

Premio
A.I.R.C.M.O.
O N L U S



**Associazione Italiana
per la Ricerca e la Cura
delle Malattie degli Occhi**

Milano, 29 maggio 2011

Istituto Scientifico Universitario San Raffaele
Faculty Club

LE SCELTE E GLI OBIETTIVI

AIRCMO - Associazione Italiana per la Ricerca e la Cura delle Malattie degli Occhi - è sorta nel 1997. L'originaria idea dei soci fondatori di AIRCMO è stata concretizzata dalla volontà oltre che dei privati, che in gran numero e con generosità ed entusiasmo hanno aderito, dalle maggiori aziende del settore ottico che si sono impegnate a sostenerne finanziariamente l'operato. Senza fini di lucro, l'Associazione si occupa dello sviluppo e del sostegno alla ricerca scientifica e medica nel campo delle malattie degli occhi.

Progetto di vita, scopo principale e fine ultimo dell'Associazione è il sostegno alla ricerca. Uno slancio non più, o non solo, umanitario ma, bensì, concreto e rivolto al miglioramento della vita. Un contributo di speranza per l'esistenza di tanti uomini.

Tutelare ed aiutare, quindi, i talenti scientifici, contribuire al miglioramento della ricerca italiana e partecipare concretamente allo sviluppo di benemerite istituzioni di cura sono gli scopi primari dell'Associazione.

Un'attenta analisi dei progetti e valutazioni scientifiche sono i canoni portanti delle scelte che l'Associazione opera. Obiettivi chiari, individuabili, concreti e realizzabili sono i parametri per ottenere il patrocinio dell'Ente.

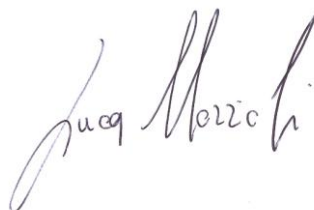
Nell'ambito delle iniziative volte a favorire la ricerca scientifica e medica, l'Associazione istituisce un premio biennale, giunto alla sua settima edizione, da assegnare a un'equipe di ricercatori che si sia particolarmente distinta nell'ambito specifico di studio applicativo; finanzia inoltre centri di ricerca e favorisce la costituzione di comitati scientifici ad hoc.

Nel nostro Paese, oggi, affrontare e dedicarsi alla ricerca spesso significa accettare sacrifici personali e, in qualche caso, anche economici. Tutti sappiamo come la crescita economica e le "aspettative" di miglioramento della vita, in quasi tutti i settori, siano legate allo sviluppo scientifico e alla ricerca applicata; per questi motivi i promotori dell'Associazione hanno ritenuto importante, con il conferimento di un concreto contributo a questa quotidiana lotta, la sensibilizzazione dell'opinione pubblica in generale e del mondo scientifico in particolare.

Oltre al Premio alla ricerca scientifica, AIRCMO contribuisce al sostegno di enti con un contributo per il loro benemerito operato. Tra questi ricordiamo: la Fondazione Banca degli Occhi del Veneto, il Centro Riabilitazione non Vedenti “Gli Angeli di Padre Pio”, la Fondazione ANT che si occupa di assistenza domiciliare ai malati oncologici. AIRCMO è anche attiva nelle operazioni di solidarietà per il terzo mondo attraverso la fornitura di lenti, montature e macchinari tecnici per le missioni francescane in Africa e per il Nava Drushti Charity Eye Centre in India.

Uno spirito, quello di AIRCMO, che ha avuto un importante momento di consacrazione nel 2003 in occasione dell’incontro in Vaticano con Giovanni Paolo II; incontro dal quale Valter Lamperti e tutto il gruppo dei volontari dell'Associazione hanno “ricevuto” una grande fonte di entusiasmo e di slancio verso le “sfide” del futuro.

Nelle sue diverse edizioni il Premio AIRCMO ha “toccato” alcuni dei luoghi più importanti e simbolici d'Italia; luoghi scelti non solo per il loro prestigio ma, anche e soprattutto, per l'attinenza con quelli che sono i principi guida ed etici dell'Associazione. E' così che dopo Venezia, Rieti e Roma il Premio, nel 2011, dopo l'edizione 2008 presso l'Università Cattolica del Sacro Cuore, torna a Milano all'Istituto Scientifico Universitario San Raffaele, luogo deputato di simbiosi tra scienza e fede. Un luogo simbolo delle forte radicamento tra insegnamento accademico, apertura alla società e servizio alla comunità.

A handwritten signature in black ink, reading "Luca Marziali". The signature is fluid and cursive, with the first name "Luca" and the last name "Marziali" clearly legible.

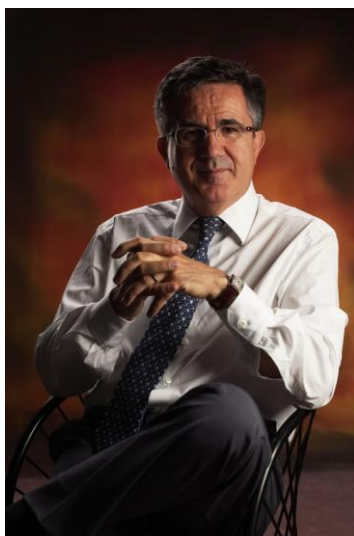
Il Presidente del Premio
Luca Marziali

LA PROSECUZIONE DI UN SOGNO

Nata da un'idea e dall'impegno sociale e cristiano di Giuseppe Lamperti, imprenditore bresciano, l'Associazione Italiana per la Ricerca e la Cura delle Malattie degli Occhi è stata sviluppata e realizzata dal figlio Valter.

Valter Lamperti ha reso concreta questa idea, l'ha portata nella società, l'ha concretizzata in un modo impareggiabile. Suo è stato lo sviluppo legale e organizzativo, suo lo slancio verso il mondo accademico e della ricerca, suo l'approccio pragmatico ma, al tempo stesso, di alto profilo intellettuale all'abbinamento tra imprenditorialità e mondo scientifico.

Con lui A.I.R.C.M.O. è diventata grande e si è data una struttura di prim'ordine nel panorama delle Onlus nazionali.



Valter Lamperti 1951-2009

Quella odierna è la prima edizione senza di lui. Purtroppo Valter ci ha lasciato. Ma quello che ci manca è lui, come uomo e come amico mentre la sua traccia operativa è solida e senza nessun problema.

Per questo l'edizione 2011 del premio A.I.R.C.M.O. è, e resterà per sempre, merito suo.

