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Corneal Limbal Stem-cell Technology: Expanding the Options for Cell Cultivation and Transplantation

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Stem-cell technology is an exciting treatment in medicine, wherein ophthalmology is leading the way. With our anatomical and histopathological knowledge, the visibility of the eye allows for ease of assessment of stem-cell treatments compared to other neurological and visceral organs of the human body. Most of the ophthalmic stem-cell headlines come from treatment for posterior segment conditions, but there is still much to learn about the complexities of retinal-cell differentiation, their safety, and the proper integration of these cells into the highly complex neuroretinal circuitry.[1] Anterior-segment stem-cell technology, due to its already well-defined corneal limbal stem cells (LSCs) with greater ease of evaluation, has been at the forefront of ophthalmic stem-cell treatment and technology since 1997.[2] This issue of *Ophthalmology Rounds* provides an overview of the current standard of care for treatment of LSC-deficient conditions and reviews recent treatment technologies using *ex vivo* expansion of cultivated LSCs of the cornea.

Corneal Limbal Stem-cell Deficiency

Corneal epithelial cells are self-renewing: the squamous epithelial cells renew themselves every 7 days. The corneal LSCs are made up of nonkeratinizing stratified squamous epithelium located at the basal layer of the epithelium in the transition zone between corneal and conjunctival epithelial cells. LSCs prevent the conjunctival epithelium from invading upon the corneal epithelium. They are also thought to provide the source for corneal epithelial renewal.[3]

Clinical symptoms of LSC deficiency include decreased vision, photophobia, tearing, blepharospasm, chronic inflammation and hyperemia, and recurrent episodes of pain.[4] Slit-lamp examination shows recurrent and persistent epithelial defects, scarring, calcification, conjunctivalization of the cornea, superficial neovascularization of the cornea, decreased mucin and aqueous tear production, keratinization of the entire ocular surface, ulceration, melting, and perforation.[4]

LSC deficiency can occur from many causes and severity can range from mild, as seen in contact lens overwear (Figure 1), to severe, as in chemical burns (Figure 2) and ocular cicatricial pemphigoid (OCP; Figure 3). Causes of LSC deficiency include: aniridia, ectodermal dysplasia, toxicity from topical medications, chemical or thermal injury, radiation, Stevens-Johnson syndrome (SJS), OCP, cryotherapy, multiple surgeries, contact lens wear, conjunctival intraepithelial neoplasia, and microbial keratitis.[4]

The Evolution of Limbal Stem-cell Transplantation

In 1965, Barraquer[5] reported the first stem-cell autograft using conjunctival-limbal-corneal epithelium harvested from the healthy fellow eyes of patients with unilateral

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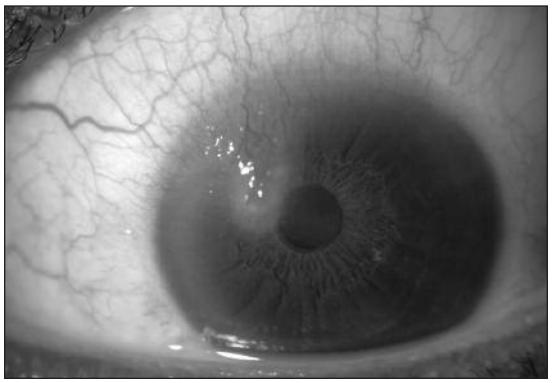
Figure 1: Mild partial limbal stem cell deficiency secondary to contact lens wear. Note conjunctivalization with superficial neovascularization of the cornea at the 11 o'clock position.



chemical burns. Keratoepithelioplasty was reported by Thoft[6] in 1984 using corneal lenticules from whole globes. The first modern LSC transplant was reported in 1989 by Kenyon and Tseng[7] on patients with unilateral LSC disease with ocular surface stabilization in 19 of 20 eyes. Further refinements to this technique maximized the harvesting and transplantation of larger amounts of conjunctiva and LSCs to the ocular surface of the recipient.[8-10] In 1997, Pellegrini et al[2] were the first to report on the successful use of autologous cultivated corneal epithelium for restoring the ocular surface in 2 patients with severe unilateral alkali burns. This has led to the most recent advances in LSC technology involving the *ex vivo* expansion of cultivated corneal epithelial LSCs for transplantation.

Current Management of LSC Deficiency

Partial LSC deficiency is defined as deficiency of <50% of the entire LSC population, subtotal LSC deficiency is >50%, while total LSC deficiency is defined as loss of the entire LSC population. Mild partial LSC deficiency, where the central visual axis is not involved and there is good visual acuity, requires only observation. Conservative management such as lubrication and removal of any potential inciting causes (ie, contact lens wear) should be addressed. Partial LSC deficiency that is symptomatic with irritation, reduction in vision, and signs of conjunctivalization of the cornea, requires mechanical débridement of the conjunctival epithelium from the corneal surface with frequent follow-up.[11] This allows the corneal epithelium to heal into the débrided area.

