

**Treatment of total limbal stem cell deficiency  
without the use of limbal stem cells  
A review**

Aje Paul Singh Dhillon

Simen Daniel Birkenes

Project

Supervisor: Tor Paaske Utheim

FACULTY OF MEDICINE, OSLO

April 09

# Treatment of total limbal stem cell deficiency without the use of limbal stem cells

## Contents

### 1. Abstract

### 2. Key words

### 3. Introduction

### 4. Limbal epithelial stem cells and corneal epithelial homeostasis

#### 4.1 Limbal stem cell deficiency

##### 4.1.1 Etiology

##### 4.1.2 Diagnosis and classification

##### 4.1.3 Management

4.1.3.1 Keratolimbal allograft (KLAL)

4.1.3.2 Conjunctival limbal autograft (CLAU)

4.1.3.3 Living related conjunctival limbal allograft (LR-CLAL)

4.1.3.4 Combined conjunctival and keratolimbal allograft (C-KLAL)

4.1.3.5 Homologous penetrating central limbo-keratoplasty (HPCLK)

4.1.3.6 Amniotic membrane transplantation

4.1.3.7 *Ex vivo* expansion of limbal epithelial cells

4.1.3.8 *Ex vivo* expansion of oral mucosal epithelial cells

4.1.3.9 *Ex vivo* expansion of conjunctival epithelial cells

4.1.3.10 *Ex vivo* expansion of bone marrow stem cells

4.1.3.11 *Ex vivo* expansion of epidermal epithelial cells

## **5. Characteristics of reported studies**

**5.1 Table 1**

**5.2 Number of patients treated**

**5.3 Number of animals treated**

**5.4 Disorders treated**

## **6. Methods employed by studies**

**6.1 Method of diagnosing and classifying stem cell failure**

**6.2 Biopsy techniques**

**6.2.1 Oral epithelial cells**

**6.2.2 Conjunctival epithelial cells**

**6.2.3 Bone marrow stem cells**

**6.2.4 Epidermal epithelial cells**

**6.3 Explant culture systems**

**6.3.1 Oral epithelial cells**

**6.3.2 Conjunctival epithelial cells**

**6.3.3 Bone marrow stem cells**

**6.3.4 Epidermal epithelial cells**

**6.4 Suspension culture systems**

**6.4.1 Oral epithelial cells**

**6.4.2 Conjunctival epithelial cells**

**6.4.3 Bone marrow stem cells**

**6.4.4 Epidermal epithelial cells**

**6.5 Culture media used**

**6.5.1 Oral epithelial cells**

**6.5.2 Conjunctival epithelial cells**

**6.5.3 Bone marrow stem cells**

**6.5.4 Epidermal epithelial cells**

**7. Evidence for presence of stem cells in cultures and grafts**

**8. Surgical transplantation of ex vivo cultured epithelial sheets**

8.1 Oral epithelial sheets

8.2 Conjunctival epithelial sheets

8.3 Bone marrow stem cells

8.4 Epidermal epithelial sheets

**9. Postoperative management**

**10. Donor screening**

**11. Outcomes**

11.1 Clinical outcomes

11.1.1 Improvement in ocular surface

11.1.2 Visual acuity

11.1.3 Subjective symptoms

11.1.4 Results with transplantation of ex vivo cultured epithelial sheets

11.1.4.1 Oral epithelium

11.1.4.2 Conjunctival epithelium

11.1.4.3 Bone marrow stem cells

11.1.4.4 Epidermal epithelium

11.2 Further surgical procedures

11.3 Evidence of donor cell survival

11.4 Complications and adverse events

**12. Regulatory considerations**

12.1 Animal subjects

12.2 Human subjects

**13. Discussion and Future perspectives**

**14. Conclusion**

**15. Method of literature search**

**Reference list**

# Treatment of total limbal stem cell deficiency without the use of limbal stem cells.

## **1. Abstract**

**PURPOSE:** Until recently, total limbal stem cell deficiency could not be treated by autologous sources of stem cells. Our study is a review of recent progress in utilizing oral, conjunctival, mesenchymal and epidermal stem cells as autologous sources of stem cells in treating total limbal stem cell deficiency.

**METHOD:** We have identified 14 clinical trials including both human and animal subjects which are the basis of our review. We studied these articles with emphasis on cultivating techniques, transplantation methods, success rates, complications, visual outcomes and followup.

**RESULTS:** Due to the lack of a common definition of success in the individual studies, comparison between the studies is of little use. The underlying pathologies described and the subjects (animals and humans) are diverse, and in addition there is a lack of a sufficient number of studies.

**CONCLUSION:** Despite the large lack of comparability, we conclude that these methods show promising results deserving further investigations and refinement.

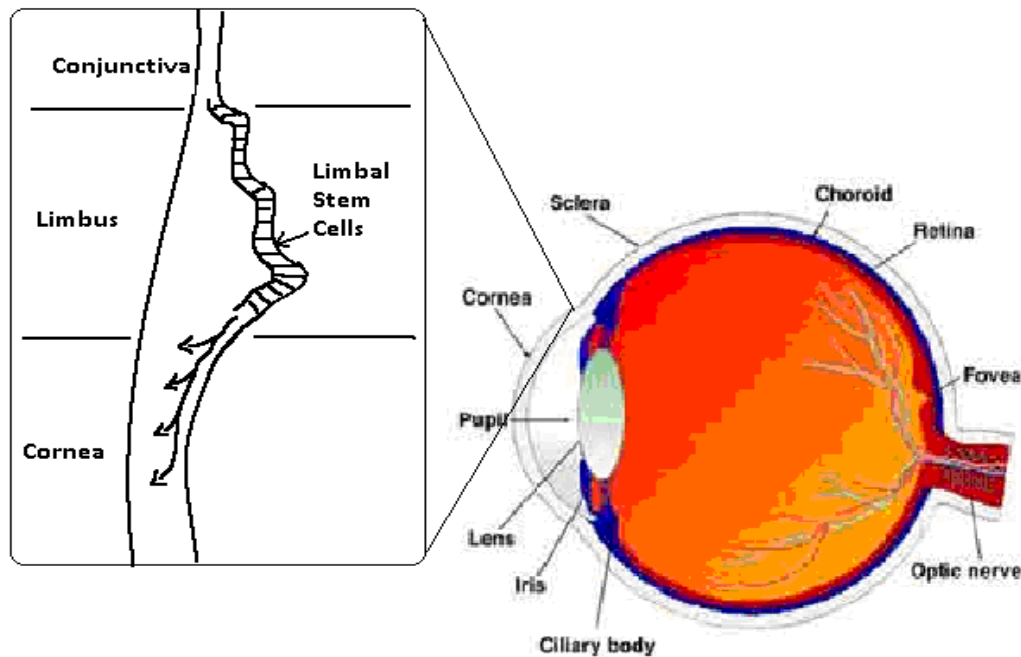
## **2. Key words**

*Limbal epithelial stem cell deficiency, Ocular surface disease ,Corneal reconstruction, Amniotic membrane, Cornea, Ex vivo cultivation, Oral, Conjunctiva ,Bone marrow, Mesenchymal, Epidermal, Transplantation, Tissue engineering.*

## **3. Introduction**

A clear image perception of the world around us is dependent of a number of structures: the tear film, the cornea, the anterior chamber, the iris, the lens, the vitreous body, the retina and the optic nerve, to mention the most important. The focus of this article will be how to treat corneal diseases due to stem cell deficiency.

Limbal stem cell deficiency (LSCD) refers to a condition in which there is a deficiency of stem cells that reside in the corneoscleral junction in the palisades of Vogt. These stem cells are of importance to the homeostasis and reparative processes of the corneal epithelium.<sup>1,2</sup> As in other epithelial surfaces, there is a continuous desquamation of epithelial cells from the corneal epithelium. These desquamated epithelial cells are replenished by descendants of limbal stem cells (Figure 1).



*Figure 1 : Location of the Limbal Stem Cells and how they migrate to produce the epithelialized corneal surface.*

There are several disorders that give rise to a deficiency of limbal stem cells, among which are Stevens Johnson Syndrome, cicatricial ocular pemphigoid, chemical and thermal burns, and trauma. The destruction of these stem cells in turn results in deficient reparative processes of the corneal epithelium. This can result in scarring of the cornea and ingrowth of conjunctival epithelium, which in turn results in severe corneal opacification and loss of visual acuity (VA).<sup>3-8</sup>

During recent years there has been great progress in treatment of LSCD, among which are the use of xenologous, allogeneous and autologous limbal stem cells from a healthy limbus.<sup>9</sup> However, this treatment posts a problem whenever both eyes of a patient are affected with total LSCD, in which there are no limbal stem cells remaining. Therefore, during recent years

techniques have been developed to overcome this problem by culturing and transplanting limbal stem cells from other sources than the limbus. These techniques are to be discussed in this article.