Grazie Valter

gli amici di A.I.R.C.M.O.

IL COMITATO DIRETTIVO

La gestione e il coordinamento di tutte le attività di AIRCMO è affidato ad un Comitato Direttivo composto da professionisti che, gratuitamente e senza fini di lucro, offrono la propria opera e la propria esperienza per la gestione di tutte le diverse fasi operative dell'Associazione.

Sotto la guida del Presidente di Aircmo, Grazia Lamperti gli interventi specialistici vengono coordinati e affidati, volta per volta, alle professionalità più adatte alle diverse esigenze.

Tramite il controllo del Comitato Direttivo operano così professionalità specifiche nella fase di analisi, selezione e valutazioni delle candidature per l'assegnazione del Premio, ma anche professionisti delle problematiche legali e amministrative, in quelle della gestione e del coordinamento degli sponsor, in quelle del marketing, della comunicazione e delle pubbliche relazioni.

A questi interventi "specialistici" si aggiungono poi decine di aziende del settore ottico e moltissimi privati che dedicano tempo ed energie al sostegno dell'Associazione.

IL COMITATO ETICO

Spirito francescano e moderna razionalità animano il Comitato Etico di A.I.R.C.M.O. che vigila e controlla sull'Associazione affinché quest'ultima non venga mai meno ai suoi principi di trasparenza. Compito e fine del Comitato Etico composto da persone super-partes, sia francescani che laici, è quello di valutare e consigliare nelle scelte il Comitato direttivo dell'Associazione affinché la stessa mantenga, sempre, il suo spirito originario e la sua vocazione verso il sostegno alla ricerca scientifica ed alla solidarietà sociale.

Le parole e l'esempio di Francesco, fatto di spiritualità ma anche di tanta concretezza e lungimiranza, restano il tratto distintivo e peculiare del Comitato Etico di Aircmo e di tutti volontari che in essa si impegnano.

Durante il soggiorno a Fontecolombo nel 1225, un anno prima della morte, Francesco fu convinto da frate Elia a lasciarsi operare. Il cauterio lo attendeva. Il ferro rovente doveva posarsi sugli occhi devastati dall'infezione. Francesco stupì il medico e i suoi fratelli, rivolgendo una preghiera al fuoco:

"O mio fratello fuoco, l'Altissimo ti ha creato splendido e invidiabile per tutte le altre creature, forte, bello ed utile. In questo momento sii buono con me, sii gentile. Io prego il grande Signore che ti ha creato, perché moderi per me il tuo calore"

(Leg. Mag. FF. 1097)

I frati fuggirono dalla stanza impressionati, mentre il medico affrontava il ferro rovente dagli occhi fino alle orecchie. Francesco non sentì dolore. Intonò un cantico per Frate Fuoco e tutti i camini accesi di Rieti scoppiettarono allegramente all'unisono con la sua voce.

IL PREMIO ALLA RICERCA SCIENTIFICA

Valutazione scientifica e di impatto “sociale/operativo” sono i cardini su cui ruota e vive la scelta delle ricerche destinate ad essere selezionate per il Premio A.I.R.C.M.O..

Una giuria composta da affermati professionisti in ambito medico-scientifico, in forma completamente anonima, opera la scelta della ricerca migliore basandosi su una precedente selezione effettuata da consulenti scientifici.

Solo dopo questa approfondita selezione viene individuata la ricerca che verrà premiata nel corso della cerimonia ufficiale.

Trasparenza, obiettività e ricerca della qualità sono gli elementi fondamentali di qualsiasi decisione di AIRCMO.

Solo in questo modo, solo grazie al mantenimento dei propri principi costitutivi l'Associazione potrà mantenere, per tutti gli anni a venire, il suo ruolo di stimolo alla crescita professionale della ricerca italiana e la sua missione di aiuto concreto alla stessa.

Il Premio AIRCMO 2011
di € 50.000,00 viene assegnato alla ricerca:

Limbal Stem-Cell Therapy and Long-Term Corneal Regeneration

U.O. Oculistica - Unità Cornea e Superficie Oculare
dell'Istituto Scientifico Universitario San Raffaele di Milano
Responsabile: Dottor Paolo Rama

SINTESI DEGLI OBIETTIVI E DEI RISULTATI DELLA RICERCA

In questo lavoro sono stati studiati 112 pazienti operati di innesto di cellule staminali dell'epitelio corneale coltivate negli anni 1998-2007.

I pazienti presentavano tutti una opacità corneale con vascolarizzazione secondaria ad un danno grave delle cellule staminali corneali. La causa più frequente era l'ustione da agenti chimici. La capacità visiva era fortemente compromessa, in tutti i casi con un visus inferiore a 1/10. La gran parte dei pazienti inclusi nello studio aveva già fatto interventi precedenti, compreso il trapianto tradizionale della cornea, senza alcun risultato. Il trapianto tradizionale di cornea non può avere successo in questi casi perché il lembo di cornea che viene innestato deve essere successivamente ricoperto dall'epitelio del ricevente. Se mancano le cellule staminali epiteliali, come nei pazienti considerati, anche il lembo trapiantato verrà nuovamente ricoperto da un tessuto vascolarizzato di origine congiuntivale con fallimento dell'intervento.

I pazienti sono stati seguiti negli anni e rivalutati tutti nel corso del 2008, in modo da avere un dato molto importante di sopravvivenza a lungo termine delle cellule staminali innestate.

Il risultato finale ha dimostrato una percentuale di successo nel 76.6% dei casi.

I successi e i fallimenti sono stati inoltre correlati con la percentuale di cellule staminali presenti nella coltura: si è osservato che, nelle colture che contenevano una percentuale di cellule staminali superiore al 3%, era stato possibile ottenere un risultato positivo nell'80% dei casi; al contrario, nelle colture povere di cellule staminali (>3%), la percentuale di successo scendeva sotto il 30%.

In conclusione, possiamo affermare che questo lavoro ha confermato la possibilità e fattibilità di coltivare e trapiantare, con un alta percentuale di successo, cellule staminali dell'epitelio corneale; così come è stato fatto per la ricostruzione dell'epidermide nelle ustioni cutanee. Abbiamo dimostrato inoltre che la percentuale di cellule staminali presenti nella coltura è fondamentale per il successo finale e di conseguenza che la procedura di espansione delle cellule coltivate in laboratorio è cruciale.

Per questo motivo è importantissimo affidare la responsabilità della coltura delle cellule solo a laboratori altamente qualificati e selezionati, per poter garantire la sicurezza e il successo a lungo termine.