Figure 2: Failed penetrating keratoplasty secondary to subtotal limbal stem cell deficiency from chemical burn.

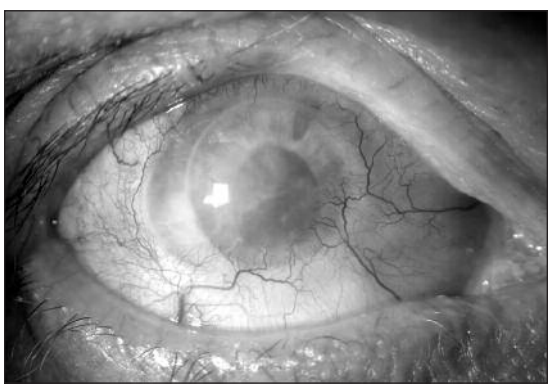
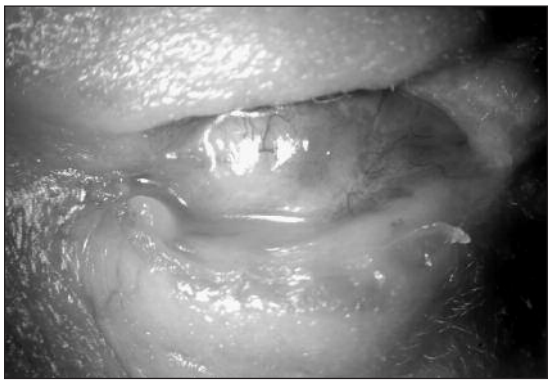


Figure 3: Total limbal stem cell deficiency secondary to ocular cicatricial pemphigoid. Complete keratinization of the ocular surface with loss of the fornices.



In severe LSC-deficient states, there are various surgical procedures to transplant LSCs to form a new source of corneal epithelium once the host's irregular epithelium and surface neovascularization have been débrided. In 1996, Holland et al[8] developed a classification system for the surgical management of severe ocular surface disease based on 3 factors: the source of tissue used, whether the source is an autograft or allograft, and (if using an allograft) whether it is derived from a living relative or a cadaveric donor. The LSC transplant also requires carrier tissue, as it is impossible to transplant LSCs alone, hence the need for conjunctival tissue (conjunctival limbal graft), corneal tissue (keratolimbal graft), or both as a carrier for LSCs.[4] The following 4 sections describe each group within this classification system.

Conjunctival limbal autograft (CLAU)

In CLAU, limbal tissue with a conjunctival carrier is harvested from the healthy contralateral eye and transplanted to the LSC-deficient eye. Since this is an autograft, there is no need for systemic immunosuppression, which is a tremendous advantage over allograft transplantation. CLAU can only be used for cases of unilateral LSC deficiency such as unilateral burns. Caution must be exercised due to the risks to the healthy donor eye of iatrogenically becoming LSC deficient secondary to harvesting of its LSCs for transplan-

tation.[12] CLAU can only be used when the contralateral donor eye has a healthy ocular surface with no risk of future LSC deficiency (eg, asymmetrical OCP or SJS, or prior history of ocular surface trauma).

Two trapezoid-shaped CLAUs are harvested, each approximately 6 mm at the limbus, extending 5-8 mm posteriorly in the bulbar conjunctiva, with anterior extension into the cornea anterior to the palisades of Vogt. They are usually harvested from the 12 o'clock and 6 o'clock meridians of the donor eye. Attention is directed towards the recipient eye. A 360° peritomy is performed with excision of the bulbar conjunctiva that is extended posteriorly in the superior and inferior quadrants to allow for placement of the 2 trapezoidal CLAUs. Superficial keratectomy of the entire corneal surface ensues, débriding irregular epithelium and pannus. The CLAUs are secured into position using 10-0 nylon interrupted sutures.[13,14]

Living-related conjunctival limbal allograft (LR-CLAL)

In LR-CLAL, healthy limbal tissue with a conjunctival carrier is harvested from a living relative and transplanted to the patient. Surgically, the technique is similar to the CLAU, but 2 separate surgeries are required. Compared to keratolimbal allografts (KLALs), LR-CLAL transplants conjunctival tissue that would be beneficial for patients with compromised conjunctiva. The advantage in using LR-CLAL over CLAU is that it can be used in patients with bilateral LSC deficiency, such as in SJS and OCP.[15,16] However, unlike autografts, allografts are at risk for rejection, and thus require both topical and systemic immunosuppression.[17]