#### **4. Limbal epithelial stem cells and corneal epithelial homeostasis**

##### **4.1 Limbal stem cell deficiency**

###### **4.1.1 Etiology**

The surface of the cornea is formed by stratified nonkeratinized squamous epithelium that regenerates quickly when injured. Epithelial defects are closed by cell migration and rapid cell division of limbal stem cells.<sup>9</sup> However, this demands that the stem cells in the limbus of the cornea are undamaged. Regular corneal regeneration will no longer be possible when these cells are compromised.<sup>9</sup>

There are many causes of LSCD, dominated by the acquired ones, like chemical and thermal burns, ionizing and ultraviolet radiation, autoimmune diseases like ocular cicatricial pemphigoid and Stevens Johnson Syndrome, other trauma to the ocular surface like surgery, lens wear and invasive infections.<sup>3-5, 7, 10-15</sup> Some causes are genetic and thus inheritable, like aniridia, where the PAX6 gene is thought to be defect.<sup>16, 17</sup>

LSCD can be partial or total, referring to the extent of damage to the LESC. Total LESC deficiency refers to a loss of LESC in all areas, while partial LESC deficiency refers to a loss of LESC in just some areas of the corneal limbus. The latter condition clinically displays sectoral ingrowth of conjunctival epithelium on the corneal surface, whilst the former shows a total conjunctivalization of the cornea.<sup>9, 18, 19</sup>

If the LESC are depleted, the conjunctival epithelial cells grow into the cornea and along with it comes increased vascularization that is inherent to the conjunctival epithelium.<sup>10, 12, 15, 16, 18</sup>

#### **4.1.2 Diagnosis and classification**

Pathognomonic for LSCD is the ingrowth of conjunctival epithelium onto the cornea, a process called conjunctivalization. The affected eye may display recurrent or persistent epithelial defects, ulceration, corneal vascularization, chronic inflammation scarring, and the loss of the clear demarcation between the cornea and conjunctiva.<sup>9,19</sup>

LSCD is a clinical diagnosis which may be confirmed by impression cytology. The presence of goblet cells on the cornea indicates conjunctival ingrowth. The clinical clues of conjunctivalization can be suggested by the loss of limbal palisades of Vogt under slit-lamp examination or by late fluorescein staining of the cornea, which reflects poor epithelial barrier function.<sup>18</sup>

Impression cytology confirms the clinical diagnosis and tissue change by displaying the presence of goblet cells inherent to the conjunctival epithelium.<sup>9,18,19</sup>

#### **4.1.3 Management**

Management and treatment of LSCD is depending on the extent of the disease, whether the LSCD is partial or total, unilateral or bilateral. The conservative management of LSCD is intensive lubrication, bandage contact lenses or autologous serum eye drops<sup>9</sup> in which only the latter is evidence based.<sup>20,21</sup> To treat partial or total LSCD however, one of the following techniques may be used:

##### **4.1.3.1 Keratolimbal allograft (KLAL)**

Keratolimbal allograft is a procedure in which limbal epithelial cells along with a small part of the adjacent corneal and conjunctival epithelium are extracted from cadaveric eyes and transplanted onto the diseased eye of the patient. Depending on the extent of the disease, a sector or a whole circular tissue is transplanted onto the diseased eye. With this procedure the graft contains a large number of stem cells. KLAL is considered for patients who have severe bilateral ocular surface disease or in patients where there is a risk developing LSCD in the healthy eye.<sup>22-24</sup>

##### **4.1.3.2 Conjunctival limbal autograft (CLAU)**

In this technique limbal epithelium attached to a conjunctival carrier is grafted from the healthy eye of the patient onto the diseased eye. This procedure, however, has its limitations in that it is only considered for patients with unilateral LSCD and since the number of stem cells transplanted is relatively small, this procedure is unsuitable for total LSCD.<sup>23,25</sup>



#### 4.1.3.3 Living related conjunctival limbal allograft (LR-CLAL)

In this procedure the same principles as in CLAU are used, the difference is in the source of the graft, which in this procedure is from a living relative.

#### 4.1.3.4 Combined conjunctival and keratolimbal allograft (C-KLAL)

This procedure is a combination of the aforementioned techniques in that cadaveric keratolimbal allograft and conjunctival limbal allo/autograft are both transplanted onto the diseased eye of a patient suffering from LSCD. This technique provides a substantial number of stem cells, thus this is a procedure of choice for the patients with the most severe LSCD, as in Stevens Johnson Syndrome, thermal and chemical burns, and cicatricial ocular pemphigoid.

#### 4.1.3.5 Homologous penetrating central limbo-keratoplasty (HPCLK)

This technique implies transplanting an eccentrically trephined keratolimbal graft to include limbal stem cells in the periphery of the graft.<sup>26, 27</sup>

#### 4.1.3.6 Amniotic membrane transplantation

Amniotic membrane can be used to restore the corneal surface in partial LSCD. The advantage in using amniotic membrane is that it does not weakly display leukocytic receptors, thus preventing a host versus graft reaction. When used in partial limbal stem cell deficiency, amniotic membrane transplantation can promote the migration and proliferation of remaining stem cells. The amniotic membrane also possesses growth factors that encourage healing.<sup>9, 9, 19, 28</sup>

#### 4.1.3.7 *Ex vivo* expansion of limbal epithelial cells

This procedure involves taking a small limbal epithelial biopsy from a healthy eye (i.e. autologous, living relative, cadaveric), cultivating this limbal epithelium *ex vivo* using various techniques and then transplanting an epithelial sheet to the diseased eye. The advantage in using this technique is that it requires a relatively small amount of specimen from a healthy eye compared to the methods above.<sup>9</sup>

#### 4.1.3.8 *Ex vivo* expansion of oral mucosal epithelial cells

This procedure requires a biopsy from the oral mucosal epithelium, and the source may be of autologous<sup>3-6, 29-31</sup> or xenologous<sup>31</sup> origin. The biopsy is cultured *ex vivo* to produce an epithelial sheet which is ultimately transplanted onto the diseased eye of the patient. Oral mucosal epithelium has shown to be a viable source of epithelial stem cells. As mentioned above, this technique involves taking a biopsy from the oral mucosa, which is a non-invasive procedure that can be carried out with minimal sequelae to the patient. The biopsy is cultivated *ex vivo* on a denuded amniotic membrane<sup>8, 11, 32-35</sup> or on a collagen membrane<sup>36</sup> using various forms of culture media. The different culture systems described are cultures with bovine serum,<sup>8, 11</sup> autologous serum,<sup>8, 11</sup> or in other special culture medium like an HCE medium<sup>34</sup> or a FAD medium.<sup>37</sup> Growth and development of the epithelium in studies using autologous serum are comparable to studies using bovine serum, the advantage being that the former is a xenobiotic free medium.<sup>4, 8</sup>

The epithelium can be co-cultured with 3T3 fibroblasts<sup>8, 32, 33, 35, 38</sup> and air-lifting techniques (an air-liquid interphase)<sup>33</sup> have been used in studies to promote stratification of the epithelium. Clinical studies applying this procedure show promising results (see table 1).<sup>3-6, 29-31, 33</sup>

#### 4.1.3.9 *Ex vivo* expansion of conjunctival epithelial cells

Similarly to oral and limbal techniques, a biopsy is taken from the conjunctiva. Preferably from the fornix region, an area in which conjunctival stem cells are abundant.<sup>1, 39, 40</sup> The conjunctival stem cells are then cultivated *ex vivo* to produce an epithelial sheet. Conjunctival epithelium can be used to produce an epithelial sheet for transplantation purposes to treat LSCD<sup>7, 12, 41, 42</sup> or conjunctival defects.<sup>14, 43</sup> This procedure however requires a biopsy taken from the conjunctiva of the healthy eye which, although minimal, is an invasive procedure as compared to the other methods using oral mucosa and epidermis. Various cultivating techniques have been described, including the use of autologous serum,<sup>44</sup> bovine serum<sup>44, 45</sup> or serum free<sup>44-49</sup> cultures. The advantage in using a human serum is described above. The proliferative capability of a serum free system has proved equal to or greater than an autologous serum containing system.<sup>47</sup>

One study, however, has assessed the problem of apoptosis induced by serum containing systems and has proposed a caspase inhibitor containing system as a solution.<sup>45</sup> The epithelium can be produced on a denuded human amniotic membrane<sup>12, 39, 44, 47</sup> or on a synthetically produced membrane.<sup>46, 48</sup>

#### 4.1.3.10 *Ex vivo* expansion of bone marrow stem cells

This procedure implies harvesting bone marrow derived mesenchymal stem cells (MSC) and cultivating these cells on a human amniotic membrane. MSCs are easy to isolate and have the potential to easily differentiate not only into epithelial cells, but also cardiomyocytes and neurons.<sup>50-56</sup> The resultant epithelial sheet is then transplanted onto a diseased ocular surface. This procedure involves harvesting bone marrow stem cells, which is an invasive procedure but with minimal sequelae to the patient.

#### 4.1.3.11 *Ex vivo* expansion of epidermal epithelial cells

In this procedure, epidermal stem cells are harvested from a biopsy from the epidermis. The biopsy is then cultured *ex vivo* and ultimately transplanted onto the diseased eye.<sup>57</sup> Although there is little reported research on this procedure,<sup>57, 58</sup> this is a promising modality in creating an epithelial sheet for treatment of LSCD. The procedure employs the multipotency of epidermal adult stem cells (epiASC) to produce an epithelial sheet for transplantation. These cells have the properties of embryonic stem cells and are shown to give rise to tissues such as neurons, cardiomyocytes and osteoblasts,<sup>58</sup> thus standing out as a technique not only to be enhanced by ophthalmology, but also other medical fields. The procedures described involve cultivation on denuded human amniotic membrane in an alternating submerged and air-liquid interphase.

