EXPERT REVIEWS

Developing novel anti-fibrotic therapeutics to modulate post-surgical wound healing in glaucoma: big potential for small molecules

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Ocular fibrosis leads to significant visual impairment and blindness in millions of people worldwide, and is one of the largest areas of unmet need in clinical ophthalmology. The antimetabolites, mitomycin C and 5-fluorouracil, are the current gold standards used primarily to prevent fibrosis after glaucoma surgery, but have potentially blinding complications like tissue damage, breakdown and infection. This review thus focuses on the development of new classes of small molecule therapeutics to prevent post-surgical fibrosis in the eye, especially in the context of glaucoma filtration surgery. We discuss recent advances and innovations in ophthalmic wound healing research, including antibodies, RNAi, gene therapy, nanoparticles, liposomes, dendrimers, proteoglycans and small molecule inhibitors. We also review the challenges involved in terms of drug delivery, duration of action and potential toxicity of new anti-fibrotic agents in the eye.

KEYWORDS: antibody • gene therapy • glaucoma filtration surgery • inhibitor • nanoparticle • ocular fibrosis • RNAi • wound healing

Fibrosis & wound healing

Fibrosis and scarring are involved in the pathogenesis or failure of treatment of virtually all the major blinding diseases. Glaucoma is the second leading cause of blindness in the world. By 2020, its prevalence is estimated to reach around 79.6 million people worldwide, with more than 11 million individuals suffering from bilateral blindness [1]. Glaucoma filtration surgery is the mainstay of surgical treatment for medically uncontrolled glaucoma. Even with new surgical techniques, postoperative scarring remains a critical determinant of the long-term surgical outcome and intraocular pressure after drainage surgery [2]. The antimetabolites, mitomycin C (MMC) and 5-fluorouracil (5-FU), have improved the surgical outcome of glaucoma filtration surgery, but lead to non-specific cytotoxicity and the risk of sight-threatening complications like tissue damage, breakdown and infection. In addition, some patients still scar and fail surgery despite antimetabolite therapy. There is thus a large unmet need to develop new anti-fibrotic therapeutics to prevent scarring and post-surgical fibrosis in the eye.

Wound healing is a complex multifactorial process consisting of a cascade of overlapping events, including hemostasis, inflammation, cell proliferation and tissue remodeling (FIGURE 1). After injury, hemostasis takes place, and blood and fibrin clots are formed to reduce blood loss. This leads to an inflammatory phase where neutrophils, macrophages and lymphocytes are attracted to the wound site as part of the
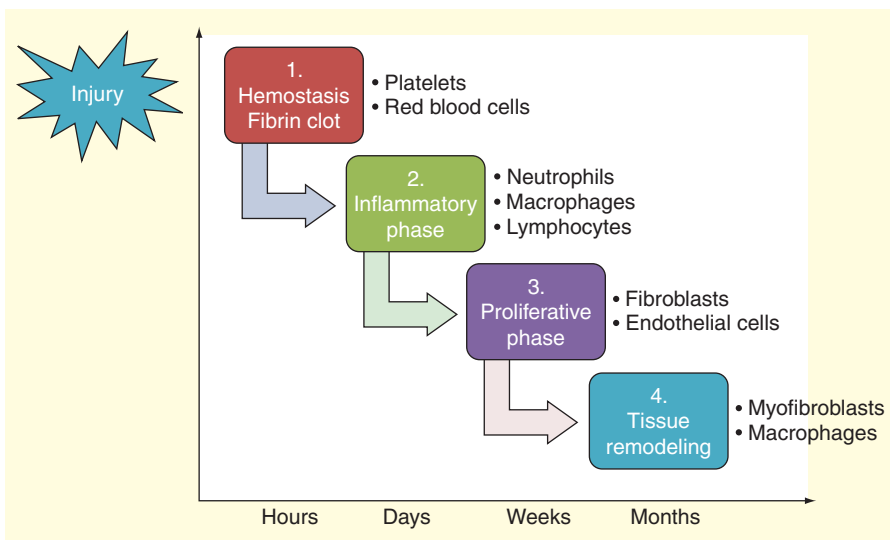
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Figure 1. The general phases of wound healing.

immune response. This is then followed by a proliferative phase where fibroblasts are activated to lay down extracellular matrix, leading to re-epithelialization, angiogenesis and granulation tissue formation. The final step is tissue remodeling and scar formation, and matrix metalloproteinases (MMPs) play an important role in the process.

In this review, we focus on the development of novel small molecule therapeutics to modulate ophthalmic wound healing, especially in the context of post-surgical scarring after glaucoma filtration surgery. We discuss the recent advances and also the pitfalls of different types of therapeutic molecules, including antibodies, RNAi, gene therapy, nanoparticles, liposomes, dendrimers, proteoglycans and small molecule inhibitors, to prevent scarring and promote healthy cell regeneration in the eye.

