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Corneal Stromal Regeneration: Current Status and Future Therapeutic Potential

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ABSTRACT

The corneal stroma comprises 90% of the corneal thickness and is critical for the cornea’s transparency and refractive function necessary for vision. When the corneal stroma is altered by disease, injury, or scarring, however, an irreversible loss of transparency can occur. Corneal stromal pathology is the cause of millions of cases of blindness globally, and although corneal transplantation is the standard therapy, a severe global deficit of donor corneal tissue and eye banking infrastructure exists, and is unable to meet the overwhelming need. An alternative approach is to harness the endogenous regenerative ability of the corneal stroma, which exhibits self-renewal of the collagenous extracellular matrix under appropriate conditions. To mimic endogenous stromal regeneration, however, is a challenge. Unlike the corneal epithelium and endothelium, the corneal stroma is an exquisitely organized extracellular matrix containing stromal cells, proteoglycans, and corneal nerves that is difficult to recapitulate in vitro. Nevertheless, much progress has recently been made in developing stromal equivalents, and in this review the most recent approaches to stromal regeneration therapy are described and discussed. Novel approaches for stromal regeneration include human or animal corneal and/or non-corneal tissue that is acellular or is decellularized and/or re-cellularized, acellular bioengineered stromal scaffolds, tissue adhesives, 3D bioprinting and stromal stem cell therapy. This review highlights the techniques and advances that have achieved first clinical use or are close to translation for eventual therapeutic application in repairing and regenerating the corneal stroma, while the potential of these novel therapies for achieving effective stromal regeneration is discussed.

Introduction

Therapeutic regeneration of the corneal epithelium primarily using stem cells of limbal origin in various forms has received much attention and gained widespread clinical use, including the first European Medicines Agency approved stem cell product, Holoclar. Likewise, endothelial regeneration therapy promoted through the use of a donor endothelial cell suspension has entered first clinical trials for bullous keratopathy. The bulk of the corneal tissue, however, consists of the corneal stroma, which comprises over 90% of the corneal thickness and imparts to the cornea its transparency, curvature and strength, and additionally protects the inner eye structures from the external environment. In clinical situations where the corneal epithelium and endothelium are healthy and functioning, the cornea can still lose its transparency by various corneal stromal pathologies that include infection, keratoconus, inflammation, neurodegeneration, neovascularization and corneal dystrophies. In these cases collectively affecting millions worldwide, the main therapeutic option has been to replace the corneal stroma with human donor tissue, either by full-thickness replacement of the cornea (eg., penetrating keratoplasty where epithelium, stroma, and endothelium are replaced) or by partial thickness stromal replacement (eg., anterior lamellar keratoplasty where epithelium and the anterior stroma are replaced; or posterior lamellar keratoplasty where posterior stroma and endothelium are replaced). In cases where human donor tissue transplantation has failed repeatedly or is contraindicated – for example due to a chronically inflamed recipient eye or high expression of enzymes that would degrade the corneal stroma – a keratoprosthesis such as the Boston KPro or the osteo-odontokeratoprosthesis could be used. These prosthetic devices are used in rare cases of severe pathology or trauma, and often only after repeated failed transplantation with a donor cornea, and are thus not suitable for the majority of cases of corneal stromal pathology; therefore, in this review we focus only on primary replacements for the corneal stroma.

Stromal replacement as implemented today, however, has its limitations. Implantation of a donor cornea into a recipient eye induces scarring at the donor-recipient tissue interfaces, results in incomplete nerve regeneration, incomplete cellular repopulation of the graft, changes in refraction due to suboptimal curvature of the implanted tissue, and carries the potential for immunologic rejection due to the foreign cells contained within the donor tissue. Although these potential drawbacks of the use of human donor stromal tissue can be significant, the main drawback of stromal replacement with human donor tissue is the global shortage of donated corneas and the need for infrastructure to identify, harvest, transport, store and distribute donor corneas within a limited time window. The need for donated corneal tissue and eye banking is a global problem that leaves millions of cases of avoidable corneal blindness unaddressed.
Similar to epithelial and endothelial regeneration, it would be ideal if the stroma could be regenerated, assisted by an implant, scaffold, cells or other factors. Although no single regenerative approach for the corneal stroma has yet emerged as an approved therapy for clinical use, several alternatives have been proposed and are the subject of intensive research, with some having reached the clinical trial stage. The purpose of this review is to summarize the various approaches for regenerating or replacing the corneal stroma, either partially or fully, in order to treat corneal pathology. These approaches aim to supplement or completely circumvent the use of human donor corneas from eye banks, which are in short supply and are an inadequate option for the future treatment of corneal stromal pathology in many parts of the world. In this review, the concept of stromal regeneration is summarized, while highlighting those approaches and technologies for stromal regeneration that are closest to clinical application.

**Evidence for endogenous stromal regeneration**

The regenerative capability of the corneal stroma (including the maintenance of corneal transparency during regeneration) is linked to the presence of various anatomic features, all serving to maintain a strong and transparent corneal stroma (Figure 1).

