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REVIEW

Corneal stem cells and tissue engineering: Current advances and future perspectives

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Abstract

Major advances are currently being made in regenerative medicine for cornea. Stem cell-based therapies represent a novel strategy that may substitute conventional corneal transplantation, albeit there are many challenges ahead given the singularities of each cellular layer of the cornea. This review recapitulates the current data on corneal epithelial stem cells, corneal stromal stem cells and corneal endothelial cell progenitors. Corneal limbal autografts containing epithelial stem cells have been transplanted in humans for more than 20 years with great successful rates, and researchers now focus on *ex vivo* cultures and other cell lineages to transplant to the ocular surface. A small population of cells in the corneal endothelium was recently reported to have self-renewal capacity, although they do not proliferate *in vivo*. Two main obstacles have hindered endothelial cell transplantation to date: culture protocols and cell delivery methods to the posterior cornea *in vivo*. Human corneal stromal stem cells have been identified shortly after the recognition of precursors of endothelial cells. Stromal stem cells may have the potential to provide a direct cell-based therapeutic approach when injected to corneal scars. Furthermore, they exhibit the ability to deposit organized connective tissue *in vitro* and may be useful in corneal stroma engineering in the future. Recent advances and future perspectives in the field are discussed.

Key words: Cornea; Stem cells; Cell-based therapy; Limbal transplantation; Tissue engineering; Corneal endothelium

Core tip: Corneal stem cell-based therapies represent a novel strategy that may substitute conventional corneal transplantation, albeit there are many challenges ahead given the singularities of each cellular layer of the cornea. To date, the most widespread clinical approach to regenerative medicine in the eye has been the utilization of limbal stem cells for ocular surface diseases. New therapeutic strategies are under development due to the recent identification

of corneal stromal stem cells and corneal endothelial cell progenitors. This review recapitulates the current advances and discusses future perspectives in the field.

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INTRODUCTION

The human cornea consists of 5 recognized layers, 3 of them are cellular (epithelium, stroma and endothelium) and 2 are interface layers (Bowman's layer and Descemet membrane)[1]. Corneal epithelium is a stratified, non-keratinizing squamous layer characterized by extreme uniformity from limbus to limbus[2]. Epithelial cells are regularly shed from the surface and replaced by new cells that are ultimately provided by stem cells located at the limbal area of the peripheral cornea[2]. Corneal stroma provides the bulk of the structural framework of the cornea and comprises approximately 80% to 90% of its thickness. Keratocytes are the major cell type of the stroma and are involved in maintaining its extracellular matrix[1]. An intricate collagen network in the extracellular matrix accounts for the cornea's shape, stability, mechanical strength and transparency[3]. Posteriorly, a thin monolayer of polygonal cells constitutes the corneal endothelium. Since it is in contact with the aqueous humor, the endothelium regulates corneal hydration through active cell pumps, thereby being crucial to maintain corneal transparency[4]. Except for oxygen, all corneal nutrients come from the aqueous humor through endothelial cells[5].

Regenerative medicine for the cornea represents a novel treatment strategy for patients with corneal diseases. Enormous promise has been ascribed to this modality because it offers a paradigm shift from conventional corneal transplantation to stem cell-based therapeutic strategies[6]. Cellular therapies are challenging given the singularities of each cellular layer of the cornea. To date, the most widespread clinical approach to regenerative medicine in the eye has been the utilization of limbal stem cells for ocular surface diseases. In 1989, Kenyon and Tseng[7] reported successful autologous transplantation of limbal tissue from the healthy fellow eye in 26 cases of unilateral limbal stem cell deficiency. Many reports and studies of limbal stem cell transplantation have followed, from heterologous donors to *ex vivo* cellular cultures.

In the present article, we discuss the current status in corneal stem cell research and the perspectives regarding corneal regenerative therapies and tissue engineering.

CORNEAL EPITHELIUM

Corneal epithelial stem cells are located at the corneoscleral limbus and are the ultimate source for regeneration of the entire corneal epithelium under normal and injured states[2,8,9]. Multiple diseases that destroy the limbal epithelial stem cell, its environment, or a combination of both can lead to a clinical condition known as limbal stem cell deficiency (LSCD)[10]. The clinical picture of LSCD is a conjunctivalized corneal surface with neovessels and recurrent or persistent epithelial defects[11]. As a result, patients with LSCD have decreased vision, photophobia, tearing, and recurrent pain episodes[2,11,12].

