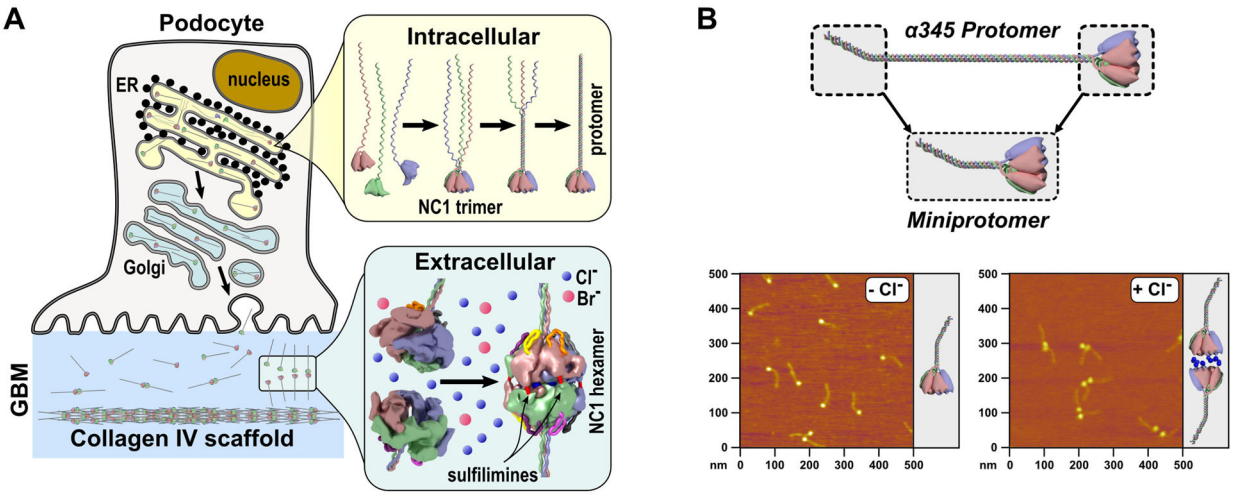

Figure 3. Collagen IV$^{\alpha 345}$ synthesis, folding, and assembly.

A. Schematic representation of collagen IV synthesis followed by intracellular and extracellular assembly steps. The NC1 domain guides individual chains to assemble into the trimeric protomer inside the cell and further initiate the assembly of the scaffold via hexamerization outside the cell. Chloride ions trigger and stabilize scaffold assembly and bromide ions act as co-factors in formation of sulfilimine bonds which reinforce hexamer stability. **B**. Schematic representation of miniprotomer and atomic force microscopy (AFM) images of miniprotomers (previously published in [23••]), which display the role of Cl^- ions in hexamer formation (protomer-to-protomer interactions forming a scaffold).

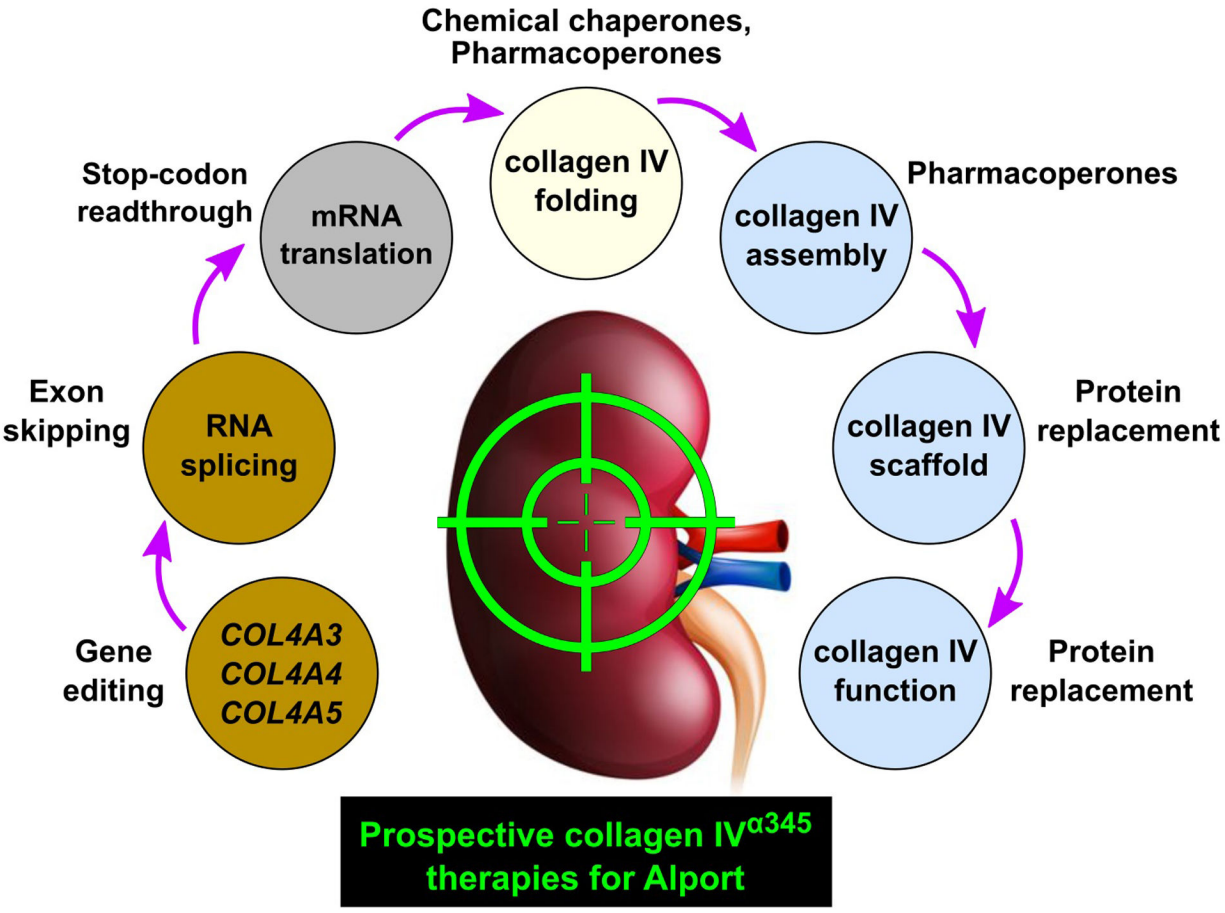

Figure 4.
Prospective collagen IV$^{\alpha 345}$ therapies for Alport syndrome.