The most anterior part of the stroma consists of Bowman’s layer, an approximately 10 µm thick layer of compacted, randomly oriented collagen fibrils that merges continuously with the underlying corneal stromal lamellar structure. Bowman’s layer is non-regenerating, but when present is believed to facilitate a more rapid and complete anterior corneal nerve and anterior stromal regeneration and healing after injury. Below Bowman’s layer is the corneal stroma proper, consisting of approximately 300 distinct lamellae, each in turn consisting of parallel-oriented bundles of collagen fibers, mainly consisting of type I collagen. The major stromal cell, the keratocyte, is present throughout the stroma with the cell body often localized between stromal lamellae. Keratocytes have multiple functions including collagen synthesis, collagen degradation, and participating in wound healing through transformation to a (myo) fibroblast phenotype. Natural stromal regeneration through the normal keratocyte-mediated turnover of collagen is a relatively slow process, occurring over at least several years. This long turnover time may be a prerequisite for the regenerated collagen to adopt a proper lamellar structure and orientation to maintain corneal strength, structure and transparency (stromal regeneration); by contrast, short collagen turnover times are often associated with aberrant collagen production by myofibroblasts leading to stromal haze and permanent scar tissue deposition (stromal repair).

Besides keratocytes (and other smaller cell populations not discussed here), the corneal stroma contains proteoglycans, which are produced by keratocytes and consist of dermatan and keratan sulfate components. These components interact with collagen fibrils to maintain fibril assembly and corneal transparency. As they are synthesized by keratocytes, the proteoglycan population of the stroma can regenerate. Finally, corneal nerves (axons originating from the trigeminal nerve) are present throughout the corneal stroma, with high densities present in the anterior stroma within the subepithelial and subbasal nerve plexi. Corneal nerve regeneration is an important factor in maintaining stromal integrity, restoring a protective blink reflex and perception of pain, temperature and chemical stimuli, and for helping to ensure proper tear film production and adequate wetting of the ocular surface. Corneal nerves additionally facilitate epithelial wound

![Figure 1. The human corneal stroma. A 10 µm-thick Bowman’s layer consisting of randomly oriented compacted collagen fibrils comprises the anterior portion of the stroma. Posterior to Bowman’s layer lies the corneal stroma proper, consisting of less compact but regularly arranged collagen fibrils organized in lamellae. Stromal cells called keratocytes are regularly interspersed throughout the stroma, preferentially between adjacent lamellae. Distributed within the lamellae in the extracellular matrix are proteoglycans produced by the keratocytes. Finally, corneal nerves traverse through the stroma and anteriorly form a dense subbasal nerve plexus.](image-url)
healing; in corneas with a neurotrophic deficit, epithelial wounds do not heal adequately, leading to stromal degradation (ulceration). Corneal nerves within the normal, healthy subbasal nerve plexus (subbasal nerves) are continuously regenerating, and this regeneration even occurs in the context of corneal injury or transplantation, although the regeneration may take several years (Figure 2). Given their physiological importance, corneal nerves and their regeneration should be an integral part of strategies aiming to regenerate the stroma.

Human donor stromal lenticules

The refractive surgery procedure of small-incision lenticule extraction (SMILE) has been gaining popularity in the past decade due to the availability of accurate, high frequency pulsed femtosecond lasers and excellent refractive results postoperatively. The procedure involves removal of a small disc (lenticule) of stromal tissue approximately 10–50 µm thick and 6–8 mm in diameter from the central stroma (at least 100 µm from the most anterior stromal layer), in order to flatten the cornea and correct for myopia (Figure 3). To date over one million SMILE procedures have been performed, potentially making a large pool of thin stromal lenticules available for treating stromal pathology. To facilitate this approach, Ganesh and colleagues developed a tissue processing and cryopreservation technique for the extracted lenticules, making long-term storage possible in a cryobank at liquid nitrogen temperatures (−196°C). The lenticules extracted from myopic patients after SMILE were cryopreserved and subsequently implanted into hyperopic corneas, with no reported allogeneic rejection or loss of best corrected visual acuity. In a separate study, Ganesh and colleagues used similarly extracted stromal lenticules to treat a series of cases of corneal micro-perforation, corneal defect and traumatic corneal tear, by using the lenticule as a bandage patch by attaching it to the recipient cornea using fibrin glue. The procedure was safe and maintained corneal transparency and integrity. Extending the application of this concept, Wu and colleagues used SMILE lenticules with thickness over 100 µm to seal corneal perforations by suturing the lenticules over the perforation sites. Interestingly, a few weeks following the operation, lenticules became incorporated into the corneal stroma, indicating a partial regenerative effect. In another study by Ganesh and colleagues, the center of the extracted lenticules was trephined to form a donut-shaped lenticule that was subsequently implanted into the cornea of a small series of keratoconus patients, to achieve effective central corneal flattening.

The inherent biocompatibility of human-to-human tissue implantation, with potential reduction of immunogenicity of the allograft tissue by cryopreservation, is expected to be beneficial for integration of the implanted lenticule with the surrounding recipient stromal tissue. A regenerative effect could be achieved by long-term quiescent stromal cell infiltration and turnover of the implanted collagen. This effect, however, has not yet been convincingly demonstrated in the case of human lenticule implantation, and thus the long-term fate of the lenticules is unknown. Would the thin lenticules degrade (and therefore corneal thickness decrease) over longer time periods or would the lenticules persist? Another question to be addressed with this technique is its extendibility to replace a thicker proportion of the stroma (Figure 3). Could multiple lenticules be implanted simultaneously, and would such an approach be technically feasible and result in an adequate transparency, strength and corneal shape? Future research could address these questions. Furthermore, it has been proposed that a chemical decellularization method could be an alternative to cryopreservation of donor lenticules.