The classic limbal stem cell transplantation procedure involves grafting of a healthy limbal biopsy onto the recipient corneal surface. The technique was developed in the late 1980s and early 1990s and proved useful in LSCD[13]. Limbal grafts are obtained either from an unaffected fellow eye[7,14], from a live relative[14,15] or from cadaveric donors[14,16,17]. For patients inflicted with unilateral total LSCD, where the entire limbal circumference is damaged in only one eye, transplantation of limbal stem cells from the fellow eye has shown success rates greater than 80%[18,19]. Due to the autologous source of cells, there is no risk of immune rejection in these cases. Concerns regarding irreversible changes that may occur at the limbal site of the donor eye, as reported in rabbits[20,21], prompted clinicians to consider cadaveric donors. However, in this procedure, there is an increased risk of allograft rejection and therefore systemic immunosuppression is mandatory[14,16,17]. Although larger grafts can be procured from cadaveric eyes (360 ring grafts), a recent study[22] found living-related limbal allografts to have better results in terms of vision gain and ocular surface restoration than cadaveric keratolimbal allografts. In a series of 39 eyes that received keratolimbal allografts from cadaveric donors, the overall survival of ambulatory vision, namely 20/200 or better, was 53.6% at 3 years, but a progressive decline of the visual outcome and graft survival became evident with time[23]. The failure of the graft is related to loss of donor cells overtime, probably due to recipient's preoperative conditions such as dry eye and/or lid abnormalities, persistent inflammation or postoperative asymptomatic and progressive allograft rejection[23].

Regardless of the source of epithelial stem cells, amniotic membrane transplantation (AMT) has been widely used in an attempt to improve limbal grafts success[24-26]. Nevertheless, a recent study interestingly showed no differences in the outcomes of chemically injured eyes treated with or without AMT as an adjunctive procedure to limbal transplantation[27]. Amniotic membrane is a biologic, avascular tissue with anti-angiogenic, anti-scarring and anti-inflammatory characteristics[28,29]. Since the process of

conjunctivalization in LSCD is invariably associated with destruction of the basement membrane[10], amniotic membrane transplanted onto the corneal surface facilitates epithelial expansion *in vivo*. Amniotic membrane has a thick basement membrane that serves as a substrate upon which epithelial cells can migrate and regenerate to form new and healthy tissue[28]. In cases of partial LSCD, AMT has been successfully used alone, without stem cell transplantation[30]. Amniotic membrane can also be used as a biologic bandage in the acute phase of inflammatory disorders, such as ocular burns[31,32] and Stevens-Johnson syndrome[33-36], in order to prevent development of LSCD and other surface complications. Nevertheless, most of the studies are non-comparative, and a clinical trial that addressed the potential benefits of AMT did not find definite advantage of early AMT over conservative medical treatment in preventing scarring sequelae in eyes with chemical burns[37].

With the development of cell therapy, amniotic membrane began to be employed in *ex vivo* expansion of limbal stem cells or their progeny[38]. Pellegrini and colleagues published the first clinical report in the field of tissue engineering for the cornea in 1997[39]. They reported an autologous cultivated epithelial cell transplantation that successfully restored the ocular surface in 2 cases of LSCD due to chemical burns. Other series of cultivated epithelial limbal transplantation (CLET) have followed[38,40-46] and efforts to culture corneal cells *in vitro* are currently being made in different centers worldwide. Protocols use either an explant or cell suspension for culturing progenitor cells. The benefits of using explants are that they are easy to prepare and there is no danger of damaging the donor's corneal epithelium through enzyme exposure[40]. However, limbal stem cells may poorly migrate from the limbal explants onto the carrier[40,47]. Some protocols include co-cultivation with murine 3T3 feeder layers[48], which are believed to promote niche regulation of limbal stem cells and thus delay epithelial differentiation[49]. The use of animal-derived products carries a theoretical risk of infection, rejection or acquisition of prion diseases[50]. By using autologous human serum instead of fetal bovine serum[51] and eliminating feeder cells, complete xeno-free cultivation for CLET is possible[52].