Dott. Paolo Rama
CURRICULUM VITAE



Paolo Rama, Primario dell'Unità Operativa di Oculistica - Cornea e Superficie Oculare dell'Istituto Scientifico San Raffaele (Mi), è nato a Feltre il 6 marzo 1959. Si è laureato in Medicina e Chirurgia a Padova nel 1985 e successivamente specializzato in Oftalmologia a Modena nel 1989. Ha conseguito un "Research Fellowship" nel 1991/92 al *Wilmer Institute* della *Johns Hopkins University* di Baltimora.

È stato aiuto ospedaliero prima a Mestre, dal 1988 al 1993, e in seguito a Venezia dal 1993 al 2000. Nel 2000, per potersi dedicare alla ricerca, si è trasferito all'Ospedale San Raffaele di Milano a dirigere il Servizio di Malattie della Cornea e della Superficie Oculare. Nel 2005 è stato nominato Primario dell'Unità di Oculistica e dal 2009, con lo sdoppiamento dell'Unità, dirige con incarico primariale, la nuova Unità Operativa di Oculistica - Cornea e Superficie Oculare. Ha prestato servizio volontario in Africa in più occasioni dal 1986 al 1996.

I suoi campi di interesse comprendono le malattie della cornea, della superficie oculare e la patologia infettiva. La sua casistica operatoria comprende più di 7.000 interventi e di questi più di 3.000 sono trapianti di cornea.

Nel settore della ricerca si è interessato del *Nerve Growth Factor* (NGF) in oculistica e nel 1998 ha pubblicato sul *New England Journal of Medicine* la prima dimostrazione di applicazione clinica di questo fattore di crescita. Si è poi interessato di cellule staminali e recentemente ha pubblicato, sempre sul *New England Journal of Medicine*, i risultati a lungo termine dell'innesto di cellule staminali coltivate nel trattamento delle ustioni corneali. Si è impegnato anche nella divulgazione scientifica con numerosi articoli pubblicati nelle riviste di medicina più prestigiose e tenendo relazioni ai più importanti congressi nazionali e internazionali. Ha svolto anche insegnamento come professore a contratto per l'Università "Tor Vergata" di Roma, per il Campus Biomedico di Roma e per l'Università Vita-Salute San Raffaele di Milano.

**RICERCATORI PREMIATI
DELLE PRECEDENTI EDIZIONI**

Prof. Giuseppe Giuffrè

Istituto di Clinica Oculistica, Facoltà di Medicina e Chirurgia Università degli Studi di Palermo
“Gli effetti della prostaglandina F2a nell’ occhio umano”

Prof. Carlo Enrico Traverso

Istituto di Clinica Oculistica, Facoltà di Medicina e Chirurgia Università degli Studi di Genova
“Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium”

Dott. Paolo Rama

Servizio di Malattie della Cornea e della superficie oculare, IRCCS San Raffaele, Milano
“Topical treatment with nerve growth factor for corneal neurotrophic ulcers”

Dott.ssa Graziella Pellegrini

Chimico Coadiutore, Laboratorio di Ingegneria dei Tessuti, Istituto Dermatologico dell'Immacolata di Roma
“Location and Clonal Analysis of Stem Cells
and Their Differentiated Progeny in the Human Ocular Surface”

Dr. Napoleone Ferrara

Genentech Fellow – Department. of Molecular Oncology (South San Francisco, California)
“Suppression of retinal neovascularization *in vivo* by inhibition of vascular endothelial growth factor
(VEGF) using soluble VEGF-receptor chimeric proteins”

Università Cattolica del Sacro Cuore di Roma

Istituto di Oftalmologia, Facoltà di Medicina e Chirurgia “Agostino Gemelli”
“Brachiterapia con placche episclerali di Iodio 125 versus Brachiterapia con Rutenio 106
associata a Termoterapia Transpupillare nel trattamento
dei melanomi uveali di medie dimensioni”

SAN RAFFAELE



La Fondazione Centro San Raffaele del Monte Tabor è un ente privato non profit, fondato nel 1971 dal Presidente Don Luigi Maria Verzé e riconosciuto nel 1972 dal Ministero della Sanità e dal Ministero della Pubblica Istruzione *Istituto di Ricovero e Cura a Carattere Scientifico*. Sua caratteristica principale e punto di forza è l'integrazione tra la componente Sanitaria (Ospedale e Poliambulatori), la Ricerca (Divisioni e Istituti) e la Didattica (Università e Liceo).

Il San Raffaele è una struttura accreditata con il Servizio Sanitario Nazionale e attrezzata per il ricovero in solvenza che conta più di 700 medici e 1300 infermieri. Ogni anno registra oltre 60 mila ricoveri e quasi 8 milioni tra prestazioni ambulatoriali ed esami di laboratorio. Sono 47 le specialità cliniche di riferimento, organizzate in Dipartimenti, suddivisi tra la sede di via Olgettina e la sede distaccata di Turro. La sinergia con l'Università Vita-Salute fa sì che il San Raffaele sia il primo polo ospedaliero universitario in Italia; a sua volta l'Ateneo fondato da Don Luigi Maria Verzé ha tre Facoltà: Medicina e Chirurgia, Filosofia e Psicologia.

Nel 2009 il San Raffaele, grazie all'intensa attività di ricerca, è stato il primo Istituto Scientifico in Italia, sia per numero di pubblicazioni scientifiche (785 lavori sulle maggiori riviste internazionali), sia per impact factor normalizzato (3.675 punti). La struttura multidisciplinare tecnologicamente all'avanguardia e l'interazione continua tra ricercatori e clinici specializzati in diverse aree della medicina permettono di ottenere risultati tali da rendere il San Raffaele punto di riferimento in Italia, in Europa e nel mondo per lo studio e la cura di molte patologie. Specializzato in medicina molecolare, comprende aree di eccellenza nell'ambito delle malattie cardiovascolari e metaboliche, neurologiche, oncologiche, immunologiche, infettive e genetiche.

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ORIGINAL ARTICLE

Limbal Stem-Cell Therapy and Long-Term Corneal Regeneration

Paolo Rama, M.D., Stanislav Matuska, M.D., Giorgio Paganoni, M.D.,
Alessandra Spinelli, M.D., Michele De Luca, M.D., and Graziella Pellegrini, Ph.D.

ABSTRACT

BACKGROUND

Corneal renewal and repair are mediated by stem cells of the limbus, the narrow zone between the cornea and the bulbar conjunctiva. Ocular burns may destroy the limbus, causing limbal stem-cell deficiency. We investigated the long-term clinical results of cell therapy in patients with burn-related corneal destruction associated with limbal stem-cell deficiency, a highly disabling ocular disease.