Acellular porcine corneal stroma

Decellularization methods for supplementing the supply of donor corneas have gained popularity in recent years, specifically in the use of non-human (typically porcine) corneal tissue for human corneal transplantation. The use of non-human corneas presents an alternative to reliance on human cornea donation, as porcine corneas are readily obtainable.
from the food processing industry and have similar characteristics to the human cornea in terms of size, thickness and biomechanical properties. Decellularization is required to mitigate the possibility of xenogenic immunoreactivity to the foreign porcine cells and the possibility of porcine-to-human disease transmission, and as such, applications are currently limited to stromal replacement and anterior lamellar keratoplasty in cases where recipient endothelium is functional and the recipient epithelium is able to regenerate to cover the graft.

Porcine corneas have been subjected to various decellularization protocols including sodium chloride (NaCl), sodium dodecyl sulfate (SDS), hypoxic nitrogen, ethylene diamine tetra-acetic acid (EDTA), aprotinin, gamma irradiation and hypotonic tris buffer. The chemical treatments are often followed by DNAse and RNAse treatments. Such treatments can substantially remove porcine cells and DNA; however, removal of the foreign immunogenic material is often not 100% complete and thus the possibility of transmission of porcine pathogens remains. Despite this, results of decellularized porcine cornea xenotransplantation in a rabbit model indicated absence of immune rejection and good biocompatibility after one year. In another report, porcine corneal stroma was decellularized by NaCl treatment and implanted into the rabbit corneal stroma for six months with or without the addition of cultured human corneal keratocytes. Without cells, a 100 µm thick stromal implant remained stable but without reported evidence of host cellular infiltration or stromal regeneration, while the addition of human keratocytes sandwiched between five 20 µm thick porcine stromal sheets resulted in an initially transparent and stable cornea postoperatively, but rendered the implanted sheets undetectable at the experimental endpoint.

Decellularized porcine corneas have also been used clinically. A Chinese team in Wuhan reported a series of 47 patients with prior fungal corneal infections who underwent anterior lamellar keratoplasty, receiving acellular porcine corneal stromal grafts. No recurrence of infection was noted and healing of ulcers was reported in most cases, although with the return of neovascularization and non-epithelialization leading to graft melting in four cases. In another Chinese study, acellular porcine corneal stroma was used in lamellar keratoplasty to treat 13 patients for herpes simplex keratitis. After a mean of 15 months of postoperative follow-up, seven cases had mostly transparent corneas but 11 of 13 cases had neovascularization, with graft melting occurring in three cases. Currently, a prospective clinical trial is ongoing in Guangzhou, using the acellular porcine cornea as a deep anterior lamellar graft in cases of infective keratitis. These reports, principally from China, indicate a potential future role for decellularized porcine corneas to meet the global demand for suitable stromal tissue for transplantation, which is particularly acute in China. Based on the reported results, future attention needs to be directed towards improving epithelialization, transparency, and avoidance of neovascularization of the graft. Detailed postoperative analysis (or ex vivo analysis of explanted, failed grafts), however, is currently lacking. It is unclear whether the porcine corneal stroma can stably permit stromal regeneration. Ideally for
regeneration, host keratocytes should migrate into the graft and produce new human collagen as the porcine collagen is gradually degraded, while corneal thickness and transparency are maintained. Conversely, a non-regenerative reaction would consist of inflammatory cell and fibroblast invasion, stromal edema, neovascularization and collagen degradation, resulting in corneal thinning and topographic irregularities. A further consideration of importance is the postoperative regeneration of corneal nerves. Does the porcine stroma permit nerve regeneration and to what degree? Could outcomes be improved with the infiltration of host nerves into the porcine stroma?

Full-thickness corneas based on porcine corneal stroma

Since the acellular porcine corneal stroma can only be used therapeutically when the corneal epithelium and endothelium are functional, attention is being given to repopulating the stromal scaffold with human cells, to enable full-thickness penetrating keratoplasty. A Chinese team from Shandong has demonstrated the ability to seed human embryonic stem cell (hESC)-derived limbal epithelial-like cells on one side of the porcine scaffold and hESC-derived corneal endothelial-like cells on the other. After transplantation into rabbits, corneal transparency was gradually improved through 8 weeks of follow-up, although residual haze and neovascularization were present. Nevertheless, along with vessels and inflammatory cells, stromal cells were observed to repopulate the implanted porcine stroma at only 8 weeks post-implantation. Whether stromal regeneration would eventually occur is unclear, but the given the ethical debate surrounding the use of hESC, alternatives are sought. One alternative to the use of hESC, involves a similar porcine stromal construct that was developed using human donor cornea-derived epithelial, stromal and endothelial cells that were seeded into the construct and prepared by cell culture methods. One week after implantation by penetrating keratoplasty in rabbits, the construct remained almost completely transparent, before an eventual immune response led to rejection and opacification of the xenogenic graft at four weeks.