Antibodies

Monoclonal antibodies are only a few nanometers in size, but currently represent one of the largest classes of therapeutic molecules. Unlike antimetabolites, one of the key advantages of an antibody is its target specificity in ocular therapeutics (TABLE 1). TGF-β plays a pivotal role in wound healing as it increases fibroblast proliferation [3], controls extracellular matrix synthesis [4] and accelerates myofibroblast differentiation [5]. Our group has previously shown that subconjunctival monoclonal antibody to TGF-β2 decreased conjunctival scarring in an experimental model of glaucoma surgery [6], but did not significantly prolong bleb survival after glaucoma filtration surgery in a randomized clinical trial (RCT) [7]. However, it is likely that the doses used were not high enough for a sufficiently long period of tissue exposure, and that a prolonged or slow release dosing regimen might have been required [8]. Furthermore, it is likely that a broader coverage of the different isoforms might also have been necessary.

Connective tissue growth factor (CTGF) is a key downstream mediator of TGF-β-induced fibrosis, and can accelerate fibroblast proliferation and secretion of extracellular matrix [9]. Wang et al. have shown that subconjunctival injection of a CTGF antibody

can maintain larger bleb areas and lower intraocular pressures in a rabbit model of trabeculectomy [10]. Yuan et al. have also found that CTGF is overexpressed in filtration blebs, suggesting that CTGF might play an important role in the process of wound healing after trabeculectomy [11].

VEGF is a crucial mediator of angiogenesis, and stimulates both fibroblasts and endothelial cells in wound healing [12,13]. Bevacizumab is a recombinant humanized monoclonal antibody against VEGF, and several studies have shown that it decreases scar formation and prolongs bleb survival after experimental glaucoma filtration surgery [12,14,15]. However, Rodríguez-Agirretxe et al. reported that combined MMC and bevacizumab implants decreased intraocular pressure to a lesser extent than MMC alone, and that bevacizumab could interact with MMC [16]. In the first RCT, Nilforushan et al. found that the MMC group had better intraocular pressure control but similar bleb morphology to the subconjunctival bevacizumab group [17], and other studies have also suggested that the antibody alone is currently less effective than MMC [18]. Vandewalle et al. recently reported that perioperative administration of intracameral bevacizumab significantly reduced the need for needling interventions, and led to a higher success rate after trabeculectomy [19]. Further RCTs are needed to investigate the best way of using anti-VEGF therapies in glaucoma surgery, and the optimal doses and combinations with other anti-scarring agents.

PlGF is a VEGF-homolog that binds to VEGF-R1 and acts on pathological angiogenesis and inflammation [20]. Bergen et al. showed that intracameral injection of a monoclonal PlGF antibody increased bleb area and survival in a mouse model of glaucoma surgery [21]. Furthermore, anti-PlGF treatment seemed to be more effective than anti-VEGF-R2 treatment in improving the surgical outcome, possibly due to its additional effect on inflammation.

Lysyl oxidase (LOX) is another important class of enzymes that catalyses the covalent crosslinking of collagens and elastin in the extracellular matrix [22]. Bergen et al. have found that LOX and LOX-like 2 (LOXL2) are both upregulated in Tenon's capsule [23]. The authors also showed that targeting LOXL2 with an inhibitory monoclonal antibody, GS-607601, reduced pathological angiogenesis, inflammation, fibrosis and prolonged bleb survival in a rabbit model of trabeculectomy [23].

Integrins are protein heterodimers consisting of non-covalently associated α- and β-subunits, and the adhesive interactions mediated by integrins are necessary for cell proliferation [24]. Paikal et al. reported that different integrin antibodies inhibited the attachment and proliferation of human Tenon's fibroblasts in vitro [25]. Further in vivo studies are needed to determine whether integrin antibodies can significantly limit scar formation after glaucoma surgery without significant toxicity.

Table 1. Summary of properties of different therapeutic molecules in ocular fibrosis.

Therapeutic molecule	Size	Specificity	Tested drug delivery route	Duration of action	Toxicity (potential side effects)
Antibody	10^{-9} m	High	Subconjunctival Intracameral	>30 days (end of study)	Low (immunogenic)
siRNA	20–25 base pairs	High	Subconjunctival Intravitreal	>14 days (end of study)	Low (off-target effect, immunogenic)
miRNA	21–25 nucleotides	High	Locked nucleic acid	Decreases fibroblast activity (in vitro)	Low
Viral vector	10^{-9} m	High	Subconjunctival Intravitreal	>30 days (end of study)	Moderate (oncogenic, immunogenic)
Nanoparticle	10^{-9} m	Moderate	Topical Subconjunctival Intravitreal	>42 days (end of study)	Low (cytotoxic, genotoxic)
Liposome	10^{-6} m	Low	Subconjunctival Intravitreal	>14 days (end of study)	Low
Drug inhibitor	10^{-6}–10^{-9} m	Low	Topical Subconjunctival Intravitreal	>30 days (end of study)	Variable (cytotoxic)
Proteoglycan	10^{-3} Da	Low	Subconjunctival Intravitreal	>14 days (end of study)	Low
Dendrimer	10^{-9} m	Low	Subconjunctival	28 days (maximal effect)	Low