METHODS

We used autologous limbal stem cells cultivated on fibrin to treat 112 patients with corneal damage, most of whom had burn-dependent limbal stem-cell deficiency. Clinical results were assessed by means of Kaplan–Meier, Kruskal–Wallis, and univariate and multivariate logistic-regression analyses. We also assessed the clinical outcome according to the percentage of holoclone-forming stem cells, detected as cells that stain intensely (p63-bright cells) in the cultures.

RESULTS

Permanent restoration of a transparent, renewing corneal epithelium was attained in 76.6% of eyes. The failures occurred within the first year. Restored eyes remained stable over time, with up to 10 years of follow-up (mean, 2.91±1.99; median, 1.93). In post hoc analyses, success — that is, the generation of normal epithelium on donor stroma — was associated with the percentage of p63-bright holoclone-forming stem cells in culture. Cultures in which p63-bright cells constituted more than 3% of the total number of clonogenic cells were associated with successful transplantation in 78% of patients. In contrast, cultures in which such cells made up 3% or less of the total number of cells were associated with successful transplantation in only 11% of patients. Graft failure was also associated with the type of initial ocular damage and postoperative complications.

CONCLUSIONS

Cultures of limbal stem cells represent a source of cells for transplantation in the treatment of destruction of the human cornea due to burns.

From San Raffaele Scientific Institute, Ophthalmology Unit, Milan (P.R., S.M., G.P., A.S.); and the Center for Regenerative Medicine Stefano Ferrari, University of Modena and Reggio Emilia, Modena, Italy (M.D.L., G.P.). Address reprint requests to Professor Pellegrini at the Center for Regenerative Medicine Stefano Ferrari, University of Modena and Reggio Emilia, Via Glauco Gottardi 100, Modena, Italy, or at graziella.pellegrini@unimore.it.

This article (10.1056/NEJMoa0905955) was published on June 23, 2010, at NEJM.org.

N Engl J Med 2010.

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A CLEAR CORNEA IS ESSENTIAL TO VISUAL acuity and depends on stromal avascularity and epithelial integrity.¹ Corneal renewal and repair are mediated by stem cells of the limbus, the narrow zone between the cornea and the bulbar conjunctiva.² Ocular burns may destroy the limbus, causing limbal stem-cell deficiency. In such cases, the cornea acquires an epithelium through the invasion of bulbar conjunctival cells. This process leads to neovascularization, chronic inflammation, and stromal scarring, with corneal opacity and loss of vision.³ Allogeneic corneal transplantation (keratoplasty) restores transparency temporarily, but eventually, the conjunctival cells begin to invade and resurface the cornea. The only way to prevent this invasion is to restore the limbus. Such restoration has been attained in cases of unilateral limbal stem-cell deficiency through the grafting of limbal fragments obtained from the uninjured eye.⁴

Under appropriate culture conditions, human keratinocytes generate holoclones, meroclones, and paraclones, all of which are capable of proliferation.⁵ Holoclone-forming cells are the stem cells of virtually all human squamous epithelia^{2,6,7} and are required to permanently restore massive epithelial defects.⁶⁻⁸ In humans, holoclone-forming cells are located in the limbus but not in the central cornea.^{9,10} Paraclones, which are derived from holoclones, have the properties of transient amplifying progenitor cells.^{5,9} Meroclones have intermediate proliferative and clonogenic potential; they give rise to and are a reservoir for paraclones.^{5,9}

Human limbal stem cells can be identified, both in vivo and in vitro, through their expression of the p63 transcription factor.¹¹⁻¹³ The *TP63* gene generates six isoforms.¹⁴ Transcription from different promoters generates two different pre-messenger RNAs: *TP63* and $\Delta Np63$. Alternative splicing of each transcript produces α , β , and γ isoforms.¹⁴ Ocular keratinocytes may contain all the ΔN isoforms, but $\Delta Np63\alpha$ (hereafter referred to as p63) is by far the most abundant^{11,12}; it is present in the limbus but not in the uninjured central cornea^{12,13} and is expressed in holoclones but not in paraclones.¹² The protein p63 sustains the proliferative potential of limbal stem cells, and the *C/EBP δ* transcription factor maintains the self-renewal of these cells and regulates their mitotic rate.¹³ During corneal repair, limbal stem cells that stain intensely (hereafter referred to

as p63-bright cells) are released from *C/EBP δ* -dependent mitotic constraints, multiply, and migrate to regenerate corneal epithelium.¹³

The finding that human limbal cell cultures¹⁵ contain holoclones⁹ led to the first therapeutic use of such cultures in the regeneration of corneal epithelium.¹⁶ Autologous limbal cell cultures also offer an opportunity to treat patients who have severe bilateral loss of corneal epithelium, provided that a tiny part of the limbus is spared in one of the two eyes; thus, it is not applicable in cases of total bilateral limbal stem-cell deficiency. Related studies have been reported,^{9,17} but they have been limited because of the comparatively small numbers of cases, the relatively short follow-up, and the heterogeneity among causes of the disorders and among the sources of transplants and cell cultures. We report the long-term results of a study in which we used this approach to treat patients with corneal damage from common causes.

METHODS

PATIENTS

Patients with severe or total, unilateral or partial burn-related lateral limbal stem-cell deficiency were included in the study. (For details, see Table 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org.)

CELL CULTURE AND TREATMENT

Autologous limbal stem cells were obtained from a biopsy specimen (1 to 2 mm²) taken from the patient's contralateral eye, and the cells were cultured on fibrin, a natural substrate that preserves holoclone-forming cells.¹⁸⁻²⁰ Clinical-grade-certified, lethally irradiated 3T3-J2 cells from a master cell bank were used as feeder cells. Details of cell culture, graft preparations, immunohistochemical tests, and surgical and postoperative procedures are described in the Supplementary Appendix.

FOLLOW-UP AND ASSESSMENT OF OUTCOME

Follow-up evaluations were performed according to standard procedures; for details, see the Supplementary Appendix. The outcome of treatment was assessed at 1 year. The treatment was considered to be successful if all symptoms had disappeared and a transparent, avascular, and stable corneal surface had been restored and was con-

sidered to be partially successful if most symptoms had disappeared but superficial neovascularization had recurred, even if it was not as extensive as at the time of admission. Treatment failure was defined as the presence of symptoms, recurrent epithelial defects, pannus, and inflammation at 1 year. Three observers assessed the clinical results; two were involved in the patients' care, and the third did not participate in any biologic or clinical procedure.