Surgeons should be conservative when selecting recipient patients and donors. The recipient must be medically fit to withstand systemic, and most likely lifelong, immunosuppression. The patient must be compliant with medications and postoperative appointments with the transplant surgeon and transplant physicians, and must also be able to comply with the rigorous monitoring and bloodwork schedules postoperatively. Donors must be screened for potential risk of iatrogenic LSC deficiency in the future. Only a limited amount of LSCs can be harvested from the donor; thus, fewer LSCs can be transplanted so patients with limited LSC deficiency are better suited for LR-CLAL compared to those with complete LSC deficiency.[13,14]

Keratolimbal allograft (KLAL)

In KLAL, the recipient receives limbal tissue harvested from cadaveric eyes using corneal tissue as a carrier. This allows for a greater quantity of LSCs transplanted to the recipient eye. KLAL is ideal for severe bilateral LSC deficiency, patients with unilateral LSC deficiency unwilling to risk LSC deficiency in their better eye with a CLAU, or in patients who are unable to obtain a willing and living relative for an LR-CLAL. Conditions such as aniridia and iatrogenic LSC deficiency with mild to moderate conjunctival involvement[18] are ideal for KLAL. However, KLAL alone should not be used in recipients with inadequate tear film,[19] significant active inflammation, and/or severe conjunctival involvement with keratinization of the ocular surface secondary to loss of both LSCs and conjunctival epithelial stem cells.[20] As mentioned previously, systemic immunosuppression is required with any allograft. The surgical technique for this procedure has been described in detail.[13,14,21]

Combined conjunctival and keratolimbal limbal allograft (C-KLAL)

In C-KLAL, the recipient receives transplantation of KLAL as well as LR-CLAL. This is the preferred procedure in cases with cicatrizing ocular-surface disease such as SJS, OCP, and severe chemical burns. The conjunctival transplantation provides additional functional goblet cells to improve the production of mucin in the tear layer. These patients require reconstruction of the conjunctival fornices and lids. Collaboration with an oculoplastics surgeon and possibly an otolaryngologist (for harvesting of nasal mucosa from the inferior turbinates) is required.[13,14] Systemic immunosuppression is required.

Stem-cell Technology and Corneal Epithelial Therapy

There are 3 types of stem cell lines: human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs), and tissue stem cells. The original stem cells, the hESCs, come from human blastocysts and are pluripotent; however, these cells carry tumorigenesis and immunological rejection issues. The iPSCs are further differentiated from the hESCs; however, cultivation of these cells has shown low yield with inconsistent tissue lines. Tissue stem cells are further differentiated stem cells and are unipotent, progenitor cells with minimal tumorigenicity and immunological reaction.

The corneal LSCs are an example of tissue stem cells that have been successfully identified and used to repair ocular-surface disease. The LSCs provide the source for corneal epithelial renewal. They are made up of nonkeratinizing stratified squamous epithelium located at the basal layer of the epithelium in the transition zone between corneal and conjunctival epithelial cells – the LSC niche. Confocal microscopy has shown how injury to the limbus has affected the LSC niche.[22]

Ex vivo *corneal epithelial limbal stem-cell expansion*

Penetrating keratoplasty has a poor prognosis in patients with severe LSC deficiency. LSC transplantation can optimize the success rate; however, there are

associated risks of inducing LSC deficiency in the donor eye, as well as the need for systemic immunosuppression if the donor is an allograft – either from a living related or cadaveric donor.

In 1997, Pellegrini et al[2] were the first to successfully treat 2 patients with total LSC deficiency by cultivating autologous corneal epithelium. A 1-mm^2 LSC donor biopsy was taken from the patients' healthy contralateral eye. The biopsy was then minced, treated with trypsin, and the LSCs were isolated and expanded on culture plastic using lethally irradiated mouse 3t3 fibroblast feeder cells. The cultivated epithelial progenitor cells were then successfully transplanted to the recipient eye using a soft contact lens as a carrier. With follow-up beyond 2 years, both patients had achieved re-epithelialization of the entire cornea. This landmark study introduced a new perspective in the treatment of LSC deficiency by reducing the risk of morbidity in the donor and maintaining an autologous source.