siRNA & shRNA therapy

RNAi is another promising therapeutic approach as it can be used to silence the expression of unwanted genes in fibrosis (TABLE 2). RNAi is mediated by siRNAs, shRNAs and miRNAs. siRNAs are small double-stranded exogeneous RNA molecules (20–25 base pairs) [26], and shRNAs are short sequences of RNA with a tight hairpin turn that can be used in post-transcriptional gene silencing [27].

Transcription factors are key molecules that regulate gene expression by controlling the transcription of DNA into messenger RNA. NF-κB is a transcription factor that activates fibroblasts and is regulated by IκB kinase subunit b. Duan et al. showed that siRNAs targeting IκB kinase subunit b effectively downregulated NF-κB in human Tenon's fibroblasts and suppressed fibroblast proliferation [28].

Growth factors, especially TGF-β, also play a pivotal role in ocular fibrosis. Nakamura et al. reported that siRNAs efficiently knocked down TGF-βRII expression in human corneal fibroblasts, and that direct ocular application of TGF-βRII siRNAs could be used to decrease scarring in the subconjunctival space in the mouse [29].

Cell cycle regulators also represent potential anti-fibrotic gene therapeutic targets. S phase kinase-interacting protein 2 is a key cell cycle regulator that targets p27. Wang et al. found that siRNA silencing of S phase kinase-interacting protein 2 decreased proliferation and cell viability in rabbit Tenon's fibroblasts, and had potential as an anti-scarring therapy in glaucoma filtration surgery [30].

Proteins are also important components of the extracellular matrix in the eye. Seet et al. showed that knocking down secreted protein, acidic, rich in cysteine (SPARC) impaired contraction in human Tenon's fibroblasts and reduced the expression of profibrotic genes, namely collagen I, MMP-2, MMP-9, MMP-14, IL-8 and TGF-β [31]. Yuan et al. found that shRNAs effectively suppressed the expression of keratoepithelin and myocilin in trabecular meshwork cells [32]. Comes and Borrás also reported that siRNAs effectively silenced Matrix GLA protein expression in trabecular meshwork cells, and that intracameral delivery of siRNAs could become a future drug delivery technique in glaucoma [33].

The success of siRNA therapeutics will depend largely on the efficient ocular delivery of siRNAs. In a Phase I study, Kaiser et al. showed that intravitreal injections of siRNA-027 were well tolerated in the eye, and led to an improvement in visual acuity and foveal thickness in patients with wet age-related macular degeneration [34]. Ocular delivery of naked siRNAs is, however, unlikely to be sufficient in ocular fibrosis as siRNAs are not permeable across cell membranes and are not resistant against enzymatic degradation. Viral delivery systems, including adenoviral vectors [35] and lentiviral vectors [36], have shown good transfection efficacy. Non-viral delivery systems have also been used, including cationic liposomes [37] and cationic copolymers like CS-g-(PEI-b-mPEG) [28].

miRNA therapy

There is now increasing evidence of the key role of miRNAs in fibrosis (TABLE 2). miRNAs are small single-stranded endogenous RNA transcripts (21–25 nucleotides) that are involved in post-transcriptional gene modulation [38]. One of the most studied

Table 2. Tested anti-fibrotic gene therapeutic targets in the eye.

Therapeutic target	Cell type	Delivery
Transcription factors		
NF-κB	Human Tenon's fibroblasts	siRNA
E2F	Human Tenon's fibroblasts	Hemagglutinating virus of Japan
Cell cycle regulators		
p53	Human Tenon's fibroblasts	Recombinant adenovirus
p21	Rabbit Tenon's fibroblasts	Recombinant adenovirus
Skp2/p27	Rabbit Tenon's fibroblasts	siRNA
Growth factors		
TGF-β	Human corneal fibroblasts	siRNA
Proteins		
Keratoepithelin	Human trabecular meshwork cells	shRNA
Myocilin	Human trabecular meshwork cells	shRNA
SPARC	Human Tenon's fibroblasts	siRNA
Matrix GLA protein	Human trabecular meshwork cells	siRNA
miRNAs		
miR-29b	Human Tenon's fibroblasts	miRNA
miR-29b	Human trabecular meshwork cells	miRNA
Others		
Smad7	Human conjunctival fibroblasts	Recombinant adenovirus
p38MAPK	Mouse subconjunctival fibroblasts	Adenovirus
Cytosine deaminase	Rabbit Tenon's fibroblasts	Recombinant adenovirus

SPARC: Secreted protein, acidic, rich in cysteine.

miRNAs in fibrosis is miR-29, and downregulation of miR-29 has been linked to tissue scarring in various body systems, namely cardiac fibrosis [39], pulmonary fibrosis [40], liver fibrosis [41], renal fibrosis [42], skin fibrosis [43] and fibrosis in cancer [44,45].

