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New developments in analysis of ocular surface diseases|Nieuwe ontwikkelingen in analyse van ziekten van het oogoppervlak

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PART I

LIMBAL STEM CELL DEFICIENCY

INTRODUCTION

INTRODUCTION

The cornea is the highly specialized part in front of the eye and its clarity and refractive ability are crucial for vision. A damaged corneal surface will reduce visual acuity and can ultimately lead to blindness. Most commonly the corneal epithelium is involved, but sometimes the corneal epithelial stem cells are also involved. These stem cells, located in the limbal region, play an important role in the continuous replacement of the basal corneal epithelial cells and thus in the maintenance of the corneal surface. Corneal stem cell dysfunction can lead to blindness, because the normal clear corneal epithelium is replaced by an opaque and vascularised conjunctival epithelium. Currently, there is no drug treatment for corneal stem cell deficiency and treatment has therefore been limited to transplantation of healthy limbal tissue. In patients with a healthy contralateral eye, an autologous transplant is usually successful; when both eyes are involved, allograft transplantation must be performed.^{1,2} However, allograft transplants are less successful, and need intensive systemic and local immuno-suppressive treatment to prevent rejection. Cyclosporin and corticosteroids are being used to prevent transplant rejection,^{3,4} but these drugs have serious side effects such as infections and tumor development in the long term.⁵ Since total limbal deficiency is a rare disease and human tissue for research is limited, animal models are being used to study limbal transplant rejection to find less harmful treatments. Using current animal models it is difficult to assess transplant rejection; even the model described by Mills et al.⁶ cannot predict transplant survival accurately. In this model the limbal transplants were followed both clinically and with PCR techniques. Sometimes no clinical rejection was seen but, unexpectedly, PCR analysis failed to prove transplant survival. However, in these cases the reliability of the PCR-test can be questioned, since at most only 200 corneal epithelial cells were available for analysis. The objective of this part of the thesis was to establish a new limbal transplant animal model in which transplant survival can be followed accurately in vivo.

CORNEAL EPITHELIAL STEM CELLS

Anatomically the cornea consists of five layers: epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium; 90% of the corneal thickness is made up by the stroma. The cornea is an unique tissue, and its transparency is due to its regular lamellar stromal structure together with its avascularity and its relative dehydrated state. The corneal epithelium and endothelium help the cornea to maintain these characteristics. The central and peripheral corneal epithelium consists of 5 to 6 layers of epithelial cells, while at the limbal region the epithelium is 3-4 cell layers thicker. The limbus, a transitional region, marked on one side by the transparent cornea and on the other side by the whitish opaque and vascularised sclera and conjunctiva, is a circular zone characterized by the palisades of Vogt.^{7,8} The limbus is the place where the corneal epithelial stem cells have their residence:^{9,10,11,12} and the stem cells are thought to be located in the palisades of Vogt. However, recent research suggests that the corneal stem cells are located in 5 to 7 crypts in the limbal region. These so called limbal epithelial crypts are groups of cells that extend from the interpalisade rete ridge to the conjunctival stroma.¹³ Stem cells are unique cells that are slow-cycling during homeostasis, poorly differentiated, have a high capacity for error-free self renewal, and a long life span.^{14,10} The limbal basal cells are known to have some of these character-

istics^{9,10,15,16,17} Corneal epithelial cell migration and differentiation starts at the limbus and occurs in a centripetal and vertical direction.¹⁸ It starts with an asymmetrical cell division of the limbal stem cell. One daughter cell remains undifferentiated and will replenish the stem cell pool (self renewal), the other daughter cell is a transient amplifying cell (TAC) that will further divide and differentiate. The TACs have limited self renewal capacity and will give rise to post-mitotic cells (PMC) that eventually become terminally-differentiated corneal epithelial cells (TDC) (Figure 1).¹⁹ For the protection and nourishment of the corneal epithelial stem cells the limbus is pigmented, forms palisades of Vogt, and is highly vascularised and innervated.⁸ The limbal region is also the place where the largest numbers of Langerhans cells (dendritic cells) are found.²⁰