STATISTICAL ANALYSIS

We analyzed results with the use of the Kaplan–Meier, chi-square, Mann–Whitney, McNemar, and Kruskal–Wallis tests as well as univariate and multivariate stepwise logistic-regression analyses (as described in the Supplementary Appendix).

RESULTS

CHARACTERISTICS OF THE PATIENTS

We studied 113 eyes from 112 patients enrolled between 1998 and 2006. The mean (\pm SD) age of the patients was 46.5 ± 14.4 years (range, 14 to 80), and 78.6% of them were men. The limbal stem-cell deficiency ranged from severe (in 35.4% of the patients) to total (in 64.6%) (Table 1 in the Supplementary Appendix). The most common causes of limbal stem-cell deficiency (in 97.3% of cases) were chemical burns (83 alkali, 16 acid, and 4 other) and thermal burns (7). Two patients had a bacterial infection and one patient had undergone orbit irradiation. Patients had unilateral (87.5%) or bilateral (12.5%) burns; only two patients had bilateral severe limbal stem-cell deficiency (Patients 24 and 83 in Table 1 in the Supplementary Appendix). Most of the eyes (84%) had been treated previously with surgery (in some cases multiple surgeries), but all surgical treatments had failed, probably because of an insufficient

number of residual limbal stem cells to regenerate corneal epithelium. Twenty-eight patients had undergone a single unsuccessful keratoplasty, and 20 had undergone multiple unsuccessful keratoplasties. Before this study, optimally corrected visual acuity was less than 0.1 (i.e., light perception, hand movement, and counting fingers) in 88.5% of the patients and was 0.1 to 0.5 in 11.5% of the patients. The baseline features of the patients, including diagnoses and grading of limbal stem-cell deficiency, are provided in the Supplementary Appendix.

GRAFTS

A total of 125 cultures were grafted in 113 eyes (112 patients) between July 1998 and December 2007. These included a bilateral graft in Patient 24 plus 12 re-grafts. Both eyes of Patient 24 and the left eye of Patient 83 were grafted with cultures prepared from a single biopsy specimen taken from a spared limbal area.

The clinical outcome of limbal stem-cell transplantation was judged to be successful in 76.6% of the eyes (Table 1).

The mean time between injury and the first graft was 18.3 ± 16.7 years (median, 12.9; range, 0.8 to 56.4). No intraoperative complications were reported. Postoperative adverse events included hemorrhage (in 12 patients), residual fibrin on the third postoperative day (in 11 patients), inflammation (in 59 patients), blepharitis with epithelial involvement (in 35 patients), and herpetic keratitis (in 3 patients) (Table 1 in the Supplementary Appendix), all of which were treated with the use of standard therapy.

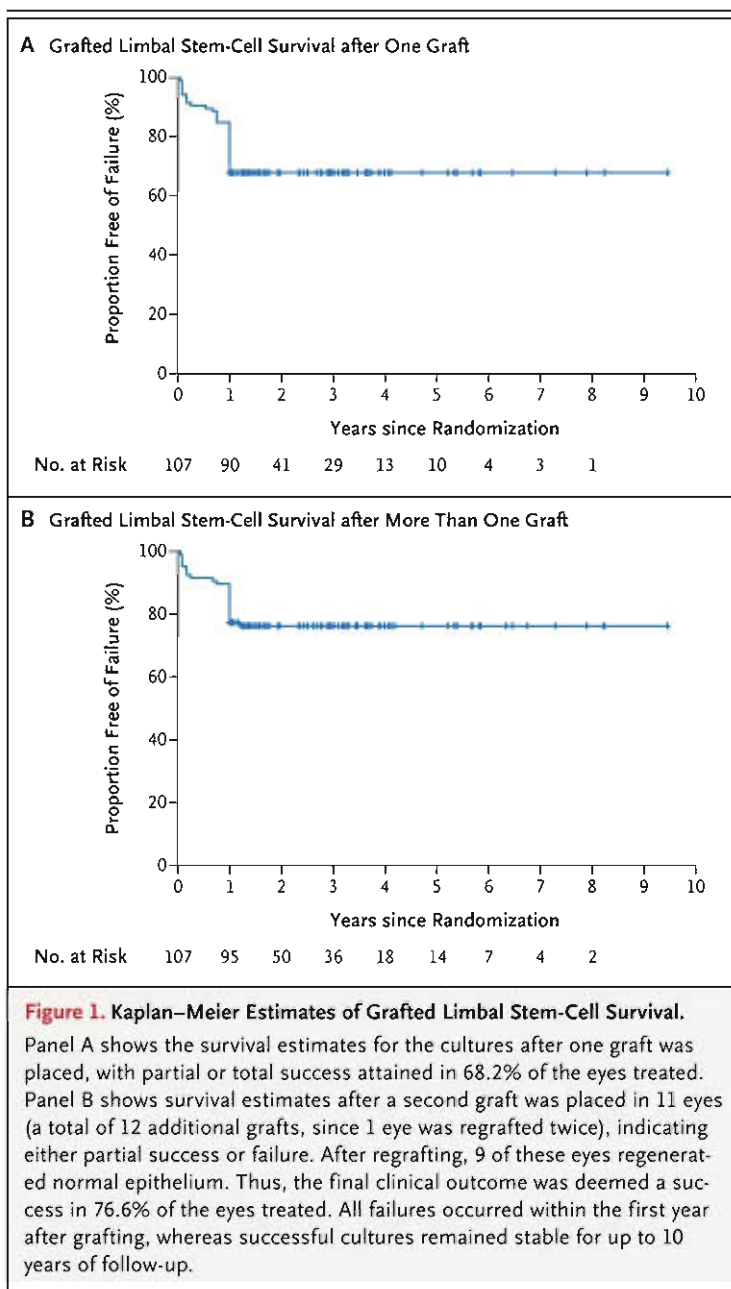
REGENERATION OF CORNEAL EPITHELIUM

The human corneal epithelium is renewed approximately every 9 to 12 months.¹ Since we therefore surmised that the regenerated epithelium would

Table 1. Clinical Outcomes of Limbal Stem-Cell Grafts in 112 Patients.*

Variable	Number of Eyes	Success	Partial Success	Failure
			number of eyes (percent)	
Outcome of first graft	107	73 (68.2)	18 (16.8)	16 (15.0)
Outcome of subsequent graft	12	9 (75.0)	2 (16.7)	1 (8.3)
Final outcome	107	82 (76.6)	14 (13.1)	11 (10.3)

* Six of the 112 patients were excluded because they did not complete the study. One patient had bilateral limbal stem-cell deficiency and received two grafts.



persist for 1 year after transplantation only if the stem cells had successfully engrafted,⁸ we carried out the first conclusive clinical evaluation after at least 1 year of follow-up. Six patients did not complete the study and were excluded from the analysis. We report the results obtained during a maximum of 10 years of follow-up for the remaining 106 patients, with a mean follow-up period of 2.91±1.99 years and a median of 1.93 years (interquartile range, 1.02 to 3.67).