Li *et al.* showed that miR-29b suppressed collagen type I gene expression and was significantly downregulated in TGF-β1-activated human Tenon's fibroblasts [46]. The authors also reported that increasing intracellular miR-29b expression, by exogenous miR-29b delivery, effectively decreased fibroblast activity *in vitro* [46].

In addition, Luna *et al.* showed that miR-29b downregulated the expression of multiple genes involved in the synthesis of extracellular matrix in human trabecular meshwork cells, and decreased cell cytotoxicity in the presence of chronic oxidative stress [47]. Increasing miR-29b expression might thus represent a therapeutic approach to limit extracellular matrix deposition, prevent trabecular meshwork cell loss and facilitate aqueous humor outflow in glaucoma [47].

miR-29 is one of the well-described TGF-β-associated miR-NAs involved in fibrosis [39,40]. miR-29 regulates multiple genes encoding for extracellular matrix proteins, including *COL1A1, COL1A2, COL3A1, FBN1, ELN* and *SPARC* [47,48]. Some level of cross-talk has also been reported between miR-29 and TGF-β in trabecular meshwork cells, suggesting that miR-29 could represent an important modulator of TGF-β in aqueous outflow in glaucoma [49].

The tumor suppressor gene *p53* enhances the rate of transcription of several genes that regulate cell-cycle arrest and apoptosis [50]. Park *et al.* have shown that miR-29 positively regulates p53, and might thus inhibit fibroblast proliferation in conjunctival scarring [51]. The authors have also found that miR-29 directly suppresses CDC42 (a Rho family GTPase) and p85α (the regulatory subunit of PI3K) [51]. As the Rho/MRTF/SRF (Myocardin related transcription factor/Serum response factor) pathway is increasingly being associated with fibrosis [52,53], upregulating miR-29 could also decrease ocular scarring by suppressing the Rho-actin pathway. Although specificity has been thought to be an advantage in many clinical situations, this overlap of activity may be advantageous in scarring inhibition due to the overlap of multiple pathways.

Other miRNAs that have been identified as key triggers of fibrosis driven by TGF-β include miR-21 [54,55], miR-192 [56], miR-216a and miR-217 [57]. miR-21 is expressed in the lungs of patients with idiopathic pulmonary fibrosis. Mice treated with miR-21 antisense probes were also protected from bleomycin-induced pulmonary fibrosis [54], and cardiac fibrosis induced by pressure overload [55]. miR-21 is thought to promote fibrosis by regulating TGF-β1 and MAPK signaling in activated myofibroblasts.

Future research will focus on the development and effective ocular delivery of miRNA-based therapies to prevent scarring in the eye. miR-29 mimics to increase miR-29 expression, and pharmacological inhibitors to prevent the downregulation of miR-29 expression, both have therapeutic potential. Locked nucleic acid-modified oligonucleotides are single-stranded bicyclic RNA analogs that irreversibly bind to miRNAs [58], and could potentially be used for the ocular delivery of miRNA therapies. Miravirsen is a locked nucleic acid that sequesters miR-122, and provides proof-of-concept of the efficacy of miRNA therapy in human disease [59].

Viral gene transfer & gene therapy

Instead of blocking unwanted mRNA expression like siRNAs, shRNAs and miRNAs, other methods of gene therapy involve increasing the expression of inhibitory genes on the cell

cycle (TABLE 2). Human p53 is a tumor suppressor gene that plays a key role in arresting cell cycle progression to allow DNA repair, or to induce apoptosis if the damage is too extensive [50]. Using a recombinant adenovirus (rAd) for p53, Johnson *et al.* induced overexpression of p53 in human Tenon's fibroblasts, and significantly inhibited fibroblast proliferation and DNA synthesis [60].

Perkins *et al.* also showed that a rAd containing the p21 gene, which is a crucial downstream effector of p53, inhibited the proliferation of rabbit Tenon's fibroblasts and performed similarly to MMC in terms of decrease in intraocular pressure [61]. In a primate model of glaucoma surgery, Heatley *et al.* found that rAd.p21 gene therapy prevented conjunctival scarring and achieved even better intraocular pressure control than the MMC-treated group [62]. Both rAd.p21 gene therapy studies did not report any of the tissue complications seen with MMC treatment.

In addition, Akimoto *et al.* used a rAd containing the cytosine deaminase gene to convert the pro-drug 5-fluorocytosine into 5-FU in glaucoma surgery [63]. In an earlier study, the same authors reported that blocking the transcription factor E2F using the hemagglutinating virus of Japan on cationic liposomes inhibited fibroblast proliferation in glaucoma surgery [64].