The four latter modalities of treatment have recently emerged as viable alternatives to treatment of LSCD and will be discussed in more detail.

### **5. Characteristics of reported studies**

We have included 14 clinical studies in our article, which 8 utilize cultivation of oral mucosal epithelium,<sup>3-6, 8, 29-31</sup> three utilize cultivation of conjunctival epithelium,<sup>7, 41, 42</sup> two utilize cultivation of epidermal epithelium<sup>57</sup> and one study is based on *ex vivo* expansion of bone marrow stem cells.<sup>50</sup> The characteristics of these studies are listed in table 1.

5.1 Table 1

Author, year	No. of eyes/patients/animals	Primary pathology	Harvest site	Degree of LSCD	Previous surgical interventions
Nakamura et al, 2003	NA	Induced surgically	Oral mucosa in humans	NA	NA
Nishida et al, 2004	4 eyes - 4 patients	Steven Johnson Syndrome	Oral mucosa in humans	Total	3 patients with failed allograft
Nakamura et al, 2004	6 eyes - 4 patients	SJS n=3, Chemical burns N=3	Oral mucosa in humans	Total	1 patient with AMI transplantation, 1 patient with allogeneic corneal transplant
Hayashida et al 2005	10 eyes - 10 rabbits	Induced surgically	Oral mucosa in rabbits	Total	None
Inatomi et al, 2006	15 eyes - 12 patients	SJS (n=7), Chemical (n=2) and thermal burns, Idiopathic ocular surface disorder (n=1), pseudo-ocular cicatricial pemphigoid (n=1)	Oral mucosa in humans	Total	2 AMI transplantations 1 AMI + limbal stem cell transplantation 1 KEP + AMI transplantation 1 PKP 1 bilateral allogeneous corneal epithelial sheet transplantation
Inatomi et al, 2006	2 eyes - 2 patients	SJS (n=1), chemical eye injury (n=1)	Oral mucosa in humans	Total	NA
Satake et al, 2008	3 eyes - 3 patients	SJS(n=2), pseudo-ocular cicatricial pemphigoid (n=1)	Oral mucosa in humans	Total	NA
Ang et al, 2006	10 eyes - 10 patients	SJS (n=7), Thermal injury (n=1), Chemical injury (n=2), ocular cicatricial pemphigoid (n=1).	Oral mucosa in humans	Total	NA
Sangwan et al, 2003	2 eyes - 1 patient	Acid burns	Conjunctival and limbal	Partial	Symbiopharon release and plasty
Tanaka et al, 2006	8 eyes - 8 rabbits	Induced surgically	Conjunctiva in humans	Total	NA
Ono et al, 2007	10 eyes - 10 rabbits	Induced surgically	Conjunctiva in rabbits	Total	NA
Ma et al 2006	16 eyes - 16 rats	Induced chemically	Bone marrow cells in humans	Total	NA
Yang et al, 2007	8 eyes - 8 goats	Induced surgically	Epidermis in goats	Total	NA
Yang et al, 2008	10 eyes - 10 goats	Induced surgically	Epidermis in goats	Total	NA

Author, year	Autograft /Allograft/Xenograft	Postoperative treatment	Success rate *	Improvement in visual acuity	Complications	Followup in time
Nakamura et al, 2003	Auto/Xenograft	NA	NA	NA	NA	3-4 weeks
Nishida et al, 2004	Autograft	NA	4/4 eyes	Improvement described, no details	Limited stromal vascularization	Mean followup 14 months
Nakamura et al, 2004	Autograft	1 patient treated with additional antibiotics	6/6 eyes	VA increase of 2 or more lines in all eyes	Mild peripheral neovascularization in all eyes	Mean followup 13,8 months
Hayashida et al, 2005	Autograft	NA	10/10 eyes	NA	NA	4 weeks
Inatomi et al, 2006	Autograft	reoperation because of longstanding epithelial defects.	10/15 eyes	In 10/15 eyes, VA improved by more than 2 lines	Out of 5 eyes, developed small but longstanding epithelial defects. 2 eyes healed completely. 1 eye demonstrated conjunctival replacement. 2 eyes reoperated	Mean followup 20 months
Inatomi et al, 2006	Autograft	PKP	2/2 eyes	Patient 1 4/100 Patient 2 10/125	Patient 1 minimal neovascularization.	Mean followup 22,5 months
Satake et al, 2008	Autograft	1 patient treated with antiglaucoma medication. Patient 1 and 3 PKP	3/3 eyes	Postoperative VA did not improve dramatically after COMET, because of stromal opacity	Glaucoma in patient 1	1 year
Ang et al, 2006	Autograft	NA	10/10 eyes	9/10 eyes improved 2 lines or more in VA.	Mild peripheral neovascularization in all eyes, 4 eyes small epithelial defects that healed, 1 eye bacterial infection that healed.	Mean followup 12,6 months
Sangwan et al, 2003	Autograft	NA	2/2 eyes	20/40 one eye, 1m finger count one eye	Conjunctivalization and corneal scarring	1 year
Tanioka et al, 2006	Xenograft	NA	NA	NA	NA	2 weeks
Ono et al, 2007	Autograft	NA	10/10 eyes	NA	NA	2 months
Ma et al, 2006	Xenograft	NA	NA**	Significantly improved***	NA	4 weeks
Yang et al, 2007	Autograft	NA	7/8 eyes	Normal VA****	1 goat failed to epithelialize the cornea	24 months
Yang et al, 2008	Autograft	NA	NA	NA	70% of all eyes failed to regain clear cornea in all four quadrants	30 months



## 5.2 Number of patients treated

The largest number of patients treated in our study is 15 eyes in 12 patients by Inatomi and co-workers,<sup>4</sup> with a mean followup time of 20 months and a success rate (success in our study is simply the individual studies' reported success with no standard definition) of 10 out of 15 eyes. The least amount of patients reported were two eyes in one patient by Sangwan *et al*,<sup>7</sup> who earlier have reported a considerably larger quantity of patients numbering a total of 125.<sup>12</sup> This study, is not included here due to insufficient data for the focus of our review. Ang *et al*, 2006, however, reported the highest success rate in 10 out of 10 eyes in 10 patients with a VA improvement of two lines or more.

## 5.3 Number of animals treated

The largest number of animals treated was 16 rats by Ma *et al*<sup>50</sup> with a followup time of four weeks. The longest followup period, 30 months, was reported by Yang *et al* 2008 in 10 eyes in 10 goats.<sup>57</sup>

## 5.4 Disorders treated

Of the total of 42 eyes treated in a total of 36 patients, four main groups of diseases are described. The most common is Stevens Johnson Syndrome, reported in a total of 24 eyes,<sup>3-6, 8, 29</sup> 10 eyes had chemical or acid burns.<sup>3-5, 7, 8</sup> Three eyes had Pseudo-ocular cicatricial pemphigoid<sup>4, 29</sup> and one eye idiopathic ocular surface disorder.<sup>4</sup> Of a total of 62 eyes treated in a total of 28 rabbits,<sup>30, 41, 42</sup> 16 rats<sup>50</sup> and 18 goats,<sup>57, 58</sup> all reported cases of LSCD were induced surgically by keratectomy.<sup>30, 41, 42, 57, 58</sup> In two studies, a more extensive surgical procedure is reported with superficial keratectomy followed by chemically burning the limbus.<sup>57</sup>

## 6. Methods employed by studies

### 6.1 Method of diagnosing and classifying stem cell failure

As both animal and human studies are employed here, the methods used to diagnose LSCD can be considered in two main groups. In the aforementioned experimental studies using animals (62 eyes), total LSCD was induced surgically. LSCD was diagnosed in the human trials (42 eyes) using a combination of clinical observation and a variety of other diagnostic methods. All the human eyes treated, with the exception of one patient,<sup>7</sup> reported total LSCD and used complete absence of the palisades of Vogt as a diagnostic criterion.

## 6.2 Biopsy techniques

### 6.2.1 Oral epithelial cells

With the exception of 10 eyes in a rabbit model,<sup>30</sup> all patients underwent some form of preoperative dental and oral hygiene program. This included a variety of tooth decay treatment, no alcohol or tobacco, used tooth brushing guidance and sterilization using iodine gargle.<sup>3-6, 8, 29, 31</sup> Some of the patients also had a dentist confirming the presence of a healthy oral mucosa prior to operation.<sup>4, 5, 8</sup> The oral mucosa biopsy specimen was harvested under local anesthesia in all human subjects, whereas the rabbits all received deep anesthesia.<sup>30, 31</sup> The human biopsy size varied from 2-8 mm<sup>2</sup>, and was in two cases<sup>6, 29</sup> reported to be taken from the inferior and interior buccal mucosa, respectively. The rest failed to report the harvest site. The animal biopsies were reported to be of 4-6 mm<sup>2</sup><sup>31</sup> and having a 3 mm radius.<sup>30</sup> None of the authors reported the location of the biopsy. Scissors were used to remove submucosal tissue before producing *ex vivo* cultured limbal epithelial cells (LEC) for transplantation.<sup>3-5, 8, 29</sup>

### 6.2.2 Conjunctival epithelial cells

Ono and co-workers successfully obtained 2 x 4 mm autologous biopsies from the conjunctival fornix and bulbar conjunctiva of the contralateral, normal eye in rabbits. It was then washed with 10% iodine and rinsed with PBS before the conjunctival epithelium was removed from the stroma by mechanical scraping.<sup>41</sup> A xenograft was created using normal conjunctival tissue from patients with conjunctivochalasis and transplanted on to denuded rabbit corneas in one study.<sup>42</sup> Further documentation as to where the biopsies were harvested is not reported in this case. Sangwan *et al* used a combination of conjunctival and limbal tissue in a patient with partial LSCD.<sup>7</sup> An incision was made 3 mm behind limbus and 1 mm into cornea superficially. The conjunctiva was then excised just behind the palisades of Vogt, and the limbal tissue and 1 mm of the cornea was excised.