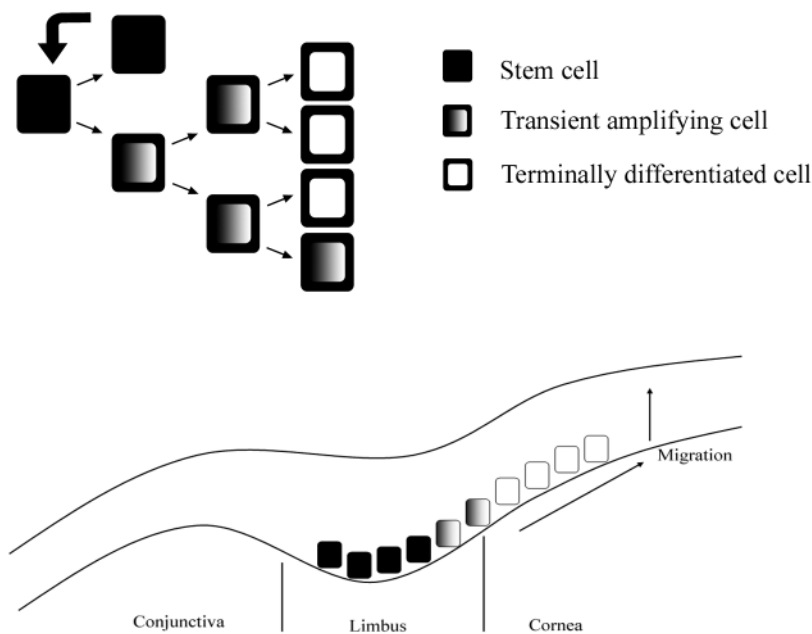
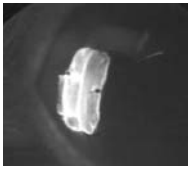


Figure 1. Limbal stem cell concept.

PROOF OF CORNEAL EPITHELIAL STEM CELLS

As has been mentioned above, the limbal epithelial basal cells have the characteristics of a stem cell. Keratin 3 and 12 are keratins that are specific for the corneal epithelium and are present at the suprabasal layers of the limbal epithelium, but not at the basal layers, indicating that the basal limbal region contains the least differentiated epithelial cells.^{9,15} Furthermore, the limbal basal epithelial cells have a higher proliferative capacity in culture than the central or peripheral corneal epithelial cells.^{16,17} Long time-intervals for thymidine incorporation further proved the long life cycle of the limbal basal cells.¹⁰ Further support for the limbal stem cell theory comes from experimental studies and clinical observations of abnormal corneal wound healing when the limbal epithelium is partly or completely re-

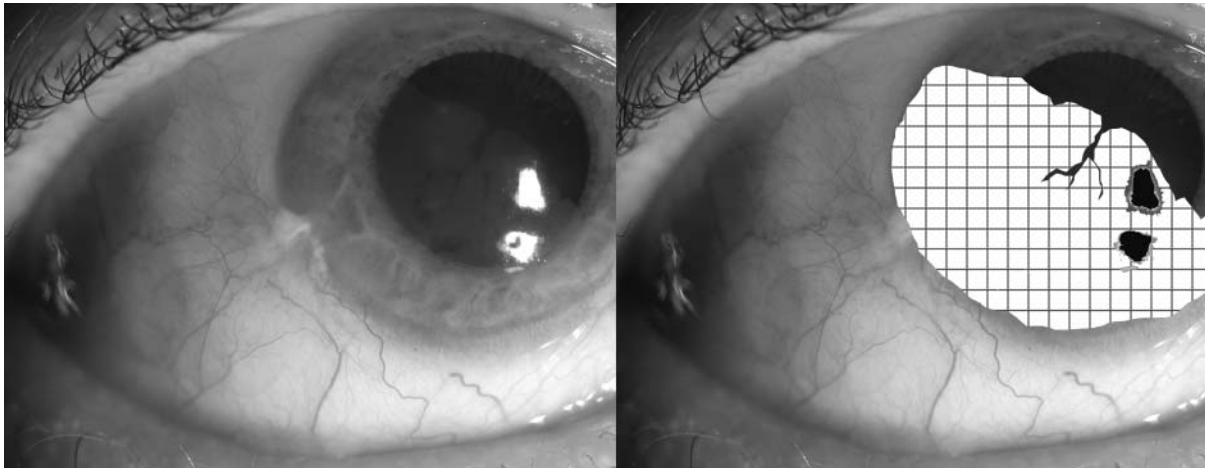


Figure 2. Partial limbal stem cell deficiency. The area with squares shows the partial limbal stem cell deficiency, where the cornea is partly opacified. The black areas inside the squares are light reflections.

moved.^{21,22,23,24} The abnormal epithelium is characterized by conjunctivalization, vascularization, and chronic inflammation, all characteristics of limbal stem cell deficiency. In addition, pigmented epithelial migration lines from the limbus to the central cornea can be seen in patients with heavily pigmented eyes and eccentric corneal epithelial defects.²⁵ Many attempts have been made to find limbal stem cell markers. The basal limbal epithelial cells are marked by high expression of p63, alpha9-integrin, alpha-enolase, ACG2 (ATP-binding cassette subfamily G member 2) and the absence of CD71, Connexin 43, gap junctions^{11,26,27,28}, but none of these markers are absolutely specific.²⁹