One year after surgery, the 107 transplants were scored as success, partial success, or failure in 73

eyes (68.2%), 18 eyes (16.8%), and 16 eyes (15%), respectively (Table 1). Of 11 eyes (representing 12 grafts, since 1 eye was regrafted twice), 6 were partially successful and 5 were considered to have failed; these 11 were all regrafted with cultures prepared from frozen cells taken from the original biopsy specimen or from cells cultured from a new biopsy specimen. In 9 of these eyes, normal epithelium was regenerated after regrafting.

Thus, the final clinical outcome was classified as a success in 82 eyes (76.6%), a partial success in 14 eyes (13.1%), and a failure in 11 eyes (10.3%) (Table 1). Grafts that were considered successful at 1 year after surgery invariably remained stable thereafter, with a transparent avascular cornea covered by normal corneal epithelium (Fig. 1). Grafts that were considered failures at 1 year did not worsen in subsequent years as compared with baseline.

Univariate logistic-regression analysis showed that failures were associated with a number of factors, including age, the cause of limbal stem-cell deficiency, previous surgical procedures, the severity of damage, culturing and graft-transport complications, postoperative complications, and inflammation (Table 2). On multivariate logistic-regression analysis, failures were still significantly associated with the severity of damage and with culturing, graft-transport, and postoperative complications (Table 2). Post hoc analyses showed no significant difference in the likelihood of success between eyes that were also treated with keratoplasty (71%) and eyes that were not (79%).

CRITERIA FOR GRAFTABLE LIMBAL CULTURES

The average percentage of clonogenic cells detected in cultures was similar for successful, partially successful, and failed transplants (37.2%, 31.1%, and 35.5%, respectively). But the percentage of colony-forming cells is inadequate as an indication of the percentage of stem cells, because it is not possible to visually identify holoclones.^{5,9}

Determining the number of holoclones by means of clonal analysis is both cumbersome as a routine procedure^{5,9} and unsuitable for preoperative quality control because the results become available only after grafting. In contrast, quantitative immunodetection of p63, a marker of holoclones, is straightforward and can be performed before grafting.^{6,21}

We were able to examine the relationship between the percentage of p63-bright cells in each culture and the clinical results in 82 patients. (We

Table 2. Association between Characteristics of the Patients and 1-Year Outcomes.*

Variable	Successful Grafts (N=73)	Partially Successful or Failed Grafts (N=34)	P Value on Univariate Analysis†	Odds Ratio (95% CI) on Multivariate Analysis‡	P Value
Age — yr	49.1±14.0	41.4±14.3	0.02		
Male sex — no. of patients (%)	54 (74)	29 (85)	0.23		
Cause of burn — no. of eyes (%)					
Acid	7 (10)	9 (26)	0.04		
Alkali	55 (75)	23 (68)			
Previous intervention — no. of eyes (%)	58 (79)	33 (97)	0.017		
Severe damage — no. of eyes (%)	41 (56)	28 (82)	0.008	4.05 (1.34–12.2)	0.013
Culture or graft-transport complications — no. of eyes (%)	8 (11)	8 (24)	0.089	3.86 (1.08–13.75)	0.037
Postoperative complications — no. of eyes (%)	43 (59)	31 (91)	<0.001	7.64 (1.99–29.33)	0.003
Inflammation — no. of eyes (%)	33 (45)	26 (76)	<0.003		
Follow-up period					
Biopsy to transplantation — days					
Median	22.5	28	0.46		
Interquartile range	16–41	17–43			
Burn to transplantation — yr					
Median	12.9	7.7	0.15		
Interquartile range	5.6–33	4.8–27			

* Plus-minus values are means ±SD. CI denotes confidence interval.

† P values were calculated with the use of the chi-square or Mann-Whitney test. Variables significantly associated with risk of failure on univariate analysis ($P<0.1$) were included in the multivariate stepwise logistic-regression model.

‡ Odds ratios are shown only for variables with significant associations. Odds ratios for continuous variables are for an increase of 1 unit.

were unable to analyze this relationship in all patients because p63 was not found to be a marker of limbal stem cells until 2001.¹¹) A post hoc Kruskal-Wallis analysis showed that cultures giving rise to successful grafts contained a significantly greater percentage of p63-bright cells than those giving rise to unsuccessful grafts ($P<0.001$) (Fig. 2A). Grafts associated with partial success were obtained from cultures with an intermediate percentage of p63-bright cells.

