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Invited Review

Epithelial stem cells in corneal regeneration and epidermal gene therapy

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Abstract

Regenerative medicine refers to innovative therapies aimed at the permanent restoration of diseased tissues and organs. Regeneration of self-renewing tissues requires specific adult stem cells, which need to be genetically modified to correct inherited genetic diseases. Cultures of epithelial stem cells permanently restore severe skin and mucosal defects, and genetically corrected epidermal stem cells regenerate a normal epidermis in patients carrying junctional epidermolysis bullosa. The keratinocyte stem cell is therefore the only cultured stem cell used both in cell therapy and gene therapy clinical protocols. Epithelial stem cell identification, fate and molecular phenotype have been extensively reviewed, but not in relation to tissue regeneration. In this paper we focus on the localization and molecular characterization of human limbal stem cells in relation to corneal regeneration, and the gene therapy of genetic skin diseases by means of genetically modified epidermal stem cells.

Keywords: stem cells; limbus; cornea; ocular surface; epidermis; skin; cell therapy; gene therapy; regenerative medicine

Introduction

Squamous epithelia are constantly renewed. Being the first protective barrier against the external environment, these epithelia receive daily assaults, such as wounds, which need timely repair. Epithelial stem cells are responsible for such regeneration and repair processes. Stem cells have the unique capacity to self-renew and to generate committed progenitors — often referred to as transient amplifying (TA) cells — that differentiate into the cell lineages of the tissue of origin after a limited number of cell divisions [1–5]. TA cells increase the number of differentiated progeny produced by each stem cell division, enabling stem cells to divide infrequently, at least under normal homeostasis. Regenerative medicine aims at the permanent restoration of damaged tissues. It thus requires tissue-specific stem cells (cell therapy), which need to be genetically modified to correct inherited genetic disorders (gene therapy). In 1984, Howard Green and his colleagues reported that the life of two children presenting full-thickness burns covering over 95% of their body surface was saved by transplantation of autologous cultured keratinocytes [6]. This masterwork initiated the age of regenerative medicine by means of *cultured* stem cells [7]. Since then, keratinocyte cultures have been used worldwide to regenerate the epidermis of thousands of victims of third-degree burns [8]. Permanent epidermal regeneration, confirmed by follow-up studies over a 20 year period, has been achieved in many of these patients [8]. Keratinocyte stem cells are currently used to regenerate many types of squamous epithelia [8]. In this paper, we will address specific issues related to limbal stem cells and corneal regeneration and the gene therapy of genetic skin diseases by means of genetically modified epidermal stem cells.

The stem cells of the corneal epithelium

The human ocular surface is covered with conjunctival and corneal squamous epithelia. The conjunctival epithelium lies on a vascularized stroma and allows the movement of the eyelid over the cornea, the maintenance of the normal lid-globe apposition and the limbal vascular supply. It contains unicellular mucin-secreting goblet glands generated as part of a specific differentiation programme of conjunctival stem cells [9,10]. The cornea is covered with a flat epithelium, devoid of goblet cells, lying on the corneal stroma by Bowman's layer [11]. The clearness of the cornea, which depends on stroma avascularity and epithelial integrity, is essential to visual acuity. The narrow zone between the cornea and the bulbar conjunctiva is referred to as the limbus. The limbal epithelium

consists of layers of cells organized in ridges known as palisades of Vogts, and is populated by Langerhans cells and melanocytes. The limbus harbours the stem cells of the corneal epithelium [10,12–18]. Limbal stem cell identification and molecular characterization have been reviewed [19,20], but not in relation to tissue regeneration. We address here some of the issues related to limbal stem cell-mediated regenerative medicine.

Location of human corneal stem cells

Experimental and clinical evidence pointing to the limbus as the source of corneal epithelial stem cells is compelling [19–22]. Relatively undifferentiated and slow-cycling epithelial cells have been found in the limbal basal layer but not in the central cornea [12–14]. Cell migration from the limbus towards a wounded cornea [14,15,20,23–26] and mathematical analyses of the maintenance of the corneal epithelial cell mass [27] strengthened the X, Y, Z hypothesis of corneal epithelial preservation by proliferating and migrating limbal stem cells [28]. Surgical removal of the limbus initially results in corneal healing [29] but subsequent corneal mechanical wounds result in a progressive vascularization and recurrent erosions of the cornea, confirming that the corneal epithelium has a limited regenerative capacity [29]. Putative limbal stem niches (crypts) have been identified as solid cords of cells that extend from the peripheral end of the palisades of Vogts into the underlying stroma [30]. Corneal malignant tumours, which are thought to arise from stem cells, are found exclusively in the limbus.