The availability of appropriate animal models to test stromal constructs containing human cells remains an issue. Additionally, initial results suggest an immune reaction of the host to possible cellular and/or DNA remnants within the porcine cornea that may compromise transparency and initiate a neovascular response. With effective strategies to remove all donor xenogenic material and sterilize the porcine corneas, along with specific postoperative regimens to suppress possible immune reaction, porcine stromal tissue could play a greater role in the future in supplementing the human donor tissue pool for applications in therapeutic keratoplasty. It remains to be determined, however, how the porcine corneal tissue (with or without cells) will be regulated. In particular, within the regulatory framework in the EU, the acellular stroma would be classified as a medical device while the addition of cells would render it an advanced therapy medicinal product (ATMP). Stringent GMP manufacturing, quality and safety controls and standards would have to be met, encompassing the entire supply, production and distribution chains.

Fish scale-derived cornea

Another innovative biological source of collagen-based stromal scaffolds are the scales of the Tilapia fish. Normally considered a waste product of fish processing, the scales of the fish can be decellularized and decalcified to produce a scaffold compatible with the culture of corneal cells. Manufactured to an appropriate thickness, a transparent stromal scaffold derived from the fish scale was evaluated after intrastromal implantation in rabbit corneas, where it was found that the scaffold could be retained in vivo for one year, maintaining transparency without eliciting an immune response. As the fish scales are mechanically robust, they additionally have the potential to be sutured onto the cornea in cases of perforating trauma. This application was tested using a model of full-thickness perforations in minipig corneas, where the fish scale-derived scaffold was successfully used as an emergency patch graft (termed the ‘Biocornea’), to temporarily seal the cornea and maintain the integrity of the anterior chamber and eye globe for up to four days (within which time a suitable donor cornea can be sourced in an emergency trauma situation). Following positive results in the minipig model, the fish scale scaffold is currently undergoing a human clinical trial as a temporary patch graft for traumatic corneal perforation (J.J. Schuitmaker, personal communication).

Bioengineered acellular corneal stroma

Another approach to stromal regeneration not involving the use of harvested tissue from humans or animals is the engineering of corneal stromal equivalents from raw starting materials. These ‘bioengineered’ stromal equivalents have the advantage that their properties can be tuned for a specific application or physiologic effect, and that they do not rely on tissue harvesting or availability of corneal tissue from animals or from humans. Furthermore, because the stroma is engineered from basic building block components, cells can be expressly excluded from manufacturing, to create a truly acellular stroma not requiring decellularization. This would greatly improve the biocompatibility of the stromal equivalent by avoiding the host immune response. As the implanted stroma is acellular, however, stromal regeneration would require host cell infiltration and new collagen production.

One such stromal scaffold is the acellular recombinant human collagen-based scaffold, termed the ‘biosynthetic’ corneal stroma. The biosynthetic scaffold is composed of human collagen (the main protein constituent of the human cornea) produced recombinantly in yeast, and is strengthened by chemically crosslinking the collagen using the non-toxic and water-soluble EDC/NHS system. We implanted this biosynthetic scaffold by anterior lamellar keratoplasty into ten Swedish patients to treat advanced keratoconus and corneal scarring. After initial limited melting and haze formation due to the softness of the scaffold (i.e., ‘cheese-wiring’ by the retaining sutures), the scaffolds remained stable and functional for at least four years postoperatively, without detectable inflammation or rejection. The bulk of the stroma was transparent as indicated by clinical examination, however,
some increased lamellar interface reflectivity was detected by optical coherence tomography (Figure 4a,b). At the cellular level, in vivo confocal microscopy revealed that after three years, host stromal cells were present at the lamellar interface regions; however, a clear migration of host stromal cells into the biosynthetic scaffold could not be detected (Figure 4c,d). Nerve regeneration, however, had occurred by three years. Regenerated subbasal nerves, visualized by in vivo confocal microscopy, were present in the anterior portion of the scaffold (Figure 4e,f). In a patient re-transplanted after four years due to suboptimal vision, histologic analysis of the excised tissue revealed regeneration of a stratified epithelium to cover the scaffold and increased density of stromal tissue at the lamellar interfaces, consistent with the presence of denser, scar-type tissue (Figure 4g). Furthermore, while stromal cells were present at the interface region, the cells did not enter the implanted scaffold.