Furthermore, Yamanaka *et al.* reported that a rAd expressing Smad7 blocked the expression of Smad2/3 and suppressed collagen type I, α-smooth muscle actin and VEGF in human conjunctival fibroblasts [65]. The authors have thus suggested that Smad7 gene transfer could become an effective strategy to prevent excessive conjunctival scarring in glaucoma filtration surgery.

Yamanaka *et al.* also showed that adenoviral gene transfer of a dominant negative p38MAPK decreased conjunctival fibrosis both *in vitro* and *in vivo* [66]. They found that p38MAPK gene transfer blocked the TGF-β increase in collagen type I expression, CTGF and the monocyte/macrophage chemoattractant protein-1. The authors have thus suggested that p38MAPK inhibition could become a future anti-scarring therapy in glaucoma filtration surgery.

A difficult challenge with gene therapy remains the delivery of the gene into the target cell. The main disadvantage of non-viral methods, including ballistic DNA injections [67] and liposomes [68], is that the gene expression is short-lived and lasts only a few days. Viral methods achieve longer gene expression, and adenovirus is a double-strain DNA virus shown to be an efficient vector for gene transfer in ocular cells [69]. There is, however, a rare but inherent risk of mutagenesis with the use of viral vectors, and at least two cases of lymphoproliferative disorders related to gene therapy have been described [70].

Nanoparticles

Nanotechnology and nanoparticle research currently represent an area of great research interest due to its translational potential in a wide variety of scientific fields. Nanoparticles typically range in size between 1 and 100 nm (TABLE 1), and several research groups worldwide are developing nanoparticles that could be used to modulate wound healing in the eye. Nanoparticles have

several key advantages: a large surface area, for example, a standard teaspoon of 10-nm diameter silica nanoparticles has more surface area than a dozen double-sized tennis courts, good penetration and improved bioavailability by enhancing aqueous solubility and targeted drug delivery to a specific location in the eye.

Using the rabbit model of glaucoma filtration surgery, Butler *et al.* showed that topical silver nanoparticles (Ag-NPs) achieved an improved and sustained reduction in intraocular pressure, and led to blebs with decreased fibrosis and ischemia compared with MMC [71]. Ag-NPs have also been shown to accelerate wound healing after skin burns, with decreased scar tissue formation and anti-inflammatory effects [72]. Ag-NPs have a good safety profile in the eye [73], but large particle diameters and higher concentrations have been associated with increased cytotoxicity and genotoxicity [74].

SPARC silencing has been shown to decrease collagen gel contraction *in vitro* [31], and to prolong bleb survival in a mouse model of glaucoma filtration surgery [75]. Tan *et al.* have shown that layer-by-layer nanoparticles could be used as an efficient delivery vehicle for SPARC silencing and siRNA therapeutics [76]. No toxic side effects were observed with the layer-by-layer nanoparticles.

Shao *et al.* have also reported that low-density lipoprotein (LDL)-MMC-chitosan nanoparticles could be used as a target drug delivery system to specifically bind to LDL receptors on activated human Tenon's fibroblasts [77]. The LDL-MMC-chitosan nanoparticles are associated with high selective targeting, good biocompatibility, low immunogenicity, decreased toxicity to normal cells and increased effectiveness of the anti-scarring agent MMC in glaucoma filtration surgery.

In addition, Santos *et al.* showed that subconjunctival administration of nanosized complexes of antisense TGF-β2 phosphorothioate oligonucleotides with polyethylenimine (PEI) increased intracellular penetration of TGF-β2 antisense oligonucleotides in conjunctival cells [78]. The authors also found that the sustained release of these nanosized complexes significantly improved bleb survival in a rabbit model of trabeculectomy [78].

Duan *et al.* also reported that cationic nano-copolymers combined with IKKβ targeting siRNA [CS-g-(PEI-b-mPEG)/ IKKβ-siRNA] inhibited fibroblast proliferation *in vitro* [28]. Ye *et al.* showed that subconjunctival injection of these nanocopolymers decreased subconjunctival scarring and increased bleb survival in a monkey model of glaucoma filtration surgery [79]. The authors also found that the cationic nanocopolymers were well tolerated and non-toxic in the eye.

Liposomes

There is now increasing research in using liposomes as a drug delivery system for anti-scarring agents in the eye. Liposomes are small composite structures composed of a lamellar phase lipid bilayer, and vary in size between low to tens of micrometers (TABLE 1). Peng *et al.* showed that subconjunctival injections of liposomes containing homoharringtonine, an alkaloid and protein synthesis inhibitor, reduced the intraocular

pressure and inhibited scarring in a rabbit model of trabeculectomy [80].