LIMBAL STEM CELL DEFICIENCY

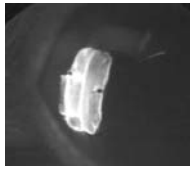
When the limbal stem cells are damaged, the corneal epithelium is slowly replaced by vascularised conjunctiva at the site of the damage (Figure 2). Since the conjunctival epithelium does not have the clarity of the corneal epithelium, the visual acuity will be decreased depending on the number and sites of limbal stem cells involved. Besides the opacification and vascularisation of the cornea, other characteristics for limbal disease are: loss of palisades of Vogt, epithelial instability resulting in ulceration or recurrent erosions, chronic inflammation, tear film dysfunction, and increased incidence of infectious keratitis.³⁰ Most patients therefore complain about reduced vision, irritation, pain, and light sensitivity, depending on the extent of limbal dysfunction.

There are a variety of causes of limbal stem cell dysfunction, which can be subdivided into genetic and acquired (Table 1). The acquired limbal stem cell disorders form the majority of cases seen clinically. To prove limbal stem cell deficiency, impression cytology can help to diagnose abnormal epithelium by detecting goblet cells or keratinisation.^{41,42} Limbal stem cell deficiency may be localized (partial), or diffuse (complete, 360°). In localized limbal stem cell deficiency, some sectors of the limbal and corneal epithelium are normal while the conjunctivalisation is restricted to the regions devoid of healthy epithelium. The stem cell dysfunction can be either unilateral or bilateral, which is of major consequence for the treat-

ment given, as will be discussed in the next paragraph.

Table 1. Causes of limbal stem cell dysfunction

Genetic causes
Aniridia ³¹
Epithelial and stromal dystrophies ³²
Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) ³³
Lacrimo-auriculo-dento-digital (LADD) syndrome ³⁴
Acquired causes
Chemical and thermal burns ³⁵
Contact lens wear ³⁶
Chronic inflammation ³⁷
Multiple surgeries at the limbus ³⁰
Cicatricial pemphigoid ³⁰
Stevens Johnson Syndrome ³⁰
Pterygium ³⁸
Drug toxicity ³⁹
Ocular surface squamous neoplasia ⁴⁰
Idiopathic ³⁰



TREATMENT OF LIMBAL STEM CELL DYSFUNCTION

Currently there is no drug therapy for limbal stem cell failure, and the treatment is therefore mostly limited to transplantation. Before the introduction of the limbal stem cell concept, corneal transplants were performed to improve visual acuity.⁴³ However, this is a solution for the short term since the actual problem, which is situated at the limbus, is not resolved. A variety of transplant techniques have been developed, aimed at solving the stem cell deficiency itself (Table 2). Conjunctival autografts were first used to treat limbal stem cell deficiency;⁴⁴ the conjunctival epithelium of these grafts was thought to be able to change its characteristics to become similar to the corneal epithelium. Later, this was called conjunctival transdifferentiation.⁴⁵ In time, the conjunctival transplants were modified to also include parts of the limbus.^{46,36} Using a rabbit model, Tsai et al. proved that transplants which included the limbus were more successful than conjunctival grafts.⁴⁷ Therefore most current transplantations involve the limbal region, with exception of transplantation of mucous membrane or amniotic membranes which will be mentioned later. The transplants that contain mostly conjunctival epithelium and limbal tissue are called conjunctival-limbal grafts, for kerato-limbal grafts the biggest part is corneal and limbal tissue. The conjunctival-limbal grafts are mostly used when there is a living donor, i.e. autologous transplants and living-related donors (a relative of the patient). The kerato-limbal transplant probably contains more limbal stem cell, however, it is more damaging to the donor eye and is therefore more often used when there is cadaveric tissue.⁵⁴

Table 2. Different transplantations performed for limbal stem cell deficiency

<i>Transplantation method</i>	
Autografts	Conjunctival graft ⁴⁴
	Conjunctival-limbal graft ⁴⁶
	Oral mucosa ⁶⁶
Allografts	Living-related conjunctival graft ⁴⁸
	Living-related conjunctival-limbal graft ^{49,50}
	Cadaveric conjunctival-limbal graft ⁵¹
	Cadaveric keratoepithelialplasty ⁵²
	Cadaveric kerato-limbal graft ⁵³