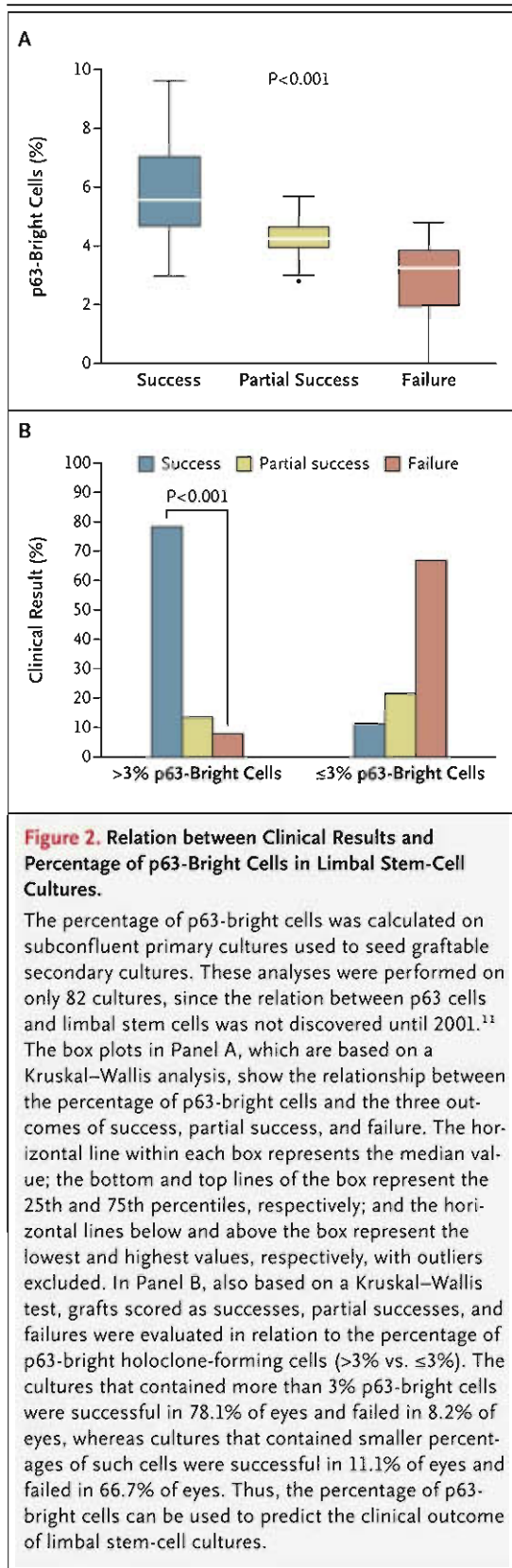
In additional post hoc analyses, we evaluated the clinical results according to culture status. Cultures that contained more than 3% p63-bright cells led to successful corneal epithelial regeneration in 78% of the eyes (Fig. 2B), and only 8% and 14% of such cultures were associated with failed or partial regeneration, respectively. In contrast, cultures in which 3% or less of the cells were p63-bright were successful in only 11% of eyes and were unsuccessful or partially successful in 67%

and 22% of eyes, respectively. Thus, the percentage of p63-bright cells in the culture was positively associated with the clinical outcome of limbal stem-cell grafting. These data are consistent with the finding that the human limbus contains at least 5% stem cells.⁹ On the basis of these data, we now use only cultures that contain more than 3% p63-bright cells to prepare grafts.

That said, a defined percentage of stem cells is necessary but not sufficient to ensure a good clinical result. Approximately 20% of cultures that contain more than 3% p63-bright cells were associated with failed or only partially successful treatment (Fig. 2), suggesting that other factors, such as the severity of injury and the presence or absence of complications, also influence outcome.

RESTORATION OF VISUAL ACUITY

The regeneration of normal avascular corneal epithelium was associated with amelioration of symp-



toms (burning, pain, and photophobia) in all patients whose grafts were considered successful (see Table 1 in the Supplementary Appendix). Normal vision was restored in only those patients with undamaged corneal stroma, however (Fig. 3, and Table 1 in the Supplementary Appendix). To improve the visual acuity of eyes with stromal scarring, we performed corrective surgical procedures 12 to 24 months after grafting the limbal stem-cell cultures: 46 patients underwent penetrating keratoplasty (89%), lamellar keratoplasty (9%), or phototherapeutic keratectomy (2%) to replace the damaged stroma. We observed no intraoperative complications and used standard therapeutic approaches for postoperative adverse events (Table 1 in the Supplementary Appendix). Histologic examination of the central cornea removed at the time of keratoplasty showed that the regenerated epithelium expressed keratin 12 (a marker of corneal epithelium) but not keratin 19 (a marker of conjunctival epithelium) (Fig. 2 in the Supplementary Appendix). We observed regeneration of the corneal epithelium that was sufficient to resurface the donor stroma in all the patients treated with keratoplasty. Permanent recovery of at least 0.6 visual acuity (range, 0.6 to 1.0) was attained in 21 patients (Table 1 in the Supplementary Appendix). The remaining 25 patients had partial recovery of vision (up to 0.5 visual acuity). Figure 3B shows the results of limbal stem-cell grafting followed by penetrating keratoplasty in the eyes of three patients who had total limbal stem-cell deficiency, complete corneal opacification, stromal scarring, and reduced visual acuity (<0.1, counting fingers and perceiving hand movements) before treatment. After keratoplasty, which was performed between 4 and 6.5 years after grafting, the cornea was transparent in each of the patients and their visual acuity ranged from 0.3 to 0.9.

Figure 3 in the Supplementary Appendix shows the eyes of Patient 24 who had severe bilateral limbal stem-cell deficiency that resulted from an alkali burn in 1948. He was treated with grafts of limbal stem-cell cultures prepared from a single biopsy specimen (taken from the left eye), followed by penetrating keratoplasty. Both corneal surfaces were restored. Follow-up at 2 years (right eye) and 5 years (left eye) showed that both eyes were stable. At follow-up his best-corrected visual acuity was 0.6 and 0.7 in the left and right eyes, respectively.

DISCUSSION

Autologous cultures of limbal cells provide an adequate long-term source of transplant tissue for the treatment of corneal damage due to burns. We observed that a minimum of approximately 3000 stem cells, detected as p63-bright holoclone-forming cells, was required to achieve clinical success. (A limbal culture contains a minimum of 3×10^5 cells,²⁰ about 30% of which are clonogenic; thus, about 3% of these clonogenic cells should be holoclones.) Analysis of our findings suggested that outcomes differ significantly depending on whether the transplanted cultures contain more than 3% p63-bright holoclone-forming stem cells or 3% or less — the success rates were 78% with the larger number of stem cells and 11% with the smaller number. On the basis of this observation, we speculate that corneal regeneration cannot be ascribed to a nonspecific stimulatory effect of epithelial cultures, fibrin, or surgical manipulation on spared residual limbal cells (if such cells are even present).

Preservation of holoclones requires culture with selected 3T3 feeder cells and fetal-calf serum,^{6,22,23} and this culture method has been used worldwide since the 1980s^{6,7} to treat patients with massive full-thickness burns.^{18,19,24} During the past 30 years, no adverse effects have been reported, and this method has been approved for use in the United States, Japan, Italy, and South Korea.^{6,7} Retention of holoclones also requires appropriate substrates for the cultivation of cells; both fibrin and plastic have been shown to preserve holoclone-forming cells.¹⁸⁻²⁰ Alternative methods involving other reagents have been proposed that obviate the use of feeder cells, serum, or both,^{6,17} since some investigators consider these reagents to be potentially harmful.²⁵ The retention of stem cells when these alternative methods are used has not been investigated.