In cases of bilateral LSC deficiency such as aniridia and SJS, an allogeneic LSC donor source, either living-related or cadaveric, is required. Reports are supportive of this technique, but long-term success has yet to be established.[23,24]

Amniotic membrane and other carrier substrates

Human amniotic membrane, the inner wall of the membranous sac surrounding the embryo during gestation, is the most common carrier substrate used for LSC culture and transplantation. Amniotic membrane provides cultured LSCs with a surrogate niche to assist with survival and function. Amniotic membrane has inherent anti-scarring, anti-angiogenic, and anti-inflammatory mediators that enhance re-epithelialization of the ocular surface.[25-29] The use of amniotic membrane simplifies manipulation and suturing, while reducing the risk of potential infection associated with using the mouse 3t3 fibroblast feeder cells. Amniotic membrane also acts as a basement membrane enabling cell migration.[30]

Amniotic membrane is a substrate that allows LSCs to survive and proliferate; however, it requires costly donor screening and there is a potential for transmission of viral disease. Amniotic membrane is also expensive, not readily available, and semi-opaque which may affect vision postoperatively. Processing methods of amniotic membrane are variable and may affect the ability of the amnion to act as a suitable substrate for LSC cultivation. The use of glycerol as a cryoprotectant when processing amniotic membrane has been shown to impair LSC expansion when compared to simple frozen amniotic membrane.[31]

Various alternative substrates have been used for corneal epithelial transplantation. These include: fibrin substrate with good results at up to 10 years of follow-up;[32,33] a novel cell-sheet manipulation technology using temperature-responsive culture dishes;[34] acrylic acid plasma polymerization to coat the inner surface of a bandage contact lens used to cultivate, transport, and immobilize the tissue;[35] carrier-free sheets using commercially available fibrin sealant;[36] and autologous serum incorporated into growth media with a Food and Drug Administration-approved soft contact lens as the substrate, carrier, and bandage to protect the eye during transplantation and healing.[37]

Alternative sources of corneal epithelial cells for transplantation

Autologous LSC transplantation and *ex vivo* expansion of cultured autologous corneal epithelial LSCs do not require systemic immunosuppression. However, in cases of bilateral, total LSC deficiency where autologous corneal epithelial LSC tissue is unavailable for harvesting and expansion, living-related or cadaveric donor allograft with long-term systemic immunosuppression are required. Immunosuppression carries a high risk of serious ocular and systemic complications.

Alternate sources of autologous epithelial cells have been studied in order to avoid the need for systemic immunosuppression in patients with severe bilateral LSC deficiency. Oral mucosal epithelial cells,[38-41] mesenchymal stem cells,[42] and hair follicle stem cells[43] may be possible alternative sources.

The *in vitro* replication of pluripotent hESC derived from blastocysts has been successfully achieved;[44] however, the translation to human therapeutic use must overcome problems with functionality, rejection, and ethical concerns. iPSCs have been generated,[45,46] but challenges remain with tumorigenicity, immune rejection, refining a specific population, and defining an appropriate model for preclinical studies. The translation of hESC and iPSC technology to human therapeutics is an exciting field that will continue to evolve and develop in the future.

Challenges in using corneal limbal stem-cell technology and therapy

There are many challenges in LSC technology and therapy. Most methods of *ex vivo* LSC expansion require the use of animal products, foreign human tissue/serum, and/or nonapproved biomaterials, all increasing the potential risk of xenobiotic infection. Ethical considerations are at the forefront in using hESC as they are harvested directly from blastocysts generated through *in vitro* fertilization. hESC and iPSC still have complexities with tumorigenicity, immune rejection, refinement to a specific population, and defining appropriate models for preclinical studies.

It is difficult to interpret and compare the efficacy of the numerous *ex vivo* expansion studies published because of variation among the studies. The main variables are: culture techniques employed; selection of recipient patients for treatment (degree of LSC deficiency in the recipients); evaluation of treatment efficacy (clinical observation versus impression cytology); lack of outcomes data (visual acuity not reported in some series); variation in follow-up; and the combined use of autologous and allogeneic transplants in some studies.[23,47] Despite these challenges, the results are favourable. A recent review summarized the results of 15 studies utilizing autografts, and revealed an 84% success rate in 292 transplants; in 9 studies with allografts, there was a 75% success rate in 48 transplants.[47] Further studies with standardized variables will allow for easier interpretation of technology and therapeutic outcomes.

Conclusion

Penetrating keratoplasty has a poor prognosis in patients with severe LSC deficiency. LSC transplantation optimizes the success rate; however, there are associated risks of inducing LSC deficiency in the donor eye, as well as the need for systemic immunosuppression if the donor is an allograft – either from a living related or cadaveric donor. The clinical use of *ex vivo* expansion of cultivated autologous LSCs was first described in 1997.[2] Modification of this technique and expansion using amniotic membrane and other carrier substrates has enhanced the translation to clinical therapy.

The recent landmark Phase I clinical study using tissue engineering to produce a biosynthetic cornea has garnered much media interest towards penetrating keratoplasty technology.[48] A biosynthetic cornea minimizes the risk of rejection, but still requires healthy endothelium and LSCs in the recipient, thus highlighting the importance of LSC technology. As LSC technology and tissue engineering continue to evolve, ophthalmologists will have a plethora of alternatives to manage severe LSC-deficient conditions such as chemical burns, SJS, and OCP without the need for systemic immunosuppression and donated cadaveric tissue.

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