Limbal autografts have the advantage of not being rejected and are successful; however, a contralateral healthy eye must be available, since a donor eye with subclinical limbal dysfunction gives limited results.³⁶ Another major drawback is the possibility to induce limbal deficiency in the contralateral donor eye.^{55,56} Recent developments made it possible to culture limbal tissue *ex vivo* from a 1-2 mm² biopsy,^{57,58,59} thereby reducing the risk of limbal deficiency of the contralateral donor eye and increasing the number of patients that are suitable for autografting. However, when patients with severe bilateral disease are not suitable for autografts, limbal allografts are an alternative. The limbal allografts can come from a deceased donor (cadaveric graft). The high concentration of Langerhans cells (dendritic cells), abundance of HLA-DR antigens, and the good vascularisation makes these transplants at risk for rejection.²⁰ HLA matching of limbal allografts might help to improve transplant survival.⁶⁰ Similarly, living-related transplants are also used to decrease the risk of transplant rejection.^{49,50} Although there are reports of a good long term clinical outcome with limbal allografts, long term persistence of donor corneal epithelial cells is still a topic of discussion.^{61,62,63,64,65,60} Since some reports failed to show surviving donor epithelial cells, it was suggested that the transplant improved the local environment to such an extent that residual limbal stem cells were able to survive and be functional, possibly by providing growth factors or other mediators for the host stem cells. On the other hand, donor cells were recently found till 3.5 years after limbal allograft transplantation.⁶⁴

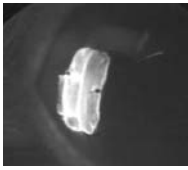
Alternatives for limbal allografts are either transplantation of amnion or autologous oral mucosa. Autologous oral mucosal epithelium has been introduced by Nishida et al,⁶⁶ who were able to improve the corneal surface and the visual acuity in four patients. Because the mucous transplant is autologous there is no rejection, while another advantage is the abundance of donor material in the oral cavity. Transplanted amnion, the most inner layer of the placental membranes, will also not reject since amnion lacks HLA-A, B or DR antigens.^{67,68}

Amnion transplants have been used in ophthalmology for corneal surface disease since 1940.⁶⁹ In 1995 amnion transplants were re-introduced in humans to treat ocular surface disease including limbal stem cell deficiency.⁷⁰ The amnion helps to improve epithelialization by providing a basement membrane and several growth factors;⁷¹ it also inhibits fibrosis, angiogenesis, and inflammation.^{72,73,74} Amnion is used in partial limbal stem cell deficient

eyes (less 50 %); when more than 50 % of the limbus is involved it can be combined with a limbal graft, however, the beneficial effect of the amnion is not clear.^{75,76,54}

Since most patients with limbal stem cell deficiency also have stromal involvement, penetrating keratoplasty is sometimes combined with a limbal transplant. There are indications that simultaneous transplantation of a limbal graft and a full thickness corneal button decreases graft survival.³ Therefore it is recommended that a limbal transplant must be performed first, and that the corneal transplant follows after three months, when the corneal epithelium is stable and the eye not inflamed.^{77,78}

Despite the variety of surgical techniques to reconstruct the ocular surface in limbal stem cell deficient eyes, it remains a disease that is difficult to treat. The success rate of a limbal transplants declines from 75-80% in the first year to 47-50% at three-year follow-up.^{75,72,73,79} Good prospective, case-controlled clinical trails for the various surgical techniques are still lacking.



TRANSPLANT REJECTION

That limbal allografts are prone to rejection has been mentioned above. Especially the vascularization and high amount of dendritic cells at the limbal region are factors that increase the chance for rejection. Transplant rejection is a T-cell mediated response to allo-antigens. In animal models high amounts of both CD4 and CD8 positive T cells and macrophages can be found.^{6,80,81} Treatment options to prevent allograft rejection may differ from a combination of topical prednisolon with systemic cyclosporine A, or systemic tacrolimus (prograf) and mycophenolatmofetil (cellcept). How long the therapy must last is not clear, but probably as long as there is a living allogeneic transplant. In patients who have a successful clinical outcome after limbal allograft transplantation, it is difficult to assess clinically whether the corneal epithelium is all donor-derived or if the host cells have repopulated the surface. Djalilian et al.⁶⁴ showed long term survival of donor cells and they support the use of long term immuno-suppression. However, others failed to establish donor cell survival and therefore question the use of systemic long term immuno-suppression.⁶⁵ Also tacrolimus, a macrolid antibiotic with immuno-suppressive characteristics and mycophenolate mofetil (CellCept) have been used to prevent rejection of limbal allografts.^{3,82,60} It must be noted that long term systemic immuno-suppressive treatment is known to be related to tumor development and susceptibility to infections.⁵ Since both can be life-threatening, questions must be raised whether the increase in visual acuity by a limbal allograft is worth the increased risk to develop cancers and infections. Therefore new less harmful therapies must be investigated.

In animal models, local clodronate liposome treatment has been used to prevent corneal graft rejection. Clodronate liposomes are liposomes which contain clodronate, which is a modified form of Ostac®. Both liposomes and clodronate itself are non-toxic. When macrophages consume and breakdown the liposomes, clodronate will be released intracellularly. Clodronate will therefore accumulate inside the macrophage, and when a certain threshold is reached, the cell is irreversibly damaged and will undergo apoptosis. In a corneal transplantation model clodronate liposomes have been used to locally deplete macrophages and thereby increase corneal transplant survival.^{83,84} This therapy has not yet

been registered for humans.