A novel possibility for delivering limbal stem cells on the corneal surface is to use contact lens as a carrier and as a substrate for cell expansion. Two studies with animal models of LSCD reported promising results with contact lens delivery, as stem cells retained their phenotype and mitotic activity after being transferred to the ocular surface[53,54]. Furthermore, the seeded contact lenses were easy to manipulate and its material is nonimmunogenic[54].

We perform transplantation of other cell lineages onto the ocular surface for LSCD when total LSCD occurs bilaterally and therefore no autologous tissue for CLET is available. Conjunctival epithelial cells are the most akin biologically to corneal epithelial ones in the human body and our group has successfully cultivated and transplanted them for ocular surface reconstruction[55]. Use of non-ocular surface epithelial cells was first made with cultivated oral mucosal epithelial transplantation in rabbits[56] and application of this technique in humans has followed thereafter[57-65]. Both conjunctival and oral mucosal epithelial cells are not expected to differentiate into corneal epithelial cells, like stem cells would. However, most studies show adequate functionality of the grafts, giving them clinical relevance in tissue engineering.

A promising source of stem cells for ocular therapy is the human deciduous teeth. These undifferentiated cells exhibit all characteristics of pluripotent adult stem cells and continuously express markers of limbal stem cells[66]. Tissue-engineered sheets of dental-pulp stem cells were effective for cornea reconstruction in a rabbit model of chemical burn[67]. Other recently proposed sources of stem cells for cornea epithelium are hair follicle[68], bone marrow[69,70] and umbilical cord[71]. It has been demonstrated that adult tissue-specific stem cells have the ability to differentiate into lineages other than the tissue of origin[72].

CORNEAL STROMA

One of the most challenging steps in engineering human corneal tissue is to produce the corneal stroma, given the difficulty in reproducing its highly ordered hierarchical ultrastructure, which relates to its exceptional biomechanical properties and optical transparency[73]. Keratocytes are native resident cells of the corneal stroma and are responsible for maintaining the extracellular matrix (ECM)[1]. The bulk of the stroma comprises approximately 200 orthogonally oriented lamellae, and each of them is made up of long uniform collagen fibrils lying parallel one to another with regular inter-fibril spacing[74,75]. Under *in vitro* expansion in serum containing media, keratocytes lose their phenotype and soon become fibroblastic. Unlike keratocytes, fibroblasts secrete a disorganized ECM typically found in opaque corneal scars[76-78]. Even in serum-free conditions, primary keratocytes maintain an *in vivo* phenotype through some population doublings, but lose their morphology, gene expression, and the ability to organize a cornea-like ECM after greater expansion[79]. A more recent study, however, demonstrated that corneal fibroblasts respond to a specific pattern of topographical cues from the substratum and regain differentiation potential, thus being able to secrete highly organized ECM typical of corneal stroma[79].

Recently, Du and coworkers identified the first human cells with keratocyte progenitor potential in the corneal stroma[80]. This small population of cells exhibited properties of stem cells, such as clonal growth *in vitro*, extended lifespan, and the ability to differentiate into several different cells types, including

keratocytes of the cornea. When cultured as floating pellets in the absence of rigid scaffolding or substratum, in a serum-free medium, the human corneal stromal stem cells (hCSSCs) differentiate into cells expressing a gene profile similar to that of human keratocytes and deposit tissue-like ECM with a composition and structure similar to that of the corneal stroma[81]. A comparative study showed that, under the same culture conditions, human corneal fibroblasts tended to differentiate into myofibroblasts and deposited a less-organized collagen-fibrillar construct, while hCSSCs successfully differentiated into keratocytes and secreted multilayered lamellae with orthogonally-oriented collagen fibrils, in a pattern mimicking human corneal stromal tissue[73]. This *in vitro* secretion of stromal-like connective tissue points to a potential use of hCSSCs in tissue bioengineering. An interesting finding is that hCSSC may exhibit immune privilege: injected into mice corneas with scars, these cells did not elicit immune T-cell response[82], as it would be expected in a xenotransplantation model. As a matter of fact, a successful restoration of corneal organization and transparency was achieved after the injection[82]. Therefore, besides the utility in bioengineering stromal tissue, hCSSC may have the potential to provide a direct cell-based therapeutic approach[83].