### 6.2.3 Bone marrow stem cells

This modality relies, as mentioned previously, on harvesting bone marrow from healthy donors, and is in that way not a biopsy per se.<sup>50</sup> 10mL of bone marrow was harvested from healthy donors in the study included in our review.



#### **6.2.4 Epidermal epithelial cells**

Yang et al reported the isolation and characterization of pluripotent stem cells from adult goat skin as described above. These primary cells have embryonic stem cell (ESC) properties. 0,5 x 0,6 cm biopsies were obtained from the ear of 3-4 years old Guanzhong goats, and cartilage and conjunctive tissue was carefully removed. The exact location from which the biopsy was taken was not reported in any more detail.<sup>57</sup>

### **6.3 Explant culture systems**

#### **6.3.1 Oral epithelial cells**

None of the clinical studies identified reported the use of this culture method. However, Madhira and coworkers cultivated oral mucosal epithelial cells to compare the results with limbal and conjunctival epithelial cells using the explant culture system.<sup>34</sup>

#### **6.3.2 Conjunctival epithelial cells**

The explants culture system is one of the two main methods of producing ex vivo cultured cell sheets for transplantation. Variations of this technique has successfully been used in several studies using LECs.<sup>9</sup> Of the 3 studies included in our article two reported using a variant of the explants culture system.<sup>7,41</sup> This method is based on the use of human amnion membranes (AM) as both a carrier and a substrate for the cultured cells. The membrane is first rinsed, frozen and stored. Immediately before use it is thawed and incubated with an enzyme (Dispase) to loosen the epithelial cell connections which are then scraped off.<sup>59</sup> The biopsies of conjunctival tissue, and in one case also limbal tissue,<sup>7</sup> is then explanted on the membrane and allowed to attach before it is submerged in culture medium. The epithelial cells are stimulated by the nutrients of the medium to proliferate and migrate out of the biopsy to cover the amnion membrane in one confluent layer (Figure 2).

#### **6.3.3 Bone marrow stem cells**

The only clinical study included in our review did not employ an explant culture system.<sup>50</sup>

### 6.3.4 Epidermal epithelial cells

Due to lack of sufficient clinical studies, explant culture systems have not yet been described in cultivation of epidermal epithelial cells.

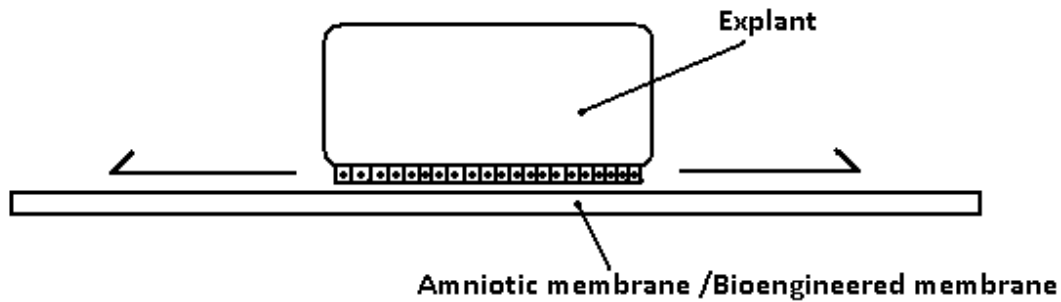


Figure 2: The explant placed on top of an amniotic membrane or a bioengineered membrane. Arrows indicate direction of migration.

## 6.4 Suspension culture systems

### 6.4.1 Oral epithelial cells

Eight of the studies reported a variant of the suspension culture system.<sup>3-6, 8, 29-31</sup> The principles of this method are to separate the cells in the biopsy using the enzyme dispase and a solution of trypsin and EDTA. Selected cells are then seeded on human AM and cocultured with mitomycin inactivated 3T3 fibroblasts in a culture medium until a confluent layer is reached. Differentiation of the cells is promoted by lowering the level of the medium, hence exposing the cells to air in the end of the period. Nishida *et al* and Hayashida *et al* reported a variation of this system.<sup>6, 30</sup> Instead of the traditional AM, the authors used a polymer membrane which alters its hydration properties according to changes in temperature. Cell adhesion and growth is facilitated at 37 °C and lowering the temperature promotes complete detachment of adherent cells without the use of enzymes or EDTA. This system also makes the grafted cell sheets easier to handle because they adhere rapidly to other surfaces (Figure 3).<sup>6, 30</sup>

#### 6.4.2 Conjunctival epithelial cells

Tanioka and co-workers is the only group using the suspension culture system to cultivate conjunctival epithelial cells (Figure 3).<sup>42</sup>

#### 6.4.3 Bone marrow stem cells

Ma and colleagues described using a suspension culture system in their study.<sup>50</sup> Human mesenchymal stem cells (MSC) were harvested from healthy donors and centrifuged and washed. The MSCs were cultured for 48 hours and analyzed by flow cytometry to confirm their MSC identity. Ultimately these MSCs were plated onto an amniotic membrane.

#### 6.4.4 Epidermal epithelial cells

Both the studies reported on epidermal epithelial cells used a variant of the suspension culture system.<sup>57,58</sup> The donor cells from the biopsy are digested enzymatically into separated single cells. The cell suspension is then cultivated on an amniotic membrane or in a growth arrested 3T3 fibroblast culture (Figure 3).

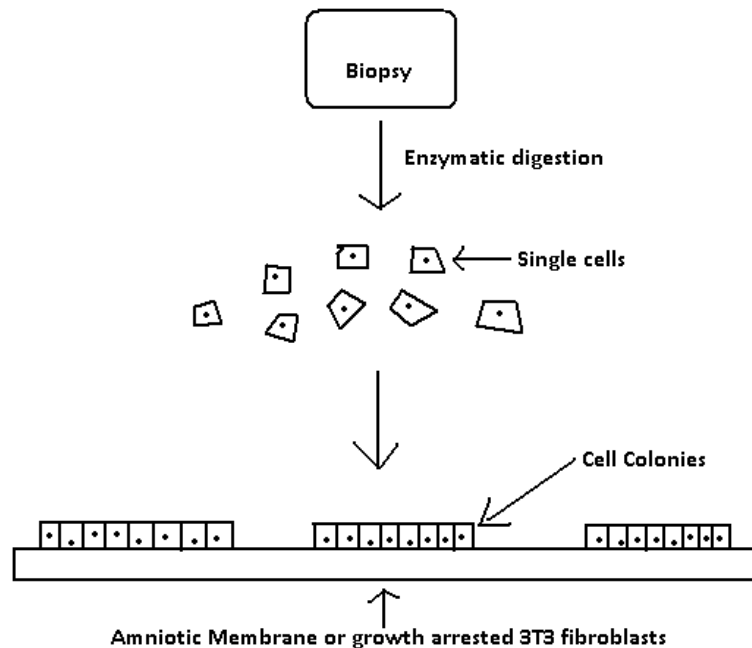


Figure 3: Digestion and cultivation of cells.

## **6.5 Culture media used**

### **6.5.1 Oral epithelial cells**

The ideal culture medium is one that is safe from disease transmission and able to support the clonal growth and serial propagation of cells. <sup>44</sup> Every clinical use of xenobiotic bioengineered ocular surface equivalents poses a risk of transmitting animal viruses and prions to human subjects.

All but one of the articles in the oral epithelial group reported using products of animal origin in their studies. <sup>30</sup> They all used fetal bovine serum (FBS) /fetal calf serum (FCS) in varying amounts. Inatomi and co-workers compared the use of 10 % autologous serum with FBS in 6 patients in one of their studies and found the former to be a viable method. <sup>4</sup> There is, to our knowledge, only one article reporting the use of a totally xenobiotic-free medium, i.e. 5% autologous serum in successful treatment of 10 patients. <sup>8</sup>

### **6.5.2 Conjunctival epithelial cells**

In two conjunctival studies on rabbit models FBS was used in various proportions in the culture medium. <sup>41, 42</sup> In the third study, Sangwan *et al* used FCS supplemented culture medium. <sup>7</sup>

### **6.5.3 Bone marrow stem cells**

The cultivating technique reported implied cultivation in a modified Eagle's medium of alpha which contained 10% FBS, Penicillin, Streptomycin and L-glutamine. <sup>50</sup> The adherent cells were washed twice and cultured for 10 to 14 days until cell clones were formed. As mentioned above, the adherent cultivated cells were analyzed using flow cytometry to confirm their MSC identity and then seeded out on a human amniotic membrane to produce confluent epithelial sheets.