In humans, peripheral and central corneal cells have a much lower clonogenic and proliferative capacity than the corresponding limbal cells [10]. When limbal epithelium is deficient, the cornea acquires an epithelium by invasion of bulbar conjunctival cells (conjunctivalization) originating beyond the destroyed limbus. This clinical entity, known as limbal stem cell deficiency, leads to neovascularization, chronic inflammation, recurrent epithelial erosions, stromal scarring, corneal opacity and loss of vision [16]. A number of ocular surface diseases, including chemical and thermal burns, Stevens–Johnson syndrome, cicatricial pemphigoid, contact lens-induced infections, chronic keratitis, repeated surgery and aniridia, share in common partial or total limbal stem cell deficiency [20]. Strikingly, the entire corneal epithelium can be permanently regenerated by a surgical technique involving the removal of free grafts of limbal tissue from the contralateral healthy eye that are then transplanted onto the diseased eye of patients with unilateral total destruction of the limbal–corneal epithelium [17].

Epithelial stem cells can be studied at a single-cell level. Clonal analysis of human keratinocytes has identified three types of clonogenic cells, giving rise to holoclones, meroclones and paraclones [31]. The holoclone-forming cell is the smallest colony-founding keratinocyte, has the highest proliferative capacity and is the stem cell of the epidermis and the hair follicle [32]. It soon became clear that the holoclone-forming keratinocyte is the stem cell of virtually all human squamous epithelia [3,8,10,33]. Holoclones have all the hallmarks of stem cells, including self-renewal capacity [32,34,35], telomerase activity and long telomeres [36], and an impressive proliferative potential. A single holoclone can indeed generate the entire epidermis or the entire corneal epithelium of a human being [10,32]. Holoclone-forming cells generate all the epithelial lineages of the tissue of origin [10,34,37,38], permanently restore massive epithelial defects [6,39–43] and can be retrieved from human epidermis regenerated from cultured keratinocytes years after grafting [8]. Finally, a defined number of genetically corrected holoclone-forming cells regenerate a normal epidermis in patients with genetic skin adhesion disorders [44]. During its clonal evolution, the holoclone produces paraclone-forming cells, which have a very limited proliferative capability, generate aborted colonies containing only terminal cells and have the properties expected of TA cells [10,31]. Meroclones have an intermediate proliferative and clonogenic potential and are a reservoir of paraclones [10,31].

Clonal analysis of the human ocular surface have shown that holoclone-forming cells are located in the limbus but not in the central cornea [10]. The corneal epithelium is formed by cells with different capacities for multiplication and the properties expected of TA cells. Clonogenic cells in the peripheral human cornea have a higher proliferative potential than those in the central cornea, which generates only aborted colonies [10].

However, a recent study has challenged the notion of corneal stem cells being located exclusively in the limbus [45]. Experiments envisaging (a) transplantation of limbal grafts from *lacZ* Rosa 26 mice onto the limbal region of athymic or SCID mice, (b) corneal healing after surgical destruction of the limbus and (c) transplantation of central corneal grafts onto the limbal region of recipient mice, have collectively demonstrated that both the limbus and the central cornea of mice can contain stem cells [45]. Accordingly, cells generating large colonies were found in the uninjured central cornea of many mammals, including mice, pigs and rabbits [45]. This study concluded that during normal homeostasis the central corneal epithelium is self-sustaining, and that limbal stem cells are recruited only in the presence of a corneal injury [45]. A similar mechanism has been postulated in the skin, where hair follicle stem cells are recruited to regenerate an epidermis only in the presence of a massive epidermal destruction [46].