Other engineering strategies for the corneal stroma include animal sources as a substitute of native tissue. Once rejection is a major obstacle to widespread use of xenotransplantation, fully acellular corneas have been proposed to overcome immune rejection. A decellularized animal tissue provides a three-dimensional ECM structure, favorable biocompatibility, adequate biomechanical tension and high transparency that mimics the native cornea and can be applied with or without the addition of a cellular component[13]. Most studies use porcine corneas[84-87], but other sources such as cow[88] and cat[89] are amenable for xenotransplantation. Since decellularized corneal stroma retains basement membrane components, it was used as a matrix scaffold for limbal stem cell expansion *in vitro*, and as a carrier for epithelial transplantation in animal models, with good results[90,91]. Decellularization may be applied not only to xenogeneic, but also to allogeneic cornea tissues for transplantation. Theoretically, this would reduce the complications associated with allogeneic corneal transplants and improve the clinical outcomes.

CORNEAL ENDOTHELIUM

Corneal endothelium is a monolayer of cells that maintains a nonproliferative state, with cell density decreasing steadily under normal circumstances throughout life[92,93]. It has been for long believed that they do not replace themselves once they are lost in aging or diseases. On the contrary of corneal epithelial cells, which readily and continuously divide from limbal stem cells, corneal endothelial cells fail to expand *in vivo*, and expand slowly *in vitro*[94,95]. Accumulating evidence, however, points to the existence of a dormant progenitor cell population located in the posterior limbus, the transitional area from the periphery of the endothelium and Schwalbe's line to the anterior portion of the trabecular meshwork. These putative progenitor cells are likely to be capable of producing both endothelial and trabecular cells[96]. Identification of a circumferential and discontinuous cord of cells with unusual ultrastructural features along the Schwalbe's line in monkeys dates from 1982[97]. Attention to these cells was drawn later, when repopulation of trabecular cells after argon laser trabeculoplasty was reported[98,99]. Possibly, these cells are invigorated and proliferate after laser trabeculoplasty through the release of growth factors and cytokines[96].

There is substantial evidence that the human corneal endothelium cells hold different characteristics according to their position in the cornea. Stained corneas revealed that cell density at the peripheral inner cornea, up to 1.6 mm from Schwalbe line, is about 30% higher than in the central cornea[100]. Additionally, specular microscopy documented the uneven cell distribution from center to periphery *in vivo*[101]. The reason for this difference is not known, albeit it has been suggested that the peripheral cornea may provide a physiologic reserve and storage region of endothelial cells for continuous, lifetime cellular supply for the central cornea[101]. Bednarz *et al*[102] showed that cells isolated from the central part are densely packed and exhibit no mitogenic activity, while cells derived from the peripheral part of the cornea are characterized by mitogenic activity but their cell-to-cell attachment seems to be less tight than *in vivo*. Konomi *et al*[103] studied human donor corneas with different donor ages and found that cells from both the central and peripheral areas retain potential proliferative capacity, although the doubling time tended to be higher for cells from the central cornea and from older donors.

Data from molecular marker studies provide support for the existence of a stem-like cell niche at the corneal endothelium. Dissected endothelial tissues from human and rabbits exhibited positive telomerase activity in the peripheral and intermediate sections, but no activity was observed in the central endothelial tissues or the limbus between the trabecular meshwork and Schwalbe's line[104]. Telomerase activity is a characteristic marker for transient amplifying cells. The same study also tested endothelial cells for alkaline phosphatase-catalyzed bromodeoxyuridine (BrdU) labeling, which demonstrates cell division. In the case of rabbit corneal endothelium, which is known to divide with regularity, BrdU staining was always seen following a mechanical wound, but in the human corneal endothelium, staining only occurred at the wound site in 10% of the corneas tested[104]. Such results corroborate a typical lack of propensity toward cell division in wounding response in the human

endothelium. Another cellular marker for proliferation is Ki67, which is detected in the nucleus of proliferating cells. Ki67 antibody staining produces characteristic patterns depending on cell position within the cycle, so it makes possible to quantify cell cycle entry and completion *in vitro*[105]. Stem cell markers that were also identified in the posterior limbus cells are nestin and alkaline phosphatase[106]. Human corneal endothelial cell precursors have been isolated using the sphere-forming assay and expressed markers of the mesenchymal (-smooth muscle actin) and neural (β3-tublin, glial fibrillary acidic protein) lineages, although none of these are specific markers of endothelial cells or their progenitors[107]. More recently, two monoclonal antibodies taken together, anti-GPC4 and anti-CD200, were found to stain HCECs with specificity[108]. The finding is useful for purification of cultures that may be contaminated by stromal fibroblasts. Since the endothelial cells are not expected to proliferate *in vivo*, isolation and stimulation of cell division *in vitro* is an important step towards cell replacement therapy[109].