Allogeneic and buccal keratinocytes have been used as sources of grafts in previous clinical experimental studies to treat limbal stem-cell deficiency.^{6,17} Allogeneic keratinocytes — even when frozen or lyophilized — improve healing of partial-thickness or small skin wounds by stimulating resident cells.^{26,27} However, engraftment of these allogeneic cells is not permanent,²⁶ and it seems unlikely that the presence of stem cells is required for their clinical effect. Donor epithelial cells have not been recovered from the ocular sur-

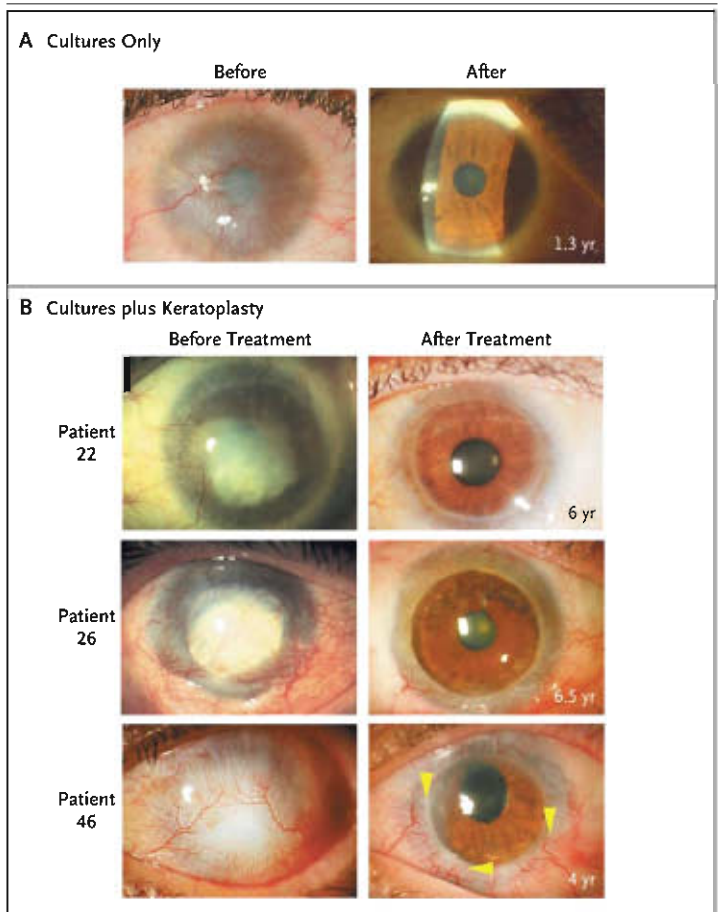


Figure 3. Regeneration of a Functional Corneal Epithelium and Restoration of Visual Acuity.

Panel A shows the left eye of Patient 93 (see Table 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org), who had total limbal stem-cell deficiency due to an acid burn (image at left). His visual acuity was reduced to counting fingers. A graft of autologous limbal cultures was sufficient to regenerate functional corneal epithelium (image at right) and to restore normal vision (visual acuity, 0.7), since the eye had no stromal scarring. Panel B shows the eyes of Patients 22, 26, and 46 (see Table 1 in the Supplementary Appendix), which were damaged by alkali burns and were treated with unsuccessful surgery 13, 30, and 3 years before admission, respectively. All three eyes had total limbal stem-cell deficiency, complete corneal opacification, and stromal scarring (images at left). Vision was reduced to counting fingers (in Patient 22) or perceiving hand movements (in Patients 26 and 46). In all three patients, autologous limbal stem-cell cultures successfully regenerated functional corneal epithelium. To improve their visual acuity after grafting, the patients underwent penetrating keratoplasty. In all three eyes, the engrafted limbal stem cells resurfaced the donor stroma. At the last follow-up visits (at 6, 6.5, and 4 years, respectively), all eyes were covered by stable corneal epithelium (images at right). The keratoplasty resulted in complete restoration of visual acuity in Patients 22 and 46 (0.9 and 0.8, respectively). The visual acuity of Patient 26 increased to only 0.3 because of a concomitant amblyopia (the alkali burn had occurred 30 years before admission). In Patient 46, the follow-up image shows that the conjunctival vessels stop at the conjunctival–corneal boundary (arrowheads); they do not invade the restored corneal surface.

face in studies that looked at long-term outcomes,^{28,29} and it seems unlikely that these cells would regenerate functional epithelium in patients with total limbal stem-cell deficiency, nor is there evidence that buccal keratinocytes can generate bona fide corneal epithelium.³⁰ If limbal stem-cell deficiency is only partial, allogeneic or oral keratinocytes may be sufficient to stimulate resident limbal cells to regenerate corneal epithelium. Additional studies to test this hypothesis are warranted. The same argument might hold true for the effect of autologous cultures on corneal regeneration. Nevertheless, the association that we observed — between the percentage of p63-bright cells and clinical success — was never related to a nonspecific stimulatory effect of the transplanted culture on resident stem cells.

In conclusion, our study shows that in patients with limbal stem-cell deficiency who received cultured limbal stem-cell grafts for corneal transplantation, the clinical results were successful at up to 10 years (at a median of 2 years) in more than 75% of the patients treated. Cultures of limbal

stem cells thus represent a source of cells for transplantation in the treatment of burn-induced destruction of the human cornea.

Supported by grants from the Italian Ministry for Education, University and Research and Ministry of Health; l'Association Française contre les Myopathies et les Maladies Neuromusculaires (AFM) Telethon; the European Commission Sixth Framework Program for Research and Technological Development (corneal engineering) and Seventh Framework Program; Optimization of Stem Cell Therapy for Degenerative Epithelial and Muscle Diseases (OptiStem, HEALTH-F5-2009-223098); Regione Emilia-Romagna (area 1b, medicina rigenerativa); and the Italian Ministry of Health.

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

This article is dedicated to the memory of Professor Stefano Ferrari, whose inspiring passion and commitment to excellence in science and education brought about the Center for Regenerative Medicine, and also to Professor Giovanni Rama, who was involved in the first phase of the project.

We thank Dr. Howard Green (Harvard Medical School, Boston) for providing the original 3T3-J2 feeder cells; Patrizia Paterna and Sergio Bondanza (Istituto Dermatologico dell'Immacolata [IDI]), and Marina Bertolin, Luca Canton, Alessandro Chiarello, Barbara Ferrari, Daniela Ferrari, Valeria Leuci, and Erika Nizza (Veneto Eye Bank Foundation [FBOV]) for their assistance with cell culture; Osvaldo Golisano (IDI) and Enzo Di Iorio and Vanessa Barbaro (FBOV) for immunohistochemical testing; and Giliola Calori and Laura Angelici for statistical analyses.

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