### **6.5.4 Epidermal epithelial cells**

The two studies on goat models employed the use of Neonatal Bovine Serum, <sup>57, 58</sup> although later in the process the cells were resuspended in serum free medium before a goatskin-fibroblast-conditioned medium was used.

## **7. Evidence for presence of stem cells in cultures and grafts**

Although markers for epithelial stem cells have been proposed, their specificity remain controversial.<sup>1</sup> Therefore the identification of stem cells relies on their proliferative capacity in vitro and immune histological analysis of the graft. A measure of the proliferative capacity of the graft is the assessment of Bromodeoxyuridine (BrdU)-ELISA Cell Proliferation Assay<sup>47, 48, 60</sup> and Colony Forming Efficiency (CFE).<sup>11, 30, 47</sup>

Proposed epithelial stem cell markers such as DeltaN isoforms of p63 have been measured by some authors.<sup>30, 34, 42</sup> Another proposed putative stem cell marker, ABCG2, was also measured by Tanioka *et al.*<sup>42</sup>

Limbal epithelial cells are characterized by a low expression of cytokeratin 3 (CK3) in the suprabasal layers of the epithelium, while it is present in all layers of corneal epithelium.<sup>61, 62</sup> Conjunctival epithelial cells are on the other hand characterized by absence of the CK3/CK12 pair, whilst expression of goblet cell gel forming mucin (MUC5AC) is present.<sup>9</sup> Normal oral mucosal epithelium is characterized by the expression of cytokeratins 3 and 13 in all but the basal layers whilst cytokeratin 4 is normally expressed in the superficial and upper half of the intermediate layer.<sup>11</sup> Studies were carried out regarding cytokeratin markers in the oral epithelial grafts,<sup>3, 10, 11, 30, 32, 34</sup> the conjunctival epithelial grafts<sup>39, 42, 44, 47, 48, 63</sup> and epidermal epithelial grafts.<sup>57</sup> Ma and coworkers found absent of CK3 and human keratin-pan staining in their grafts, indicating that the MSCs had not differentiated into epithelial cells.<sup>50</sup>

## **8. Surgical transplantation of ex vivo cultured epithelial sheets**

### **8.1 Oral epithelial sheets**

All studies describe removal of the conjunctivalized ocular surface, the most common method being a 360 degree conjunctival tissue peritomy. Mitomycin C was applied by many authors to prevent postoperative conjunctivalization followed by vigorous washing with saline solution.<sup>3-5, 8, 29</sup> The oral epithelial sheets were then transferred to the exposed corneal stroma after either being separated from the human amniotic membrane<sup>3</sup> or transplanted with the supporting amniotic membrane.<sup>5, 31</sup> Hayashida and co-workers transferred the cultivated oral mucosal epithelial sheet onto a poly (vinylidene difluoride) (PVDF) support membrane and then transferred this onto the corneal stroma, followed by removal of the PVDF membrane with surgical scissors.<sup>30</sup> An even more unique membrane has been used by Nishida and coauthors, employing a temperature sensitive membrane that separates from the adjacent epithelium by lowering the temperature.<sup>6</sup> All but Nishida *et al.*,<sup>6</sup> described securing the epithelial sheet with

sutures. The integrity of the epithelial sheet was then confirmed by applying fluorescein eye drops.<sup>3-5,8</sup> All studies described the use of a protecting contact lens.

## **8.2 Conjunctival epithelial sheets**

All studies describe removal of the conjunctivalized ocular and fibrovascular pannus. The grafts were then transplanted onto the denuded ocular surface with<sup>7,41</sup> or without<sup>42</sup> the supporting amniotic membrane. The epithelial sheets were then secured with sutures.<sup>7,42</sup>

## **8.3 Bone marrow stem cells**

Ma and colleagues transplanted grafts onto 16 eyes of 16 rats.<sup>50</sup> Seven days after induced corneal injury, cells grown on amnion membrane were transplanted. The damaged corneal surfaces were carefully keratectomized under anesthesia. The grown cells on amnion membranes were sutured onto the corneal surface. The grafted cells on amnion membrane were then covered with a blank amnion membrane with the basement membrane facing up. After surgery, the rat eyelid was sutured to avoid grasping.

## **8.4 Epidermal epithelial sheets**

The two studies described are relatively different when it comes to details regarding the surgical procedure. One study only describes that epidermal adult stem cell – human amniotic membrane sheets (epiASC-HAM) were transplanted onto the ocular surface.<sup>57</sup> The other study has a more detailed approach.<sup>58</sup> Conjunctival peritomy was performed and subconjunctival fibrotic tissue removed. The epiASC-HAM was transferred over the ocular surface and the amniotic membrane (AM) fixed to the sclera with various sutures. Finally, two to four incisions on the AM were made to make the epidermal stem cells grow outward (the rationale behind this is not described), and the excess AM was trimmed.<sup>58</sup>

## **9. Postoperative management**

Almost all authors report the use of topical antibiotics and corticosteroids postoperatively to prevent infection and inflammation, the most common combination used was 0,1% Bethametasone and 0,3% Ofloxacin or equivalents, instilled 3-5 times daily.<sup>5-8, 29, 30, 41, 42, 57, 58</sup> Doses were tapered over 1-3 months.<sup>5-8, 29-31, 41, 42, 57, 58</sup> In addition to this topical treatment many authors described the use of systemic antibiotics<sup>5, 8, 42, 57, 58</sup> and/or corticosteroids.<sup>5, 6, 8, 29, 42, 57, 58</sup> This systemic treatment was given either orally<sup>5, 6, 8, 29</sup> or via intramuscular injections.<sup>42, 57, 58</sup>

As mentioned earlier, Ma and coworkers described suturing the eyelids of the intervention eye in their subjects (rats) to avoid grasping.<sup>50</sup> The eyes were reopened after 10 days and Dexamethasone-Gentamycine was applied twice daily.

To enhance wound healing and epithelial integrity, topical preservative-free eye drops containing hyaluronate sodium or chondroitin sulphate were used.<sup>29,58</sup> Nishida and authors occluded the puncta lacrimale to further enhance tear retention.<sup>6</sup> One article described the use of autologous serum derived eye drops for epithelial management, but the rationale for this is not described.<sup>29</sup>

## **10. Donor screening**

Transplantation of tissue between individuals usually implies a risk of contamination by bacteria, viruses, prions or other pathogens. Consequently, donors are regularly screened for pathogens, the most important being Human Immunodeficiency virus (HIV), Human T-lymphocyte virus (HTLV) and Hepatitis B and C virus (HBV and HCV). Only a few of the studies reported donor screening as described above. In fact, the only satisfactory screening described was that of donor human amniotic membranes (HAM), by Yang *et al*, where HIV type 1 and 2, HTLV, HBV and HCV were screened.<sup>58</sup> This underreporting does not necessarily imply that no screening was performed. Allogeneous transplantation does not necessitate thorough donor screening, because the graft is transplanted onto the donor. Preventive measures to infections are described regarding the preparation of HAM by washing it with a solution of saline and antibiotics.<sup>4,41,57,58</sup> In some studies that implied *ex vivo* cultivation of oral epithelium, preoperative (biopsy) daily treatment with iodine containing gargle has been described.<sup>5,6,29</sup> Furthermore, Ang *et al* reported screening the donors of autologous serum for soluble Fas ligand<sup>8</sup> which is thought to play an important role apoptosis of keratinocytes in Stevens Johnson Syndrome.<sup>64</sup>

## **11. Outcomes**

### **11.1 Clinical outcomes**

To our knowledge, there is no standardized scoring system or method for measuring the clinical outcome of corneal transplantation. There is, however, certain ways of measuring the success of the procedures and some scoring systems have been proposed and used in some of the

reported articles. As reported in table 1, the success rate, defined just as reported success in the individual studies, lacking a standard definition, was 64/70 eyes.

### 11.1.1 Improvement in ocular surface

Yang and co-workers used a system proposed by Ti *et al* to score the success of the epidermal transplants.<sup>65</sup> Eyes that regained four quadrants of clear cornea were considered a success, three quadrants a partial success and two or less clear quadrants were considered a failure.

Ma *et al* employed a scoring system of transparency of the corneal surface.<sup>50</sup> Results showed that 56% had a completely transparent cornea, in 38% the iris was not clear, and in 6% the pupil could not be seen.

Inatomi *et al* monitored corneal superficial vascularization photographically and used a grading system including extent and intensity, where grade 1 indicated peripheral vascularization, grade 2 peripheral and midperipheral vascularization, grade 3 modest vascularization involving the entire cornea, and grade 4 massive vascularization of the entire cornea.<sup>4</sup> Ma and co-authors also reported using an extensive scoring system based on neovascularization,<sup>50</sup> i.e. the extent of neovascularization related to the limbus. The results showed that 50% had no neovascularization, 38% had neovascularization within 2mm from the limbus, and 12% of the subjects had neovascularization over 2mm from the limbus.