To overcome the limitations of the relatively soft biosynthetic scaffold and improve resistance of the scaffold to enzymatic degradation and the robustness for surgical implantation, we subsequently used medical-grade purified porcine collagen (extracted from porcine skin) with the EDC/NHS crosslinking system to develop mechanically stronger stromal scaffolds that were additionally more resistant to enzymatic degradation. These bioengineered porcine collagen constructs (BPC) were tested in rabbit models by intrastromal implantation.52,53 The BPC scaffolds were biocompatible and maintained corneal transparency up to the three month experimental endpoint without a host immune reaction. Implantation of the scaffold was followed by stromal (myo) fibroblasts migrating to the lamellar interface and producing small amounts of type III collagen in a thin region surrounding the scaffold (Figure 5a). These fibroblasts then entered the implant at the edges, where they continued to produce type III collagen while simultaneously degrading the scaffold (Figure 5b). The new collagen produced by these cells differed from the scaffold collagen but resembled the host stroma, based on the staining pattern. This indicates a partial regeneration of host stroma by host stromal cells, facilitated by scaffold implantation. Interestingly, the acellular collagen scaffolds retained stromal proteoglycans from the original porcine dermal source as observed by transmission electron microscopy (Figure 5c,d); however, the in vivo functionality or integrity of these proteoglycans were not examined. This bottom-up approach of scaffold fabrication from purified collagen as a raw material enables stromal scaffolds to be manufactured with a desired diameter, thickness, or in a hybrid fashion where different parts can be tuned to have differing transparency and degradation properties.53 A newer version of the BPC scaffolds have subsequently been tested in a minipig model54 and have been implanted within the stroma in patients with advanced keratoconus, with positive initial results (M. Rafat, personal communication). Besides addressing the global shortage of donor stromal tissue for transplantation, the bioengineered constructs can be manufactured and stored for up to two years prior to use, based on validated shelf life studies (M. Rafat, personal communication).

Another interesting option reported for the treatment of corneal ulcers is the use of ‘plastic compressed collagen’ as a therapeutic option where amniotic membrane grafts fail to epithelialize the ulcerated region. Schrader and colleagues report a commercial-grade CE-marked type I porcine dermal atelocollagen used to create a mechanically compressed collagen matrix that is more dense and robust compared to typical hydrogel-based scaffolds, while retaining high tensile strength and elasticity.55 The compressed collagen scaffold, however, was not crosslinked and is therefore prone to degradation. In a first clinical case, the compressed collagen matrix

Figure 4. Postoperative assessment of stromal regeneration following biosynthetic corneal implantation into keratoconus patients by anterior lamellar keratoplasty, three years postoperatively (A – F) and four years postoperatively in one patient (G). (a) The anterior lamellar scaffold remained transparent, with border region visible in the slit lamp (border indicated by arrows). (b) Optical coherence tomography reveals the intact scaffold with increased stromal density and reflectivity at implant-to-host interfaces (arrow). (c, d) By in vivo confocal microscopy, stromal cells from the host have not migrated to occupy the implanted scaffold (dark, transparent region). (e, f) Subbasal nerves anterior to the scaffold have partially regenerated (arrows). (g) Hematoxylin and eosin stained section from an excised corneal button indicates an intact scaffold (white asterisk) that remains acellular. Host stromal cells (black arrows) are present at the scaffold-to-host lamellar interface, within a region of denser, more compacted collagen (black asterisk) indicated by stronger eosinophilic staining. Stromal fibroblasts also migrated anteriorly to the implant under the epithelium and deposited a layer of denser collagen, consistent with the light-scattering scar tissue (white arrows) also observed anteriorly in the subbasal plexus (F). Images (C – F) are 400 x 400 µm.
was used to fill the ulcerated defect region (as a corneal inlay) while a second identical matrix was used to cover the cornea as a bandage (onlay). While the bandage onlay degraded within the first postoperative week, it facilitated coverage of the entire ulcerated region by recipient corneal epithelium, with the inlay portion remaining intact. During the following six month period, the inlay also degraded but with epithelium remaining intact without signs of inflammation or immune rejection. Stromal regeneration was not demonstrated, and it is not clear whether the inlay had an impact upon the host stroma. This type of stromal replacement however, although temporary, may provide a means to heal otherwise untreatable ulcers, enabling future vision rehabilitation (by for example, keratoplasty) in these cases. It is interesting to note that even a temporary stromal scaffold can promote epithelial regeneration in this context; stabilization of the plastic compressed scaffold by crosslinking the collagen could possibly provide a means to tailor the degradability in cases where a longer-lived stromal scaffold is desirable.

Cell-populated engineered scaffold

In cases where host cells would be unsuitable or cannot repopulate the scaffold, an alternative approach is to provide appropriate cells along with the scaffold. This approach is illustrated by the bioengineered human allogeneic nanostructured anterior cornea developed by a Spanish team. The construct consists of a fibrin-agarose scaffold made from a combination of human blood plasma and 0.1% agarose formed into a scaffold with nanoscale features. Donor human corneal fibroblasts are implanted into the scaffold and donor corneal limbal epithelial cells are seeded on top, to create an anterior lamellar graft under full GMP conditions in vitro, as an authorized investigational advanced therapy medicinal product. At the time of writing, a Phase I/IIA randomized multicenter clinical trial is being conducted in Spanish hospitals, to treat refractory neurotrophic ulcers in 20 patients by anterior lamellar keratoplasty. In the first five patients receiving the fibrin-agarose scaffold, complete healing