ANIMAL MODELS

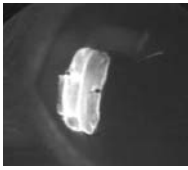
A variety of animal models have been developed, using mice, rats, and rabbits.^{6,85,86,87,88,89,90,47,81} Most animal models have disadvantages, which is probably inherent to the use of an animal model. Rabbit models are not suitable to investigate the immunological background of limbal transplantations since the animals are not inbred and there is a limited availability of antibodies. However, when compared to mice and rats, the rabbit eye probably more closely resembles the human eye. The mouse model is probably the best model to investigate the immunological process, because of the wide variety of mice strains and a huge amount of antibodies available. The size of the mouse eye and cornea are a limitation and limbal transplantations are therefore difficult to standardize. The only operation that probably can be reliably performed is the kerato-epithelial transplant. The rat model is a compromise between the mouse and rabbit models. The rat eye is large enough to perform limbal transplants, there are inbred strains, but only a limited availability of antibodies. Mills⁶ was the first to use the rat for limbal transplantation; in this model the limbal transplants were followed up both by PCR, and clinically. They showed that donor corneal epithelial cells did not survive much longer than seven days despite immuno-suppressive treatment. However, there was a discrepancy with the clinical follow-up where the treated allografts survived much longer. This could be similar to limbal transplants in humans, since clinically they perform well, while there is difficulty in showing donor epithelial cells some time after transplantation.^{61,63} The animal model by Mills⁶ is a reliable model, but we started our experiments with the hope that transplant follow-up could be improved, as is further discussed below and in Chapter 2.

ENHANCED GREEN FLUORESCENT PROTEIN

Green fluorescent protein was first discovered in the jellyfish *Aequorea* by Shimomura et al.⁹⁰ The major advantage of GFP is its intrinsic fluorescent capacity, for most other fluorescent proteins a co-factor is needed. Enhanced green fluorescent protein (E-GFP) is an enhanced version of GFP. When the protein is excited with blue light of around 488 nm it will emit green light from around 508 nm. It is a protein with a chromophore inside, which is responsible for the fluorescent characteristics. A fluorescent microscope is needed to illuminate the chromophore with pure blue light, the green light that is produced can then be recorded by the microscope. E-GFP has been widely used for research purposes, it can be used to track proteins in living cells.⁹¹ Also in animal models E-GFP is used to track tumor growth, tumor metastasis, tumor angiogenesis, and gene expression^{92,93,94,95,96}; it is considered to be the least immunogenic fluorescent protein. GFP has also been used to study corneal epithelial migration.^{18,97} In Chapter 2 we investigated whether E-GFP could be used to monitor limbal transplant behaviour, and whether it could be used to reliably detect transplant rejection.