It is important that the cornea regains a satisfactory barrier function. The structural integrity of the cornea following COMET can be measured by fluorescein stain. In one study using oral mucosal epithelium Satake and co-workers found that the transplanted oral epithelium demonstrated a sufficient barrier function to large molecules and pathogens, although the epithelium was permeable to small molecules, such as those in eye drops.<sup>29</sup>

Most of the studies used one or more of the following parameters in describing the ocular surface outcomes: Smooth surface,<sup>3, 5, 31, 42, 57</sup> stable ocular surface,<sup>29</sup> no defects,<sup>4-6, 29, 30, 42, 42</sup> integral epithelium,<sup>57</sup> complete coverage of the ocular surface,<sup>42</sup> complete re-epithelialisation,<sup>6</sup> opacity scoring,<sup>41</sup> transparency,<sup>4, 6, 42, 50</sup> normal secretion,<sup>57</sup> no goblet cells,<sup>29, 42</sup> decreased fibrotic tissue<sup>29</sup> and degree of neovascularization.<sup>4-6, 29, 41, 50</sup>

The most common method used was fluorescein staining<sup>5, 6, 29-31, 50</sup> and slit lamp examination,<sup>5, 6, 30, 50</sup> but different forms of cytology, H&E-stain, PAS and Immunocyto-chemistry are also described.<sup>57</sup> Most of the articles included pictures and descriptions of selected eyes.

The experiments portrayed on animals had the advantage of controls for comparison, whilst the human trials had to rely on preoperative data.

In general, the total success rate as reported in table 1 has to be interpreted with caution because there are great variations between the studies when it comes to methods and diseases treated.



### **11.1.2 Visual acuity**

All the human trials reported outcome in visual acuity either in detail or as an improvement in best corrected visual acuity (BCVA) of 2 or more lines (Table 1). Ma and colleagues reported using an interesting method for measuring VA in rats.<sup>50</sup> A head tracking device was fitted and the rats were tested using an optokinetic device, and showed a significant improvement in VA in the intervention group compared to the control group. Of the remaining studies Yang and co-workers used an eye patch on the healthy eye of 8 goats and found that the intervention eye had seemingly visual acuity as the goats could walk and seek food as normal.<sup>58</sup>

### **11.1.3 Subjective symptoms**

None of the clinical studies included in our review reported any information on improvement of subjective symptoms. This, however, does not necessarily implicate that no improvement occurred.

### **11.1.4 Results with transplantation of *ex vivo* cultured epithelial sheets**

#### **11.1.4.1 Oral epithelium**

With the exception of one study by Sangwan and co-workers using a combination of conjunctival and corneal epithelial cells, grafts from oral epithelium is to our knowledge the only alternative to limbal epithelial cells (LEC) demonstrated on human subjects. There are several advantages in using oral mucosal epithelium. The tissue is thought to be at a lower stage of differentiation than keratinocytes, it has short cell turn over time, it has a favorable location site for the biopsy, and has cytokeratin 3 (CK3) as a reliable marker.<sup>5</sup> Successful treatment has been reported in 45 of a total of 50 eyes in patients receiving grafted oral mucosal epithelium (table 1). The main hatch to using oral epithelium is the reports of corneal neovascularization.<sup>4</sup> As mentioned above, the transplanted oral mucosal epithelium also showed low barrier function.<sup>29</sup>

#### **11.1.4.2 Conjunctival epithelium**

Out of the 3 reported studies, only two had a measure of success.<sup>7,41</sup> Sangwan *et al*<sup>7</sup> and Ono *et al*<sup>41</sup> reported success in two out of two eyes and ten out of ten eyes, respectively. Two out of three studies were performed on animal subjects,<sup>41,42</sup> thus making the comparability to the

other methods less useful. In addition, the only human study was conducted on both eyes of one human subject.<sup>7</sup>

#### 11.1.4.3 Bone marrow stem cells

Ma and coauthors reported measures of success on many areas.<sup>50</sup> 16 eyes in 16 rats were transplanted using human mesenchymal stem cells cultivated on human amniotic membrane. As mentioned above significant differences were seen in the intervention group compared to control groups especially regarding VA. This study also measured improvement in corneal transparency, neovascularization and fluorescein staining compared to control groups. Interestingly, Ma and coworkers suggested that the intervention groups that received a MSC or a traditional limbal stem cell transplant had a lower incidence of neovascularization and inflammation because of underexpression of the inflammatory markers MMP-2, CD 45 and interleukin 2. As mentioned above, the MSC grafts did not display staining of CK3 and keratin-pan typical for human corneal epithelium, indicating that the MSCs had not differentiated into epithelium. This lack of differentiation was suggested to have a therapeutic effect on graft survival because of inhibition of inflammation and inflammation-related angiogenesis.

#### 11.1.4.4 Epidermal epithelium

This is the most recent and the least explored method reported. Yang and coworkers produced two studies on animal subjects, treating a total of 18 eyes.<sup>57,58</sup> Only one of these studies have a measure of success, indicating a successful transplantation in 7 out of 8 eyes.<sup>58</sup> The other study<sup>57</sup> reported a partial success rate of 70% according to the criteria described by Ti *et al.*<sup>65</sup>

### 11.2 Further surgical procedures

Penetrating keratoplasty (PKP) was reported as an additional procedure in four patients,<sup>3,29</sup> one study in which this was part of a two step surgical combination approximately 6 months after COMET.<sup>3</sup> Satake and coauthors performed PKP in two patients because of residual stromal opacity at 6 and 19 months and reported significant visual improvement in both patients.<sup>29</sup> Two eyes were re-operated, 12 eyes underwent cataract surgery<sup>4,8</sup> and two eyes had eyelid plastic surgery postoperatively.<sup>4</sup>

Further surgical procedures were not described in any of the animal models.

### 11.3 Evidence of donor cell survival

It is of great interest to know for how long and to what degree donor cells are capable of surviving transplantation. Nakamura *et al* and Inatomi *et al* uses characteristic differences in fluorescein staining patterns in cornea and conjunctiva as compared to oral epithelium to pose the survival of oral donor cells at 11<sup>5</sup> and 34 months.<sup>4</sup> These results strongly suggest the long survival and epithelial supply of presumed oral epithelial stem cells.<sup>4,5</sup>

A common method of detecting the surviving graft stem cells is yet to be described, although suggestions have been made. Performing immunohistochemical analysis of transplanted cultivated oral epithelium on the cornea excised during PKP at 6 months post operatively, Inatomi and coauthors found evidence that the intrinsic characteristics of the ectopically transplanted epithelium had not changed. The surviving epithelium was positive for CK3 and CK14 and negative for CK10 and CK12, resembling cultivated oral mucosal epithelium.<sup>3</sup> Satake *et al* confirmed survival of transplanted oral epithelial cells by impression cytology more than one year after transplantation.<sup>29</sup> Reconstructed corneal epithelium expressed a down regulation of CK1/10 over the course of 12 months, indirectly proving EpiASC survival in a goat model.<sup>57</sup> Tanioka *et al* and transplanted human conjunctival epithelial stem cells onto rabbit corneas and tracked these human cell with anti-human nuclear antibodies, thus proving that the epithelial cells on the cornea were of human origin, and indirectly proving survival of the graft cells.<sup>42</sup> Ma *et al* also used this technique to trace human cells in rat corneal transplants.<sup>50</sup>

### 11.4 Complications and adverse events

There is no exact definition of what is considered a complication in these cases. Some of the authors report no complications, but still describe vascularization.<sup>6,41</sup> Re- or neovascularisation was also reported by other authors,<sup>4,29</sup> but was not classified as a serious complication.<sup>8</sup> Two eyes showed increased intraocular pressure, which was successfully managed by antiglaucoma medication.<sup>3,29</sup> Two patients showed bilateral small epithelial defects, suggesting a bacterial infection, which was controlled by using of topical antibiotics.<sup>5,8</sup> Inatomi *et al* reported five eyes with small, but longstanding epithelial defects, two of which required reoperation.<sup>4</sup> One eye had a total corneal defect originating in the donor cornea after the medical contact lens fell off.<sup>3</sup> This defect gradually reepithelialized from the surrounding oral epithelium after rewear of the medical contact lens. Finally, one eye was reported having recurrence of conjunctivalization.

7

## **12. Regulatory considerations**

### **12.1 Animal subjects**

Although some authors lack description of regulatory guidelines, this does not necessarily mean that guidelines were not followed. In the studies describing guidelines, animals were treated according to the Association of Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research.<sup>30, 41</sup> Two articles state that the experimental procedure was approved by the local committee for animal research.<sup>30, 50</sup>

### **12.2 Human subjects**

Many authors state their study was approved by a local institutional review board for human research.<sup>3-6, 8, 42, 57</sup> As mentioned above, absence of such a statement does not implicate that regulatory guidelines were not followed. Most authors claim that prior informed oral and/or written consent was obtained from the human subjects in accordance to the Helsinki declaration for research involving human subjects.<sup>3-6, 8, 42, 57</sup>

## **13. Discussion and Future perspectives**

The lack of sufficient limbal stem cells to be harvested in patients suffering from bilateral ocular surface disease has paved the way for alternative sources of stem cells. This bypasses the need for immunosuppression which is necessary when using allogeneous grafts. Our article assesses recent, pioneering research of *ex vivo* cultivation of epithelial stem cells, in particular from oral, conjunctival, bone marrow and epidermal stem cells. In the existing literature, we identified a total of 14 articles using these alternative stem cell sources for cultivation and transplantation of epithelial sheets.

The treatment methods described in this article are new, but show promising future perspectives. There are some areas that need further investigation and/or improvements.