Simmons *et al.* also developed a liposomal delivery system for 5-FU to prolong the ocular levels of the drug in glaucoma filtration surgery [81]. They found that liposomal 5-FU prolonged the scleral and conjunctival concentrations of the drug in rabbits, while reducing the peak ocular concentrations. Liposomal 5-FU could thus help improve the surgical outcome of glaucoma surgery, as well as decrease the risk of ocular side effects.

Daunorubicin is an anthracycline anti-cancer drug that is used to block DNA synthesis. Varma *et al.* showed that daunorubicin was safe and effective at lowering intraocular pressure in glaucoma filtration surgery [82]. Shinohara *et al.* also showed that daunorubicin encapsulated in empty liposomes was effective in preventing proliferative vitreoretinopathy in a rabbit model [83]. The authors did not report any adverse side effects with liposomal daunorubicin.

Tilleul *et al.* also showed that a liposome formulation of the anti-mitotic mitoxantrone reduced intraocular pressure and improved the surgical outcome of glaucoma surgery, when injected subconjunctivally at the end of surgery in rabbits [84]. The effect of mitoxantrone in liposome formulation was similar to MMC application, and decreased the occurrence of corneal opacity compared with those treated with mitoxantrone in solution.

Dendrimers & proteoglycans

Other classes of therapeutic molecules that have been developed to modulate ophthalmic wound healing are dendrimers and proteoglycans (Table 1). Dendrimers are hyperbranched nanomolecules that can be chemically synthesized to have precise structural characteristics. Our group has shown that the dendrimer glucosamine [D(+)-glucosamine] and dendrimer glucosamine 6-sulfate [D(+)-glucosamine 6-sulfate] have immunomodulatory and anti-angiogenic properties respectively, and that their combined use significantly increased the long-term surgical success in a rabbit model of glaucoma filtration surgery [85]. We did not observe any local or systemic side effects with the dendrimer drugs.

Decorin is a small proteoglycan that binds to TGF-β and can inhibit its activity in wound healing [86]. Grisanti *et al.* reported that decorin significantly decreased intraocular pressure and post-surgical fibrosis in a rabbit model of trabeculectomy [87]. Decorin was also well tolerated in the experimental model of glaucoma filtration surgery, and no adverse effects, such as inflammation or blurring of the optical media, were observed.

Small molecule inhibitors & drugs

Small molecule inhibitors and drugs are showing a lot of potential as effective anti-fibrotic agents for the eye (Table 1). Honjo *et al.* showed that the Rho-associated protein kinase (ROCK) inhibitor, Y-27632, prevented fibroblast-mediated matrix contraction *in vitro*, and increased bleb survival in a rabbit model of glaucoma filtration surgery [88]. There were no adverse side effects observed with the Y-27632 inhibitor. ROCK inhibitors may block TGF-β-induced scarring by

downregulating pathways generating mechanical tension, and thus improve the long-term success of glaucoma surgery. Inoue and Tanihara have also shown that a ROCK inhibitor significantly decreased intraocular pressure *in vivo* by directly affecting the trabecular meshwork and Schlemm's canal [89].

Rac1 is a small Rho GTPase involved in regulating cytoskeletal organization, and plays an important role in protrusive activity and wound healing [90]. Xu *et al.* have shown that Rac1 inhibition reverses the phenotype of fibrotic fibroblasts in the skin [91]. Our group has previously shown that the Rac1 inhibitor, NSC23766, efficiently inhibited conjunctival tissue and fibroblast-mediated matrix contraction [92]. NSC23766 did not cause any significant toxicity, and was associated with reduced MMP1 expression during matrix contraction.

In a high-throughput screen, Evelyn *et al.* have also identified the small molecule inhibitor CCG-1423 as being more effective in reducing MRTF/SRF-regulated gene transcriptional signaling than ROCK inhibitors [93]. Bell *et al.* have more recently optimized a second-generation inhibitor, CCG-203971, that is more potent and less cytotoxic than CCG-1423 [94]. CCG inhibitors have shown efficacy in decreasing fibrosis in the skin [95] and colon [96], and it will be interesting to see their effects on wound healing in the eye.

MMPs are a large family of calcium-dependent zinc-containing endopeptidases that play a crucial role in tissue remodeling and degradation of collagens and extracellular matrix [97–99]. Our group has previously shown that MMP inhibition decreased matrix contraction and collagen production *in vitro* [98], as well as scarring in a rabbit model of glaucoma filtration surgery [97,99].

Furthermore, inhibitors for activin receptor-like kinase 5 (ALK5), also known as TGF-β receptor type I, have been studied in ocular fibrosis. Sapitro *et al.* reported that the ALK5 inhibitor, SB-505124, suppressed TGF-β activity and promoted bleb survival in a rabbit model of trabeculectomy [100]. Xiao *et al.* also showed that the ALK5 inhibitor, SB-431542, decreased post-surgical scarring and fibrosis after experimental glaucoma filtration surgery [101].