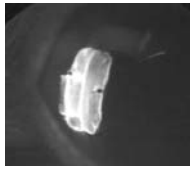
REFERENCES

1. Tseng SC, Tsubota K. Important concepts for treating ocular surface and tear disorders. *Am J Ophthalmol*. 1997;124:825-835.
2. Holland EJ, Schwartz GS. Changing concepts in the management of severe ocular surface disease over twenty-five years. *Cornea*. 2000;19:688-698.
3. Solomon A, Ellies P, Anderson DF et al. Long-term outcome of keratolimbal allograft with or without penetrating keratoplasty for total limbal stem cell deficiency. *Ophthalmology*. 2002;109:1159-1166.
4. Ilari L, Daya SM. Long-term outcomes of keratolimbal allograft for the treatment of severe ocular surface disorders. *Ophthalmology*. 2002;109:1278-1284.
5. Hojo M, Morimoto T, Maluccio M et al. Cyclosporine induces cancer progression by a cell-autonomous mechanism. *Nature*. 1999;397:530-534.
6. Mills RA, Coster DJ, Williams KA. Effect of immunosuppression on outcome measures in a model of rat limbal transplantation. *Invest Ophthalmol Vis Sci*. 2002;43:647-655.
7. Goldberg MF, Bron AJ. Limbal palisades of Vogt. *Trans Am Ophthalmol Soc*. 1982;80:155-171.
8. Townsend WM. The limbal palisades of Vogt. *Trans Am Ophthalmol Soc*. 1991;89:721-756.
9. 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.
10. Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell*. 1989;57:201-209.
11. Pellegrini G, Dellambra E, Golisano O et al. p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci U S A*. 2001;98:3156-3161.
12. Chen WY, Mui MM, Kao WW, Liu CY, Tseng SC. Conjunctival epithelial cells do not transdifferentiate in organotypic cultures: expression of K12 keratin is restricted to corneal epithelium. *Curr Eye Res*. 1994;13:765-778.
13. Dua HS, Shanmuganathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol*. 2005;89:529-532.
14. Daniels JT, Dart JK, Tuft SJ, Khaw PT. Corneal stem cells in review. *Wound Repair Regen*. 2001;9:483-494.
15. Kurpakus MA, Stock EL, Jones JC. Expression of the 55-kD/64-kD corneal keratins in ocular surface epithelium. *Invest Ophthalmol Vis Sci*. 1990;31:448-456.
16. Ebato B, Friend J, Thoft RA. Comparison of limbal and peripheral human corneal epithelium in tissue culture. *Invest Ophthalmol Vis Sci*. 1988;29:1533-1537.
17. Lindberg K, Brown ME, Chaves HV, Kenyon KR, Rheinwald JG. In vitro propagation of human ocular surface epithelial cells for transplantation. *Invest Ophthalmol Vis Sci*. 1993;34:2672-2679.
18. Nagasaki T, Zhao J. Centripetal movement of corneal epithelial cells in the normal adult mouse. *Invest Ophthalmol Vis Sci*. 2003;44:558-566.
19. Tseng SC. Concept and application of limbal stem cells. *Eye*. 1989;3 (Pt 2):141-157.
20. Gillette TE, Chandler JW, Greiner JV. Langerhans cells of the ocular surface. *Ophthalmology*. 1982;89:700-711.
21. Chen JJ, Tseng SC. Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. *Invest Ophthalmol Vis Sci*. 1991;32:2219-2233.
22. Chen JJ, Tseng SC. Corneal epithelial wound healing in partial limbal deficiency. *Invest Ophthalmol Vis Sci*. 1990;31:1301-1314.
23. Kruse FE, Chen JJ, Tsai RJ, Tseng SC. Conjunctival transdifferentiation is due to the incomplete removal of limbal basal epithelium. *Invest Ophthalmol Vis Sci*. 1990;31:1903-1913.
24. Huang AJ, Tseng SC. Corneal epithelial wound healing in the absence of limbal epithelium. *Invest Ophthalmol Vis Sci*. 1991;32:96-105.
25. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature*. 1971;229:560-561.
26. Tani H, Morris RJ, Kaur P. Enrichment for murine keratinocyte stem cells based on cell surface phenotype. *Proc Natl Acad Sci U S A*. 2000;97:10960-10965.
27. Zieske JD, Bukusoglu G, Yankauckas MA, Wasson ME, Keutmann HT. Alpha-enolase is restricted to basal