Figure 5. Evidence for stromal regeneration following implantation of a bioengineered porcine construct (BPC) scaffold into rabbit corneas for 8 weeks. (a) Immunohistochemistry of the cornea with staining for scar-type collagen III indicates limited new collagen production by host stromal fibroblasts at the lamellar interface (arrows). Note that the fibroblasts migrate along the lamellar surface until the edge of the scaffold is reached, before entering the scaffold at the edge (asterisk). (b) At the scaffold edge, fibroblasts enter the scaffold (arrows), breaking down the scaffold collagen (hematoxylin stained, gray color) and producing new host-derived collagen, as indicated by the eosinophilic pink color. (c) Transmission electron microscopy image of host rabbit stromal collagen stained for proteoglycans, visible as dark electron-dense spots (arrows). Note the distribution of proteoglycans in parallel lines, following the parallel orientation of collagen fibrils in the native rabbit stroma. (bd) In the scaffold, proteoglycans are also present but appear randomly distributed (arrows).
of the ulcer was observed without signs of infection or allogeneic rejection at a two-year follow-up, and the patients reported relief of ocular symptoms. This promising approach awaits final evaluation. It will be important to determine the fate of the scaffold and implanted cells, including signs of tissue regeneration, in terms of re-establishment of stromal architecture, structure and thickness, and stromal cell (and possibly nerve) repopulation of the lamellar graft.

Tissue adhesive gel

For the repair of corneal defects due to traumatic injury or infection that result in stromal thinning, an alternative to invasive corneal transplantation or use of pre-formed scaffolds has been recently proposed by a US-based team. The device is a stromal mimic that is a viscous gel-like substance when applied to the cornea, which adapts to the form of the defect in situ. Once the defect is filled, the gel is ‘locked’ in place and stabilized into a more rigid stromal mimic through photopolymerization. This technology, called GelCORE (gel for corneal regeneration) has been developed as an adhesive biomaterial to treat corneal stromal loss. The biomaterial is natural and acellular in that it is derived from porcine gelatin that is stabilized by addition of methacrylic anhydride. The result is a tough but elastic and transparent biomaterial that can be photopolymerized using visible blue light, thereby avoiding exposure of eye structures to the potentially damaging UV light normally used to photocure polymers. The GelCORE was shown to be compatible with human corneal fibroblast migration in vitro, without inducing cell death after visible light polymerization. In a rabbit model of keratectomy with 50% of the stromal depth removed, the GelCORE was applied with four-minute photopolymerization time, and resulted in a transparent and smooth cornea that epithelialized within a week. Histologic analysis at 14 days revealed some inflammatory cell infiltration in the implanted zone, but the bulk of the GelCORE stromal substitute was no longer detectable, indicating either a rapid transformation or degradation following epithelial coverage. Initial results are encouraging, and more detailed longer-term in vivo evaluation would yield further insights into the fate of the biomaterial and its potential to stimulate stromal regeneration.

3D bioprinting

Unlike bioengineered corneas made from raw materials that are dispensed or moulded into stromal tissue equivalents, three-dimensional bioprinting offers additional flexibility for controlling the spatial organization of the fabricated stroma. A bioprinter additionally offers the possibility to combine different biomaterials, cells and other factors into a customized matrix with high spatial resolution. As an initial step in this direction, Connolly and colleagues reported a 3D bioprinted corneal stromal equivalent fabricated using methacrylated type I collagen and sodium alginate as bioinks, followed by crosslinking using calcium chloride. Incorporation of human corneal keratocytes within a collagen-alginate bioink resulted in a 3D bioprinted stromal equivalent that retained live keratocytes for 7 days.

An alternative cell-based approach reported by Skottman and colleagues was the laser bioprinting of a 3D stromal equivalent using two bioinks, one containing type I human collagen and the other containing a mixture of human adipose stem cells (hASC), collagen, human plasma and thrombin. To create the stromal equivalent, ten alternating layers of acellular collagen and hASC were printed to form a 7x7 mm and 500 µm thick stromal equivalent. After 14 days, thickness had reduced to 300 µm but cells were viable, proliferating, and had aligned themselves in a lamellar organization mimicking the native corneal stroma. Advantages of incorporating stem cells into the stroma are their repair and proliferative capacity and the potential for non-immunogenicity, when compared to allogeneic differentiated stromal cells such as keratocytes or fibroblasts.

While the above bioprinting approaches are a promising means to achieve stromal regeneration, they await in vivo evaluation in suitable animal models, cell fate determination post-implantation, and demonstration of stromal regenerative ability such as scar-free healing and new collagen production. Nevertheless, these and similar 3D printed scaffolds are attractive as they have a highly controllable production process amenable to mass production, or alternatively could enable customized patient-specific structures to be printed, guided by the condition and topography of the patient’s cornea.

Stromal regeneration by stem cell therapy

As an alternative to incorporating cells within a stromal construct, ‘stromal cell therapy’ aims to harness the direct and/or paracrine functions of stem cell populations to repair and regenerate the existing corneal stroma. Cell therapy can be initiated for example, by direct delivery of therapeutic cells into the diseased or damaged stroma in situ. This concept has been demonstrated for treatment of corneal scars that would normally require corneal transplantation. Instead of transplantation, Basu and colleagues isolated human corneal stromal stem cells (hCSSC) from the limbal stroma of an allogenic limbal biopsy and demonstrated that these cells possess mesenchymal stem cell-like properties. Following isolation of these stem cells in cell culture, they were then introduced into the anterior stroma of the mouse cornea after stromal debridement, and kept in place using standard fibrin glue. The stem cells were still detectable four weeks post-implantation, at which time the stroma had healed without scarring. At the time of writing, this therapy is undergoing two human clinical trials in India, for the treatment of stromal injury, scars and opacities due to pathology or surgery.