Pirfenidone (PFD) is an interesting small molecule drug that downregulates a series of key profibrotic cytokines and growth factors like TGF-β [102]. Zhong *et al.* showed that postoperative topical PFD was safe and improved bleb survival in a rabbit model of glaucoma filtration surgery [103]. Other authors have also found that PFD decreased scarring and improved wound healing after corneal burns [104], strabismus surgery [105] and proliferative vitreoretinopathy [106].

Expert commentary

To date, there has been significant progress made in the development of small molecule therapeutics to modulate ophthalmic wound healing using different approaches (Figure 2). Given that fibrosis-related events play a part in most of the blinding diseases in the world and also account for over 40% of all deaths, this represents one of the largest areas of unmet need in clinical ophthalmology and clinical medicine.
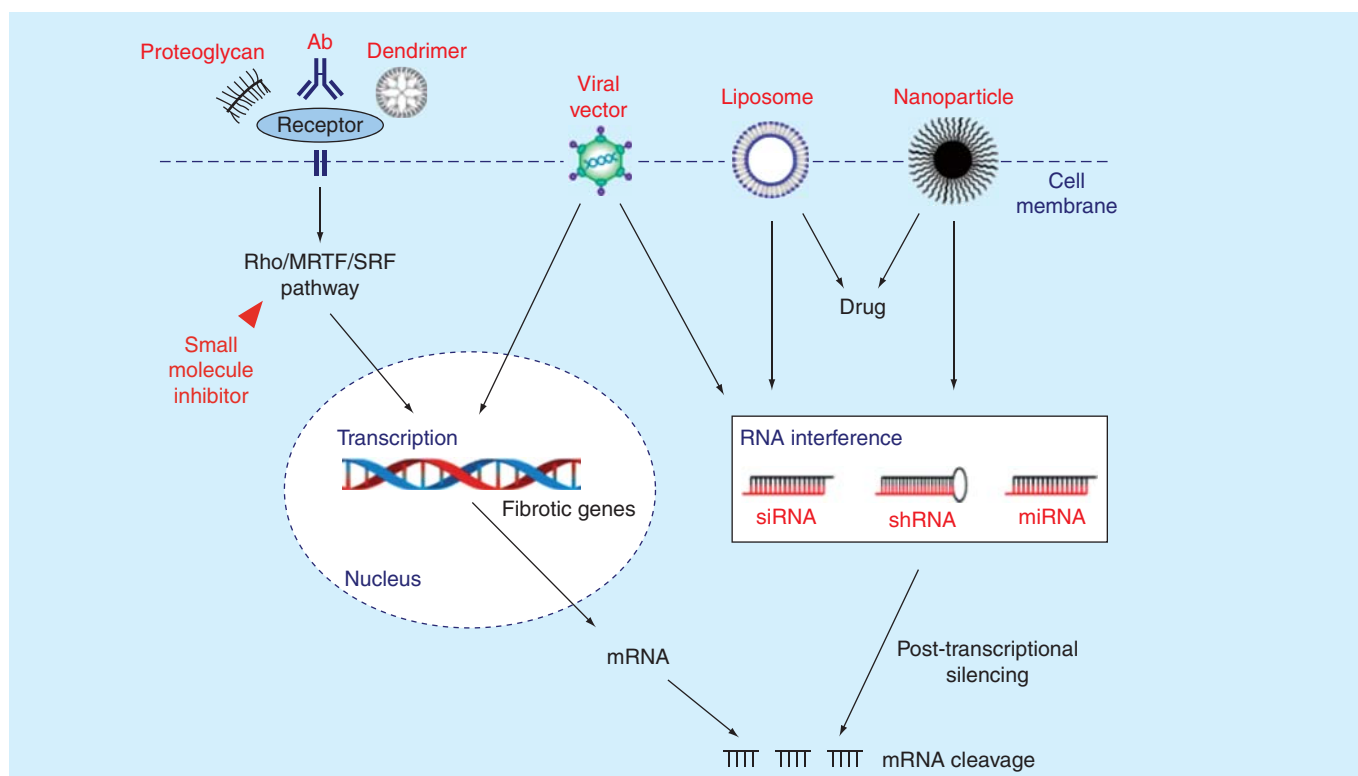
Figure 2. Schematic diagram summarizing the different therapeutic molecules in ocular fibrosis and their modes of action.
MRTF: Myocardin-related transcription factor; SRF: Serum response factor.

Box 1 describes the properties of the ideal anti-scarring agent or combination of agent(s). Antibody therapeutics is currently a fast growing area of research, and there have also been major advances in the field of gene modulating therapies, nanoparticles, liposomes, dendrimers and small molecule inhibitors in ocular fibrosis. Clinical trials using antisense oligonucleotides and ribozymes have so far proved to be disappointing, but RNAi is more potent than antisense oligonucleotides [107].