- cells of stratified squamous epithelium. *Dev Biol.* 1992;151:18-26.
28. Matic M, Petrov IN, Chen S et al. Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. *Differentiation.* 1997;61:251-260.
 29. Chee KY, Kicic A, Wiffen SJ. Limbal stem cells: the search for a marker. *Clin Experiment Ophthalmol.* 2006;34:64-73.
 30. Puangsricharern V, Tseng SC. Cytologic evidence of corneal diseases with limbal stem cell deficiency. *Ophthalmology.* 1995;102:1476-1485.
 31. Nishida K, Kinoshita S, Ohashi Y, Kuwayama Y, Yamamoto S. Ocular surface abnormalities in aniridia. *Am J Ophthalmol.* 1995;120:368-375.
 32. Dunaief JL, Ng EW, Goldberg MF. Corneal dystrophies of epithelial genesis: the possible therapeutic use of limbal stem cell transplantation. *Arch Ophthalmol.* 2001;119:120-122.
 33. Ahonen P, Myllarniemi S, Sipila I, Perheentupa J. Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *N Engl J Med.* 1990;322:1829-1836.
 34. Cortes M, Lambiase A, Sacchetti M, Aronni S, Bonini S. Limbal stem cell deficiency associated with LADD syndrome. *Arch Ophthalmol.* 2005;123:691-694.
 35. Wagoner MD. Chemical injuries of the eye: current concepts in pathophysiology and therapy. *Surv Ophthalmol.* 1997;41:275-313.
 36. Jenkins C, Tuft S, Liu C, Buckley R. Limbal transplantation in the management of chronic contact-lens-associated epitheliopathy. *Eye.* 1993;7 (Pt 5):629-633.
 37. Foster CS, Calonge M. Atopic keratoconjunctivitis. *Ophthalmology.* 1990;97:992-1000.
 38. Dushku N, Reid TW. Immunohistochemical evidence that human pterygia originate from an invasion of vimentin-expressing altered limbal epithelial basal cells. *Curr Eye Res.* 1994;13:473-481.
 39. Wilson FM. Adverse external ocular effects of topical ophthalmic therapy: an epidemiologic, laboratory, and clinical study. *Trans Am Ophthalmol Soc.* 1983;81:854-965.
 40. Lee GA, Hirst LW. Ocular surface squamous neoplasia. *Surv Ophthalmol.* 1995;39:429-450.
 41. Donisi PM, Rama P, Fasolo A, Ponzin D. Analysis of limbal stem cell deficiency by corneal impression cytology. *Cornea.* 2003;22:533-538.
 42. Sridhar MS, Vemuganti GK, Bansal AK, Rao GN. Impression cytology-proven corneal stem cell deficiency in patients after surgeries involving the limbus. *Cornea.* 2001;20:145-148.
 43. Brown SI, Bloomfield SE, Pearce DB. A follow-up report on transplantation of the alkali-burned cornea. *Am J Ophthalmol.* 1974;77:538-542.
 44. Thoft RA. Conjunctival transplantation. *Arch Ophthalmol.* 1977;95:1425-1427.
 45. Shapiro MS, Friend J, Thoft RA. Corneal re-epithelialization from the conjunctiva. *Invest Ophthalmol Vis Sci.* 1981;21:135-142.
 46. Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology.* 1989;96:709-722.
 47. Tsai RJ, Sun TT, Tseng SC. Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. *Ophthalmology.* 1990;97:446-455.
 48. Kwitko S, Marinho D, Barcaro S et al. Allograft conjunctival transplantation for bilateral ocular surface disorders. *Ophthalmology.* 1995;102:1020-1025.
 49. Daya SM, Ilari FA. Living related conjunctival limbal allograft for the treatment of stem cell deficiency.
 50. Rao SK, Rajagopal R, Sitalakshmi G, Padmanabhan P. Limbal allografting from related live donors for corneal surface reconstruction. *Ophthalmology.* 1999;106:822-828.
 51. Pfister RR. Corneal stem cell disease: concepts, categorization, and treatment by auto- and homotransplantation of limbal stem cells. *CLAO J.* 1994;20:64-72.
 52. Thoft RA. Keratoepithelioplasty. *Am J Ophthalmol.* 1984;97:1-6.
 53. Turgeon PW, Nauheim RC, Roat MI, Stopak SS, Thoft RA. Indications for keratoepithelioplasty. *Arch Ophthalmol.* 1990;108:233-236.
 54. Santos MS, Gomes JA, Hofling-Lima AL et al. Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. *Am J Ophthalmol.* 2005;140:223-230.
 55. Tan DT, Ficker LA, Buckley RJ. Limbal transplantation. *Ophthalmology.* 1996;103:29-36.
 56. Basti S, Mathur U. Unusual intermediate-term outcome in three cases of limbal autograft transplantation. *Ophthalmology.* 1999;106:958-963.

57. Rama P, Bonini S, Lambiase A et al. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. *Transplantation*. 2001;72:1478-1485.
58. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med*. 2000;343:86-93.
59. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea*. 2000;19:421-426.
60. Reinhard T, Spelsberg H, Henke L et al. Long-term results of allogeneic penetrating limbo-keratoplasty in total limbal stem cell deficiency. *Ophthalmology*. 2004;111:775-782.
61. Henderson TR, Coster DJ, Williams KA. The long term outcome of limbal allografts: the search for surviving cells. *Br J Ophthalmol*. 2001;85:604-609.
62. Shimazaki J, Kaido M, Shinozaki N et al. Evidence of long-term survival of donor-derived cells after limbal allograft transplantation. *Invest Ophthalmol Vis Sci*. 1999;40:1664-1668.
63. Williams KA, Brereton HM, Aggarwal R et al. Use of DNA polymorphisms and the polymerase chain reaction to examine the survival of a human limbal stem cell allograft. *Am J Ophthalmol*. 1995;120:342-350.
64. Djalilian AR, Mahesh SP, Koch CA et al. Survival of donor epithelial cells after limbal stem cell transplantation. *Invest Ophthalmol Vis Sci*. 2005;46:803-807.
65. Egarth M, Hellkvist J, Claesson M, Hanson C, Stenevi U. Longterm survival of transplanted human corneal epithelial cells and corneal stem cells. *Acta Ophthalmol Scand*. 2005;83:456-461.
66. 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.
67. Adinolfi M, Akle CA, McColl I et al. Expression of HLA antigens, beta 2-microglobulin and enzymes by human amniotic epithelial cells. *Nature*. 1982;295:325-327.
68. Akle CA, Adinolfi M, Welsh KI, Leibowitz S, McColl I. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. *Lancet*. 1981;2:1003-1005.
69. de Roth A. Plastic repair of conjunctival defects with fetal membrane. *Arch Ophthalmol*. 1940;23:522-525.
70. Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. *Cornea*. 1995;14:473-484.
71. Dua HS, Azuara-Blanco A. Amniotic membrane transplantation. *Br J Ophthalmol*. 1999;83:748-752.
72. Koizumi NJ, Inatomi TJ, Sotozono CJ et al. Growth factor mRNA and protein in preserved human amniotic membrane. *Curr Eye Res*. 2000;20:173-177.
73. Hao Y, Ma DH, Hwang DG, Kim WS, Zhang F. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. *Cornea*. 2000;19:348-352.
74. Tseng SC, Li DQ, Ma X. Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. *J Cell Physiol*. 1999;179:325-335.
75. Tseng SC, Prabhasawat P, Barton K, Gray T, Meller D. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. *Arch Ophthalmol*. 1998;116:431-441.
76. Ivekovic R, Tedeschi-Reiner E, Novak-Laus K et al. Limbal graft and/or amniotic membrane transplantation in the treatment of ocular burns. *Ophthalmologica*. 2005;219:297-302.
77. Sangwan VS, Matalia HP, Vemuganti GK et al. Early results of penetrating keratoplasty after cultivated limbal epithelium transplantation. *Arch Ophthalmol*. 2005;123:334-340.
78. 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.
79. Shimazaki J, Yang HY, Tsubota K. Amniotic membrane transplantation for ocular surface reconstruction in patients with chemical and thermal burns. *Ophthalmology*. 1997;104:2068-2076.
80. Miyazaki D, Inoue Y, Yao YF et al. T-cell-mediated immune responses in alloepithelial rejection after murine keratoepithelioplasty. *Invest Ophthalmol Vis Sci*. 1999;40:2590-2597.
81. Chen W, Cao L, Hara K, Yoshitomi T. Effect of immunosuppression on survival of allograft limbal stem cells. *Jpn J Ophthalmol*. 2004;48:440-447.
82. Sloper CM, Powell RJ, Dua HS. Tacrolimus (FK506) in the management of high-risk corneal and limbal grafts. *Ophthalmology*. 2001;108:1838-1844.
83. Slegers TP, van Rooijen N, van Rij G, van der GR. Delayed graft rejection in pre-vascularised corneas after subconjunctival injection of clodronate liposomes. *Curr Eye Res*. 2000;20:322-324.