While this approach is a promising means to support regenerative stromal healing, the cells are sourced from the limbal stromal region of donor corneas and are therefore still dependent on an adequate supply of donated tissue. The donated tissue, however, may not necessarily need to be from the same species, as illustrated in the xenogenic human-to-mouse cell delivery above, and in other studies of cell-based stromal repair due to their apparent ability to avoid a host immune response. Non-human derived CSSC have yet to be tested definitively for...
stromal regeneration and immunomodulatory potential in vivo, but such cells could provide a source of CSSC not dependent on human donors. hCSSC, however, have the ability to synthesize human stromal collagen and deposit this collagen in an organized manner to mimic the transparent arrangement of collagen fibrils in the native cornea. The approach of local hCSSC stromal delivery may therefore not only provide a means to aid bio-integration and scar-free healing of implanted biomaterials in the stroma, but hCSSC may also improve outcomes in xenogenic transplants (such as the acellular porcine corneal stroma described earlier) through their immunomodulatory properties. Another point worth mentioning is the lack of apparent effect of native hCSSC in cases of corneal scarring where the limbal niche is functional and intact. If CSSC exist in the stroma in close proximity to limbal epithelial stem cells (LESC) in the Palisades of Vogt region, why do they not contribute to stromal wound healing, in an analogous manner as LESC's contribute to epithelial wound healing? Suboptimal wound healing post-transplantation may lead to stromal scarring, for instance the host-to-donor interface scarring commonly seen after penetrating and lamellar keratoplasty. It appears that hCSSC are not able to effect optimal stromal wound healing while in their native niche to inhibit such interface scarring, whereas their isolation and local delivery to the wounded stroma has an observable effect. The current belief that CSSC provide an important support function in maintaining the LESC phenotype\(^70\) may therefore mean that the primary function of the in situ CSSC is to maintain the function of the limbal niche and not to repair damaged corneal stroma. Nevertheless, it would be of great interest if CSSC could be stimulated to effect stromal repair in situ, without requiring their extraction from a donor, purification and re-introduction into host stroma.

A promising source of cells for stromal therapy avoiding reliance on donor corneas is being developed by Alió and colleagues in Spain, who used autologous adipose-derived adult stem cells obtained from an elective liposuction procedure. These stem cells were introduced mid-stromally in advanced keratoconus patients with a thin corneal stroma.\(^71\) The cells were introduced via a femtosecond laser-created pocket, either alone or in combination with a 120 µm thick decellularized human donor stromal implant that had been re-cellularized with the autologous stem cells prior to implantation. 12 months postoperatively, all 9 corneas receiving the autologous adipose-derived stem cells were transparent. Five corneas receiving only the stem cells had increased in thickness by 14.5 µm while those receiving the re-cellularized stromal implant with stem cells had maintained the stromal implant thickness but with no further increase in thickness. While this study demonstrates the concept of autologous stromal cell therapy, which has the added benefit of immune compatibility, the results suggest that cell therapy alone is insufficient to replace the bulk of the corneal stroma within a reasonable time frame; instead, the cells could augment healing in cases where for example a non-donor stroma is used, such as a bioprinted or bioengineered stromal equivalent.

### Conclusions and perspectives

Many of the therapies discussed herein are not mutually exclusive; several could be combined for optimal therapeutic effect, for example acellular stromal scaffolds and therapeutic cells. Moreover, specific therapies can be personalized, for example by 3D bioprinting a scaffold designed to fit into a specific recipient bed, bioengineering a stromal equivalent to desired dimensions and with a tailored time to degradation, or using autologous cells to avoid immune reaction while promoting regenerative healing through paracrine processes. The future also holds further hope for newer advanced techniques not described here and which are still in a nascent stage, such as the use of stem-cell derived exosomes (extracellular vesicles) for promoting scar-free stromal healing\(^72\) and the use of iPSC-derived cells or organoids\(^73,74\) to promote autologous, regenerative healing. The next few years will likely see the first stromal equivalents and cell-based technologies being approved for clinical use, initially for conditions non-responsive to conventional treatments. As ophthalmic specialists and surgeons become more familiar with these new therapies and as they become more readily available and cost-effective, such niche applications over time are likely to be broadened to include additional indications affecting larger patient populations.

Although many of the proposed stromal regeneration techniques claim to be regenerative, very few demonstrate regeneration or turnover of stromal collagen, restoration of a lamellar stromal organization or regeneration of corneal nerves critical for trophic support of the stroma. In addition to anti-scarring treatments and repair of the extracellular matrix, neuro-regenerative factors should also be provided to the regenerating stroma, either in the form of specific axonal growth factors or as stem cell-derived paracrine factors providing a milieu favoring nerve growth. An overview of the various approaches for stromal regeneration and the evidence for stromal regeneration in vivo is given in Table 1. It is clear that much work is still necessary to demonstrate stromal regeneration in vivo in animal models and in humans. Beyond initial integration of implanted stromal scaffolds into host corneas without rejection or extensive tissue degradation, stromal melting or scar tissue formation, the regeneration of stromal cells, matrix proteins, nerves and proteoglycans has been difficult to demonstrate. Part of the difficulty in demonstrating stromal regeneration may be the time frame required, as normal stromal collagen turnover and nerve regeneration are typically slow processes that can take many years. In vivo detection of stromal regeneration in humans can also be challenging, but may be facilitated by careful cellular and extracellular matrix morphologic observations using high-resolution imaging techniques such as in vivo confocal microscopy and optical coherence tomography.