The eye has the distinct advantage of being a closed compartment where small molecule therapeutics can be injected close to the target tissues, thus minimizing the risks of side effects to the rest of the body. This includes both the intraocular and subconjunctival space after glaucoma surgery that is easily accessible in the outpatient clinical setting. Drug delivery and duration of action remain major hurdles in the development of novel anti-scarring therapies. New slow release drug delivery systems, like ocular tablets and *in situ* gels, are being developed to decrease the need for multiple injections in glaucoma surgery [108]. RNAi formulations (RNAi + delivery system) are also being designed to increase the cellular uptake, to protect against enzymatic degradation and to permit the long-term delivery of RNAi therapies [26].

Another key challenge with new anti-fibrotic agents is their potential toxicity in humans. Several small molecule inhibitors have shown promising results as anti-fibrotic agents, but their ocular toxicity have yet to be fully investigated. Viral vector delivery systems have also shown high transfection efficacy, but

is limited by the risks of mutagenesis and host immunity [109]. The unselective silencing of additional genes represents another potential side effect with RNAi therapeutics, and a possible solution might be to chemically modify one or both of the siRNA strands to minimize the risk of off-target effects [110].

Five-year view

It is well established that some group of patients scar much more than others. However, there is a current lack of reliable biomarkers to stratify the risk of scarring and post-surgical fibrosis in the eye. In the next 5 years, the hope is that advances in genotyping and phenotyping, using modern tissue biomarkers

Box 1. Properties of the ideal anti-scarring agent(s).

- Effective anti-fibrotic effects, but allows some healing (e.g., maintains tissue flow ~10 mmHg after glaucoma surgery).
- High specificity for target(s), but able to adequately suppress multiple pathways that constitute complex fibrosis.
- Safe to handle.
- Short initial delivery method, ideally at the time of surgery.
- Long duration of action, ideally >10 years (or easily delivered at intermittent intervals, e.g. yearly).
- Inexpensive and easily mass produced.
- Minimal toxicity.
- Applicable in many different situations in the eye and body.

and high-resolution real-time *in vivo* imaging techniques, will help to identify the groups of patients that would scar more aggressively, and thus help to develop a more personalized and stratified approach in anti-fibrotic ocular therapeutics.

Although fibroblasts underlie the scarring process after glaucoma filtration surgery, there are also other cell types, like inflammatory cells, which play an important role in the wound healing process. The aim of future research will thus be to develop combination therapies targeting several pathways that would be more efficacious than monotherapies, especially in the high-risk groups. The ability to predict the risk of scarring and to tailor the anti-fibrotic treatment regimen to each individual patient will be an extremely useful tool clinically to prevent undertreating, or exposing patients to unnecessary treatments with potential side effects. Furthermore, development of new therapies in combination with surgical devices, where scarring is currently still a significant long-term problem, further increases both the challenges and the gains to be made in clinical outcomes in many pathological situations affecting the eye and other body organs.

Finally, apart from a few studies on anti-TGF-β and anti-VEGF therapies, there is a current lack of well-designed randomized controlled trials to compare the efficacy and safety of different therapeutic molecules in ocular fibrosis. Similar to cancer therapeutics, more refined human studies with long-term follow-ups are needed, alongside carefully chosen comparative control groups. In addition, a combination of agents may afford us much better control of the scarring process, as has been the case in cancer therapy. The ultimate goal in the future will be to modulate the wound healing process in each patient toward a more regenerative repair process with no scarring, possibly combined with new bioengineered devices. This will allow us to achieve a new generation of outcome goals not previously possible. This includes audacious goals such as our 10, 10, 10 target for glaucoma surgery: a postoperative intraocular pressure of 10 mmHg, carried out in 10 minutes and lasting for at least 10 years. New therapies to prevent long-term scarring, together with new bioengineering technologies, will help to make this possible in the future.

Key issues

- Fibrosis and scarring are responsible for the pathogenesis or failure of treatment of all the major blinding diseases, with postoperative wound healing responses posing a major problem for most ocular surgery on a worldwide scale.
- Fibrosis prevention is one of the largest areas of unmet need in clinical medicine.
- Antibodies are a fast growing class of therapeutics and hold a lot of potential in the development of new anti-fibrotic treatments.
- Gene modulation and gene therapy represent an exciting and innovative approach to prevent scarring and post-surgical fibrosis in the eye.
- There have also been significant advances in small molecule therapeutics, including nanoparticles, liposomes, proteoglycans, dendrimers and inhibitors, to modulate ophthalmic wound healing.
- Optimal drug delivery and potential ocular toxicity remain major challenges in the development of effective anti-fibrotic therapies.
- Future research will focus on developing a more personalized and stratified approach in anti-fibrotic ocular therapeutics, combinations of therapies and the need for well-designed randomized controlled human trials.
- New therapies to prevent scarring together with new bioengineering technologies will also allow far better long-term clinical outcomes in the future.

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