Most of the human subjects in our material had different forms of stem cell deficiency with different prognosis. Therefore we find it difficult to make conclusive generalizations in favor of certain methods.

An important problem is the lack of standardized effect estimates. Herein is the lack of standardized markers for stem cells both in *ex vivo* cultures and in grafts during follow up.

Although objective outcome measures were described in most studies, no gold standard has been identified or followed, thus making comparison between the studies difficult. Neither did we identify any reports of resolution or exacerbation of subjective symptoms which should be of great interest. Subjective data would be useful in comparing these to alternative modes of treatment, although this was not the scope of this review.

Furthermore there is no standardized timing for evaluation, nor any standardized method for comparing success or complications, although suggestions have been made.

A reliable comparison between the different studies is also compromised by the fact that both animal and human subjects are included.

Our review includes only a few clinical studies on the different sources of cultivating epithelial stem cells, making the statistical evaluation questionable. We reviewed 14 clinical studies regarding cultivation and transplantation of epithelial stem cells onto eyes suffering from LESCD. There are advantages and drawbacks with the different techniques. The obvious universal advantage is the ability to treat limbal stem cell deficiency without the use of limbal stem cells, hence providing an autologous source of progenitor/stem cells.

*Ex vivo* cultivation of oral epithelial stem cells is minimally invasive, sparing the ocular surface of any injury. This method involves taking a biopsy from an oral mucosa, which is not necessarily healthy in systemic diseases such as Stevens Johnson Syndrome, where both the ocular surface and oral mucosa are affected.

*Ex vivo* cultivation of conjunctival epithelial stem cells compared to the other therapy modalities involves taking a biopsy from the ocular surface. This could impose a risk of further ocular surface impairment in an already diseased eye. The conjunctival epithelium is believed to be closer to that of the corneal epithelium than that of the oral mucosal epithelium, indicating that this method could be superior in restoring a corneal surface.<sup>42</sup>

*Ex vivo* cultivation of bone marrow-derived mesenchymal stem cells represents, as the use of oral mucosal epithelium, a minimal invasive procedure. Isolation and expansion of these cells are also described as relatively easy. It is, however, still questionable whether MSCs can differentiate into epithelial cells.<sup>50</sup>

*Ex vivo* cultivation of epidermal stem cells, is not only applicable in cultivating ocular surface epithelial sheets, but has also shown promising results creating neurons, cardiomyocytes and osteoblasts.<sup>58</sup> This method however lacks research on human subjects.

We have identified the need for improvement in several phases of the processes described;

#### Biopsy

There is a need to identify stem cell markers to better be able to identify and harvest tissue suitable for *ex vivo* cultivation. Furthermore, it would be a significant step forward to pinpoint the exact location, niches, in for example the oral mucosa or the conjunctiva most suitable for

stem cell harvesting. This might improve the overall success and particularly the outcome for elderly patients as it has been suggested that the number of tissue specific stem cells decreases with age.<sup>5</sup>

### Cultivation

There is still work to be done to identify key factors controlling proliferation and differentiation of adult stem cells. More knowledge about these factors would in turn help create a more suitable microenvironment for *ex vivo* differentiation. Several authors have recently reported the successful use of autologous derived serum and artificially produced membranes. These are significant steps towards creating a complete xenobiotic free culture method, which will enhance the safety of the treatment.

Recently, a Norwegian group reported a novel method for storing and transporting cultured HLEC in a serum based medium.<sup>66-69</sup> Their reports hold high applicability for storage of all kinds of epithelial cells as they found that the same principles could be used in storage of conjunctival cells (unpublished data). As more clinical studies use other epithelial cells than from the limbus and conjunctiva, this method holds the promise to significantly improve tissue availability.

### Follow up

In general, more reports from human trials are needed, as are reports on the long term survival of the transplanted grafts. The hitherto longest reported follow up times identified are 22 months for humans and 30 months for animals (Table 1).

The process of neovascularization needs more attention. Work is being done to identify angiogenic/antiangiogenic factors expressed by the oral mucosal epithelium in particular.<sup>4</sup>

## **14. Conclusion**

In sum, the studies included all report positive results. We will institute a search for more and longer studies in humans, a general consensus on classification of disease, success and complications, as well as basal research to identify stem cell markers and xenobiotic-free culture methods. As more knowledge accumulates, we also hope it may all be gathered in easy-to-use databases to guide clinicians in search for the best and safest treatment options for their patients.

## **15. Method of literature search**

The basis of our literature is formed by searching Pubmed.com using the following key words: *Limbal Stem Cell Deficiency, Ocular surface disease ,Cornea, Reconstruction, Oral, Oral mucosa, Oral culture, Oral epithelium, Conjunctiva, Conjunctival, Conjunctival culture, Conjunctival epithelium ,Bone marrow, Mesencymal, Epidermal, Epidermal culture, Epidermis, Stem cells, Transplantation, Ex vivo, Cultivation, Culture and Epithelial sheets*. Combinations of the above mentioned key words were also used. We specifically searched for trials on human and animal subjects. The reference lists in the articles mentioned in our reference list were also a basis for further literature search. All identified, relevant studies until March 2009 were included.

## Reference List

1. Pellegrini G, Golisano O, Paterna P, et al. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol.* 1999;145:769-782.
2. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol.* 1986;103:49-62.
3. Inatomi T, Nakamura T, Kojyo M, Koizumi N, Sotozono C, Kinoshita S. Ocular surface reconstruction with combination of cultivated autologous oral mucosal epithelial transplantation and penetrating keratoplasty. *Am J Ophthalmol.* 2006;142:757-764.
4. Inatomi T, Nakamura T, Koizumi N, Sotozono C, Yokoi N, Kinoshita S. Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. *Am J Ophthalmol.* 2006;141:267-275.
5. Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N, Kinoshita S. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. *Br J Ophthalmol.* 2004;88:1280-1284.
6. Nishida K, Yamato M, Hayashida Y, et al. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med.* 2004;351:1187-1196.
7. Sangwan VS, Vemuganti GK, Iftekhar G, Bansal AK, Rao GN. Use of autologous cultured limbal and conjunctival epithelium in a patient with severe bilateral ocular surface disease induced by acid injury: a case report of unique application. *Cornea.* 2003;22:478-481.
8. Ang LP, Nakamura T, Inatomi T, et al. Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. *Arch Ophthalmol.* 2006;124:1543-1551.
9. Shortt AJ, Secker GA, Notara MD, et al. Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. *Surv Ophthalmol.* 2007;52:483-502.
10. Nakamura T, Inatomi T, Cooper LJ, Rigby H, Fullwood NJ, Kinoshita S. Phenotypic investigation of human eyes with transplanted autologous cultivated oral mucosal epithelial sheets for severe ocular surface diseases. *Ophthalmology.* 2007;114:1080-1088.
11. Nakamura T, Ang LP, Rigby H, et al. The use of autologous serum in the development of corneal and oral epithelial equivalents in patients with Stevens-Johnson syndrome. *Invest Ophthalmol Vis Sci.* 2006;47:909-916.



12. Sangwan VS, Vemuganti GK, Singh S, Balasubramanian D. Successful reconstruction of damaged ocular outer surface in humans using limbal and conjunctival stem cell culture methods. *Biosci Rep.* 2003;23:169-174.
13. Scuderi N, Alfano C, Paolini G, Marchese C, Scuderi G. Transplantation of autologous cultivated conjunctival epithelium for the restoration of defects in the ocular surface. *Scand J Plast Reconstr Surg Hand Surg.* 2002;36:340-348.
14. Ang LP, Tan DT. Autologous cultivated conjunctival transplantation for recurrent viral papillomata. *Am J Ophthalmol.* 2005;140:136-138.
15. Majo F, Barrandon Y, Othenin-Girard P, Toubienc M, Hoang-Xuan T. [Corneal epithelial diseases related to limbal stem cell deficiency]. *J Fr Ophtalmol.* 2006;29:1060-1069.
16. Secker GA, Daniels JT. Corneal epithelial stem cells: deficiency and regulation. *Stem Cell Rev.* 2008;4:159-168.
17. Lopez-Garcia JS, Garcia-Lozano I, Rivas L, Martinez-Garchitorena J. [Congenital aniridia keratopathy treatment]. *Arch Soc Esp Oftalmol.* 2006;81:435-444.
18. Liang L, Sheha H, Li J, Tseng SC. Limbal stem cell transplantation: new progresses and challenges. *Eye.* 2008.
19. Ang LP, Tan DT. Ocular surface stem cells and disease: current concepts and clinical applications. *Ann Acad Med Singapore.* 2004;33:576-580.
20. Kojima T, Higuchi A, Goto E, Matsumoto Y, Dogru M, Tsubota K. Autologous serum eye drops for the treatment of dry eye diseases. *Cornea.* 2008;27 Suppl 1:S25-S30.
21. Geerling G, MacLennan S, Hartwig D. Autologous serum eye drops for ocular surface disorders. *Br J Ophthalmol.* 2004;88:1467-1474.
22. Espana EM, Di PM, Grueterich M, Solomon A, Tseng SC. Keratolimbal allograft in corneal reconstruction. *Eye.* 2004;18:406-417.
23. Cauchi PA, Ang GS, zuara-Blanco A, Burr JM. A systematic literature review of surgical interventions for limbal stem cell deficiency in humans. *Am J Ophthalmol.* 2008;146:251-259.
24. Croasdale CR, Schwartz GS, Malling JV, Holland EJ. Keratolimbal allograft: recommendations for tissue procurement and preparation by eye banks, and standard surgical technique. *Cornea.* 1999;18:52-58.
25. Grueterich M, Tseng SC. [Surgical approaches for limbal stem cell deficiency]. *Klin Monatsbl Augenheilkd.* 2002;219:333-339.
26. Reinhard T, Sundmacher R, Spelsberg H, Althaus C. Homologous penetrating central limbo-keratoplasty (HPCLK) in bilateral limbal stem cell insufficiency. *Acta Ophthalmol Scand.* 1999;77:663-667.