84. Van der Veen G, Broersma L, Dijkstra CD et al. Prevention of corneal allograft rejection in rats treated with subconjunctival injections of liposomes containing dichloromethylene diphosphonate. *Invest Ophthalmol Vis Sci.* 1994;35:3505-3515.
85. 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.
86. Xu KP, Wu Y, Zhou J, Zhang X. Survival of rabbit limbal stem cell allografts after administration of cyclosporin A. *Cornea.* 1999;18:459-465.
87. Tsai RJ, Tseng SC. Effect of stromal inflammation on the outcome of limbal transplantation for corneal surface reconstruction. *Cornea.* 1995;14:439-449.
88. Yao YF, Inoue Y, Miyazaki D et al. Ocular resurfacing and alloepithelial rejection in a murine keratoepithelioplasty model. *Invest Ophthalmol Vis Sci.* 1995;36:2623-2633.
89. Swift GJ, Aggarwal RK, Davis GJ, Coster DJ, Williams KA. Survival of rabbit limbal stem cell allografts. *Transplantation.* 1996;62:568-574.
90. Shimomura O, Johnson FH, Saiga Y. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *J Cell Comp Physiol.* 1962;59:223-239.
91. Fletcher LM, Welsh GI, Oatey PB, Tavaré JM. Role for the microtubule cytoskeleton in GLUT4 vesicle trafficking and in the regulation of insulin-stimulated glucose uptake. *Biochem J.* 2000;352 Pt 2:267-276.
92. Yang M, Jiang P, Sun FX et al. A fluorescent orthotopic bone metastasis model of human prostate cancer. *Cancer Res.* 1999;59:781-786.
93. Kan Z, Liu TJ. Video microscopy of tumor metastasis: using the green fluorescent protein (GFP) gene as a cancer-cell-labeling system. *Clin Exp Metastasis.* 1999;17:49-55.
94. Yang M, Baranov E, Jiang P et al. Whole-body optical imaging of green fluorescent protein-expressing tumors and metastases. *Proc Natl Acad Sci U S A.* 2000;97:1206-1211.
95. Yang M, Baranov E, Moossa AR, Penman S, Hoffman RM. Visualizing gene expression by whole-body fluorescence imaging. *Proc Natl Acad Sci U S A.* 2000;97:12278-12282.
96. Yang M, Baranov E, Li XM et al. Whole-body and intravital optical imaging of angiogenesis in orthotopically implanted tumors. *Proc Natl Acad Sci U S A.* 2001;98:2616-2621.
97. Moore JE, McMullen CB, Mahon G, Adamis AP. The corneal epithelial stem cell. *DNA Cell Biol.* 2002;21:443-451.