Finally, stromal regeneration may represent the final piece in the puzzle in the quest towards a fully engineered corneal substitute. The combination of engineered epithelial and stromal parts into an anterior corneal equivalent, or endothelial and stromal parts into a posterior corneal equivalent could find application in treating a wide range of corneal pathology normally requiring donor tissue transplantation. An engineered full
Table 1. Summary of various approaches for achieving stromal regeneration and evidence for regeneration in animal models and in human studies.

<table>
<thead>
<tr>
<th>Stromal Regenerative Approach</th>
<th>Key advantages</th>
<th>Stromal regeneration in animal models</th>
<th>Stromal regeneration in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decellularized scaffolds</td>
<td>Availability, native stromal architecture, strength</td>
<td>Integration with rabbit stroma and macaque stroma</td>
<td>Integration with host stroma</td>
</tr>
<tr>
<td>Human donor stromal lenticules (SMILE)</td>
<td>Abundant, human stromal tissue</td>
<td>Integration with rabbit stroma, host cells in scaffold</td>
<td>No evidence provided</td>
</tr>
<tr>
<td>Acellular porcine corneal stroma</td>
<td>Abundant, low cost, thick stromal tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recellularized scaffolds</td>
<td>Cells can actively effect stromal repair</td>
<td>No evidence provided in rabbit model</td>
<td>Integration with host stroma, cell survival</td>
</tr>
<tr>
<td>Human embryonic stem cells</td>
<td>Pluripotent, high proliferative potential</td>
<td>Integration with rabbit stroma, delivered cells remain in scaffold</td>
<td></td>
</tr>
<tr>
<td>Adipose-derived MSC</td>
<td>Abundant, autologous, non-immunogenic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human donor corneal cells</td>
<td>Corneal cell type, isolation from donor corneas</td>
<td>Scafod not detected in rabbits, no evidence in rabbits, scar suppression in mice</td>
<td>No evidence available</td>
</tr>
<tr>
<td>Cell-free bioengineered scaffolds</td>
<td>Bottom-up design, low immunogenicity, no reliance on donor eyes</td>
<td>Poor integration in rats, tolerated in rabbits with macrophage infiltration, no integration in mini-pigs</td>
<td></td>
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<tr>
<td>Fish-scale collagen</td>
<td>Abundant, low cost, natural biocompatible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bioengineered collagen</td>
<td>Natural biocompatible, biocompatibility</td>
<td>Integration with rabbit stroma, host cells in scaffold, scaffold breakdown and new collagen production</td>
<td>Integration with host stroma, nerve regeneration</td>
</tr>
<tr>
<td>Tissue adhesives</td>
<td>Strength, biocompatibility, ease of application</td>
<td>Rapid scaffold degradation in rabbits, rapid ECM deposition hypothesized</td>
<td></td>
</tr>
<tr>
<td>GelCORE</td>
<td>Natural biocompatible, biocompatibility, photopolymerization</td>
<td></td>
<td></td>
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<tr>
<td>GelCORE</td>
<td></td>
<td></td>
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<tr>
<td>Cell-based without scaffold</td>
<td>Cell-mediated, no biomaterial needed, direct delivery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone-marrow-derived MSC</td>
<td>Abundant, autologous source, stromal lineage, immunomodulatory</td>
<td>Cells survive and differentiate into keratocytes in mouse stroma</td>
<td></td>
</tr>
<tr>
<td>Adipose-derived MSC</td>
<td>Autologous MSC source, abundant</td>
<td>Cells survive in rabbit stroma, differentiate to keratocytes, new collagens produced</td>
<td>Cells survive, slight stromal thickness increase, possible new collagen production</td>
</tr>
<tr>
<td>Umbilical cord-derived MSC</td>
<td>Availability, cryopreservation for later use, young cells highly potent</td>
<td>Cells survive in mouse stroma, differentiate to keratocytes, synthesize ECM components</td>
<td></td>
</tr>
<tr>
<td>Induced pluripotent stem cells</td>
<td>Multipotent, high potency, autologous</td>
<td>Recovery of stromal transparency following wounding in mice, suppresses inflammation</td>
<td></td>
</tr>
<tr>
<td>Human corneal stromal stem cells</td>
<td>Isolation from donor cornea rims, stromal origin/fate</td>
<td>New ECM components produced in healthy and injured stroma in mice</td>
<td>No evidence available</td>
</tr>
</tbody>
</table>

Abbreviations: SMILE: small-incision lenticule extraction; MSC: mesenchymal stem cells; GelCORE: gelatin-based adhesive for corneal regeneration; ECM: extracellular matrix

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Declaration of interest
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