27. Sundmacher R, Reinhard T, Althaus C. [Homologous central limbo-keratoplasty in limbus stem cell damage. Retrospective study of 3 years' experience]. *Ophthalmologe*. 1997;94:897-901.
28. Tseng SC. Amniotic membrane transplantation for ocular surface reconstruction. *Biosci Rep*. 2001;21:481-489.
29. Satake Y, Dogru M, Yamane GY, Kinoshita S, Tsubota K, Shimazaki J. Barrier function and cytologic features of the ocular surface epithelium after autologous cultivated oral mucosal epithelial transplantation. *Arch Ophthalmol*. 2008;126:23-28.
30. Hayashida Y, Nishida K, Yamato M, et al. Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature-responsive culture surface. *Invest Ophthalmol Vis Sci*. 2005;46:1632-1639.
31. Nakamura T, Kinoshita S. Ocular surface reconstruction using cultivated mucosal epithelial stem cells. *Cornea*. 2003;22:S75-S80.
32. Kinoshita S, Koizumi N, Nakamura T. Transplantable cultivated mucosal epithelial sheet for ocular surface reconstruction. *Exp Eye Res*. 2004;78:483-491.
33. Kinoshita S, Nakamura T. Development of cultivated mucosal epithelial sheet transplantation for ocular surface reconstruction. *Artif Organs*. 2004;28:22-27.
34. Madhira SL, Vemuganti G, Bhaduri A, Gaddipati S, Sangwan VS, Ghanekar Y. Culture and characterization of oral mucosal epithelial cells on human amniotic membrane for ocular surface reconstruction. *Mol Vis*. 2008;14:189-196.
35. Yokoo S, Yamagami S, Mimura T, et al. UV absorption in human oral mucosal epithelial sheets for ocular surface reconstruction. *Ophthalmic Res*. 2006;38:350-354.
36. Imaizumi F, Asahina I, Moriyama T, Ishii M, Omura K. Cultured mucosal cell sheet with a double layer of keratinocytes and fibroblasts on a collagen membrane. *Tissue Eng*. 2004;10:657-664.
37. Costea DE, Dimba AO, Loro LL, Vintermyr OK, Johannessen AC. The phenotype of in vitro reconstituted normal human oral epithelium is essentially determined by culture medium. *J Oral Pathol Med*. 2005;34:247-252.
38. Rouabhia M, Deslauriers N. Production and characterization of an in vitro engineered human oral mucosa. *Biochem Cell Biol*. 2002;80:189-195.
39. Meller D, Dabul V, Tseng SC. Expansion of conjunctival epithelial progenitor cells on amniotic membrane. *Exp Eye Res*. 2002;74:537-545.
40. Wei ZG, Wu RL, Lavker RM, Sun TT. In vitro growth and differentiation of rabbit bulbar, fornix, and palpebral conjunctival epithelia. Implications on conjunctival epithelial transdifferentiation and stem cells. *Invest Ophthalmol Vis Sci*. 1993;34:1814-1828.

41. Ono K, Yokoo S, Mimura T, et al. Autologous transplantation of conjunctival epithelial cells cultured on amniotic membrane in a rabbit model. *Mol Vis*. 2007;13:1138-1143.
42. Tanioka H, Kawasaki S, Yamasaki K, et al. Establishment of a cultivated human conjunctival epithelium as an alternative tissue source for autologous corneal epithelial transplantation. *Invest Ophthalmol Vis Sci*. 2006;47:3820-3827.
43. Alfano C, Chiummariello S, Fioramonti P, Innocenzi D, Scuderi N. Ultrastructural study of autologous cultivated conjunctival epithelium. *Ophthalmic Surg Lasers Imaging*. 2006;37:378-382.
44. Ang LP, Tan DT, Seah CJ, Beuerman RW. The use of human serum in supporting the in vitro and in vivo proliferation of human conjunctival epithelial cells. *Br J Ophthalmol*. 2005;89:748-752.
45. Higuchi A, Shimmura S, Takeuchi T, Suematsu M, Tsubota K. Elucidation of apoptosis induced by serum deprivation in cultured conjunctival epithelial cells. *Br J Ophthalmol*. 2006;90:760-764.
46. Yoshizawa M, Feinberg SE, Marcelo CL, Elnér VM. Ex vivo produced human conjunctiva and oral mucosa equivalents grown in a serum-free culture system. *J Oral Maxillofac Surg*. 2004;62:980-988.
47. Ang LP, Tan DT, Beuerman RW, Lavker RM. Development of a conjunctival epithelial equivalent with improved proliferative properties using a multistep serum-free culture system. *Invest Ophthalmol Vis Sci*. 2004;45:1789-1795.
48. Ang LP, Cheng ZY, Beuerman RW, Teoh SH, Zhu X, Tan DT. The development of a serum-free derived bioengineered conjunctival epithelial equivalent using an ultrathin poly(epsilon-caprolactone) membrane substrate. *Invest Ophthalmol Vis Sci*. 2006;47:105-112.
49. Ang LP, Tan DT, Cajucom-Uy H, Phan TT, Beuerman RW, Lavker RM. Reconstruction of the ocular surface by transplantation of a serum free cultivated conjunctival tissue equivalent. *Ann Acad Med Singapore*. 2004;33:S55-S56.
50. Ma Y, Xu Y, Xiao Z, et al. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. *Stem Cells*. 2006;24:315-321.
51. Pittenger MF, Mackay AM, Beck SC, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999;284:143-147.
52. Hakuno D, Fukuda K, Makino S, et al. Bone marrow-derived regenerated cardiomyocytes (CMG Cells) express functional adrenergic and muscarinic receptors. *Circulation*. 2002;105:380-386.
53. Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93-98.

54. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res.* 2000;61:364-370.
55. Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc Natl Acad Sci U S A.* 1999;96:10711-10716.
56. Zhao LR, Duan WM, Reyes M, Keene CD, Verfaillie CM, Low WC. Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol.* 2002;174:11-20.
57. Yang X, Moldovan NI, Zhao Q, et al. Reconstruction of damaged cornea by autologous transplantation of epidermal adult stem cells. *Mol Vis.* 2008;14:1064-1070.
58. Yang X, Qu L, Wang X, et al. Plasticity of epidermal adult stem cells derived from adult goat ear skin. *Mol Reprod Dev.* 2007;74:386-396.
59. Koizumi N, Inatomi T, Quantock AJ, Fullwood NJ, Dota A, Kinoshita S. Amniotic membrane as a substrate for cultivating limbal corneal epithelial cells for autologous transplantation in rabbits. *Cornea.* 2000;19:65-71.
60. Papa V, Leonardi A, Getuli C, Pacelli V, Russo P, Milazzo G. Effect of ofloxacin and netilmicin on human corneal and conjunctival cells in vitro. *J Ocul Pharmacol Ther.* 2003;19:535-545.
61. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol.* 1986;103:49-62.
62. Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. *Eye.* 2003;17:877-885.
63. Paladino G, Marino C, La Terra MS, Civile C, Rusciano D, Enea V. Cytokeratin expression in primary epithelial cell culture from bovine conjunctiva. *Tissue Cell.* 2004;36:323-332.
64. Abe R, Shimizu T, Shibaki A, Nakamura H, Watanabe H, Shimizu H. Toxic epidermal necrolysis and Stevens-Johnson syndrome are induced by soluble Fas ligand. *Am J Pathol.* 2003;162:1515-1520.
65. Ti SE, Anderson D, Touhami A, Kim C, Tseng SC. Factors affecting outcome following transplantation of ex vivo expanded limbal epithelium on amniotic membrane for total limbal deficiency in rabbits. *Invest Ophthalmol Vis Sci.* 2002;43:2584-2592.
66. Utheim TP, Raeder S, Utheim OA, et al. A novel method for preserving cultured limbal epithelial cells. *Br J Ophthalmol.* 2007;91:797-800.
67. Utheim TP, Raeder S, Utheim OA, et al. Sterility control and long-term eye bank storage of cultured human limbal epithelial cells for transplantation. *Br J Ophthalmol.* 2009.

68. Raeder S, Utheim TP, Utheim OA, et al. Effects of organ culture and Optisol-GS storage on structural integrity, phenotypes, and apoptosis in cultured corneal epithelium. *Invest Ophthalmol Vis Sci.* 2007;48:5484-5493.
69. Utheim TP, Raeder S, Utheim OA, et al. A novel method for preserving cultured limbal epithelial cells. *Br J Ophthalmol.* 2007;91:797-800.