The dogma that murine corneal stem cells reside exclusively in the limbus has been, at least initially, based on the presence of label-retaining cells in the limbal but not in the corneal epithelium [13,14,47]. The study from Mayo *et al* disavows this dogma and supports the grounded opinion that mitotic quiescence

is not an obligatory property of stem cells [48]. Stem cells of intestinal crypts have been estimated to divide every 24 h [49,50] and haematopoietic stem cells are not necessarily quiescent [51]. Mayo *et al* have also shown that the limbus and central cornea of mice contain oligopotent stem cells able to acquire a corneal or a conjunctival fate, depending upon a differing stromal environment [45], challeging the notion of the existence of two independent populations of stem cells in the cornea and conjunctiva [9,10]. It is crucial to pose the question, *does all this hold true for human beings*?

Of mice and men

The model proposed by Mayo *et al* is in sharp contrast (a) with compelling clinical evidence pointing to the essential role of the limbus in maintaining human corneal integrity [19–22], (b) with the undebatable notion that conjunctival cells cannot regenerate a corneal epithelium — invasion of the conjuntival epithelium onto a corneal surface causes corneal opacification and vascularization, independently of the stromal environment [16,18–22] (see also Figure 2B, C), and (c) with the notion that only conjunctival holoclones can generate goblet glands [10].

These opposing views could be reconciled by the observation that, at variance with other mammals, holoclone-forming cells are contained in the limbus but not in the central cornea of human beings. Mayo *et al* have indeed confirmed that cells giving rise to holoclone-type colonies were detected in the central cornea of mice, rabbits, pigs and cows but not humans [45], and that the few clonogenic cells contained in the central human cornea generate only aborted colonies [10,45]. These observations explain the clinical entity known as limbal stem cell deficiency and the therapeutic approaches used to cure it (see below). Therefore, the physiology of the human corneal epithelium appears to be somehow different from that of other mammals. Of note, the avascular Bowman's layer underlying the central corneal epithelium is present in humans (and primates) but not in other mammals [11], suggesting that a different stromal environment between species could, at least in part, explain those differences.

Similarly, lineage-tracing experiments have shown that a single type of progenitor cell sustains normal homeostasis of murine tail epidermis [52]. These data challenge the stem/TA cell model [53] and are consistent with the observation that most clonogenic cells can sustain an epithelium in mice [34,38]. Obviously, lineage-tracing experiments cannot be performed in humans. Altogether: (a) the different distribution and growth characteristics of clonogenic cells in mice versus humans [45]; (b) the notion that holoclones, but not paraclones, behave as self-renewing stem cells [35,36]; (c) the existence of a repertoire of transcription factors reflecting differences in self-renewal and proliferative capabilities of human epithelial cells [35,54]; and (d) the notion that not all clonogenic cells sustain the permanent regeneration of an epithelium [8,41,42,44,55] confirm, in our opinion, the existence of stem and TA cells in human squamous epithelia. Of note, in the bulge of the murine hair follicle there are distinct clonogenic cells endowed with stem cell properties [37]. $Sox9^{+}$ bulge stem cells are instrumental in maintaining the population of $Sox9^{-}$ TA cells, essential for the formation of hair follicle lineages [56]. The single progenitor model has been shown in the mouse tail skin, which is a specialized scale-forming epidermis, but not in the mouse back skin [52,53].

Differences between mice and humans have been reported in other tissue regeneration models. In *mdx* mice lacking dystrophin, the progressive muscle-wasting disease presents itself in a much milder form than in humans. At variance with humans, *mdx* mice show little weakness and have a near-normal lifespan. This partial recovery is the result of muscle regeneration promoted by an expansion of the satellite cells and muscle hypertrophy, probably related to a higher self-renewal potency of satellite cells in mice as compared to humans. The absence of these regenerative properties in the human muscle justifies the severity and lethality of human muscular dystrophy (reviewed in [57]).

Thus, extreme caution should be used in inferring human epithelial physiology from animal data. In the case of corneal (and epidermal) regeneration, the potentially misleading extrapolation of animal data could have a deleterious impact on the choice of either surgical therapy of massive corneal defects, or quality controls assuring the clinical performance of keratinocyte cultures.