Human corneal endothelial cells proliferate and replicate under special conditions. In medium containing fetal bovine serum and fibroblast growth factor, cells from older donors proliferated but responded more slowly and to a lesser extent than cells from younger donors. When epidermal growth factor added to the medium, cells from older donors were stimulated to enter the cell cycle faster and the amount of actively cycling cells and mitotic figures increased[105]. An important factor to activate endothelial cell cycle is the disruption of cell-cell contact. Zhu and colleagues demonstrated that maturation of intercellular adherent junctions correlates with a rapid decline of the proliferative potential in endothelial cell monolayers during cultivation[110]. To overcome that, EDTA solution was used in an organ culture model once it has the ability to release endothelial cells from cell-cell contact. After treatment of the intact endothelial monolayer with EDTA, approximately 17% of the cell population entered the cell cycle[111]. The use of EDTA alone, rather than a trypsin/EDTA solution to digest cells, may afford a greater cell survival rate[112]. Also, the presence of L-ascorbic acid 2-phosphate (Asc-2P) in the culture was found to increase the proliferation of endothelial cells. Asc-2P is an antioxidant derivative of Asc that upregulates hepatocyte growth factor production *in vitro*[113].

Further advance is required for the delivering method. Different cell carriers have been tested in animal models, and the main challenges are to achieve biomaterial transparency, structural strength and integration with native tissues[13,114]. One approach to deliver cultured endothelial cells through direct injection employed magnetic attraction to attach cells to the Descemet's membrane, thus preventing them to disperse in the anterior chamber[115]. Mimura *et al*[115,116] incorporated iron powder spheres in rabbit corneal endothelial cells and a neodymium magnet was placed anterior to the eyelid to attract the particles after cell suspension injection. They demonstrated that the use of 5-10 micromoles of iron powder solution was associated with good cell survival rate and 12 mo later no noticeable toxicity was present. Since rabbit's endothelial cells are able to regenerate spontaneously, a control group that had been submitted to the same corneal injury did not receive the cell injection. The control group showed signs of endothelial failure. Magnetic field was later used in a human *ex vivo* model. Superpara-magnetic microspheres were incorporated into the cytoplasm of human corneal endothelial cells and facilitated cell migration and attachment without affecting cell viability or light transmittance[117]. Toxicity to cells and other ocular tissues from magnetite oxide particles is a concern though, and safety remains to be demonstrated. Such compounds are used as magnetic resonance imaging contrast agents, on which safety and efficacy studies have been conducted, but the toxicity profile of this agent is likely to be different when delivered to the anterior segment of the eye.

The literature to date supports that the putative endothelial cells progenitors are precursors, not stem cells *per se*. Common features of precursors and adult stem cells are that both have self-renewal capacity and the ability to differentiate into mature effector cells, but stem cells can renew themselves indefinitely and produce one or more progenies through symmetric and asymmetric division, whereas progenitor cells have limited self-renewal capacity and more restricted differentiation abilities[96,107].

CONCLUSION

Progress in corneal stem cell research provides an optimistic prospect on their use in regenerative medicine and tissue engineering. In this paper, we presented different limbal transplantation techniques, using either autologous or heterologous source of limbal stem cells, and the epithelial cells cultivated *in vitro* that are being successfully transplanted to the ocular surface. Future perspectives include the use of pluripotent stem cells or other adult stem cells to restore the corneal epithelium. However, a measured scientific approach is required to guarantee safety before transplantation can be done in patients. We discussed the growing data on human stromal stem cells, their immune privilege, and their potential to secrete organized collagen lamella that may eventually be useful in tissue engineering. We also summarized the accumulating evidence for the presence of putative stem cells in the corneal endothelium. These cells require specific conditions to show proliferative activity *in vitro* and researchers have already addressed that. Cell therapies for each corneal layer will target a specific disorder, instead of a full or partial thickness corneal transplant, which is the current treatment. Furthermore, one donor cornea will potentially treat multiple patients if the cells are successfully cultivated,

and certainly this would have a positive impact on the shortage of donor corneas worldwide.

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