Searching for a limbal stem cell marker

Integrins ($\alpha 9$ and $\beta 1$), NGF receptors (TrkA), ATP-binding cassette subfamily G, member 2 (ABCG2), CD34 and CD133, α-enolase and metabolic enzymes, vimentin and K19, have all been proposed as markers for mammalian limbal stem cells, but most of them lack stem cell specificity (reviewed in [19,20]). Integrin $\beta 1$ and TrkA are expressed by the basal cells of both limbus and cornea [58,59], whilst integrin $\alpha 9$ is expressed by TA cells [60]. Metabolic enzymes, α-enolase, vimentin and K19 are expressed by the majority of basal limbal (and in some instances corneal) cells [19,20]. CD133 is expressed in the entire corneal epithelium [61], whereas CD34 appears to be expressed in murine but not human epithelia [61,62]. Of the numerous markers investigated, the p63 transcription factor [63] is one of the more reliable we have at the present for the human limbal stem cell [20]. Although p63 has been proposed as an essential determinant of the proliferative potential of stem cells in stratified epithelia [26,35,64,65], many investigators do not regard p63 as a truly specific marker of limbal stem cells [19].

The p63 (corneal) controversy

Ablation of the *p63* gene results in the absence of stratified epithelia [66,67]. Mutations of *p63* cause disorders of the epithelia and of non-epithelial structures whose development depends on the epithelial functions, particularly in the cranio-facial region [68]. The essential function of p63 in the development of squamous epithelia is still the subject of controversy [69–73], but there is a good deal of evidence supporting the hypothesis that p63 is a determinant of stem cell proliferative potential in these epithelial cell types. The basis for this hypothesis is that, in the newborn mouse whose *p63* gene has been ablated, there are detectable terminally differentiated suprabasal keratinocytes but no proliferative basal layer containing the stem cell population necessary to sustain the epithelium [67].

In humans, p63 is expressed in patches of limbal but not corneal basal cells [26,58,74]. Smaller clonogenic cells express higher levels of p63 than larger cells [75]. The $\Delta N\alpha$ isoform of p63 is strongly expressed in holoclones (and weakly in meroclones) but is undetectable in paraclones, being the first polypeptide able to distinguish these clonal types [26,74]. Other laboratories have argued that the expression of p63 by the majority of limbal basal cells and by suprabasal cells, as assessed by the 4A4 antibody recognizing all p63 isoforms (see below), should be considered too broad to be stem cell-specific [61]. The observation that p63 can be contained in the central cornea, in groups of cells rather than in individual cells [61], and in most proliferating cells [76], further argued against limbal stem cell specificity. These opposing views could be reconciled by a careful analysis of the function and expression of p63 and the repertoire of transcription factors regulating the self-renewal and the proliferative potential of human limbal stem cells.

The *p63* gene generates six isoforms [63]. The transactivating isoforms are generated by an upstream promoter; the truncated ΔN isoforms are produced from a downstream intronic promoter and lack the transactivation domain. For both transcripts, alternative splicing gives rise to three different C termini, designated α, β and γ [63]. Depending on the conditions, limbal and corneal keratinocytes may contain all three ΔN isoforms. In the uninjured surface of the eye, $\Delta Np63\alpha$ is present in the limbus but absent from the corneal epithelium. $\Delta Np63\beta$ and $\Delta Np63\gamma$ (and $\Delta Np63\alpha$) appear upon wounding and correlate with limbal cell migration and corneal regeneration and differentiation [26]. Discrepancies in p63 expression can therefore be ascribed (at least in part) to the use of a pan-p63 antiserum (which has been used in most of the studies) and to the level of integrity of the corneal specimens. Indeed, the activation of a corneal wound-healing process, characterized by the appearance of p63 and clonogenic cells within the central corneal epithelium, can already be observed a few hours after cornea withdrawal, expecially in donors presenting with incomplete closure of the eyelids [26].

Figure 1 shows a schematic description of a model for human corneal regeneration (from Barbaro *et al* [35]). Limbal stem cells shift from a homeostatic state of relative quiescence to rapid proliferation upon central corneal wounding [14,26]. Co-expression of C/EBPδ, Bmi1 and $\Delta Np63\alpha$ identifies mitotically quiescent limbal stem cells *in vivo* and holoclones, but not meroclones and paraclones, *in vitro*, hence identifying part of the genetic programme maintaining human limbal stem cell identity [35]. Of note, only a fraction of $\Delta Np63\alpha^+$ cells contained in a holoclone co-expresses C/EBPδ. That fraction is mitotically quiescent, even in culture [35]. C/EBPδ and Bmi1 are never found in the human central corneal epithelium [35]. C/EBPδ positively regulates the expression of $\Delta Np63\alpha$, which sustains the proliferative potential of stem cells in

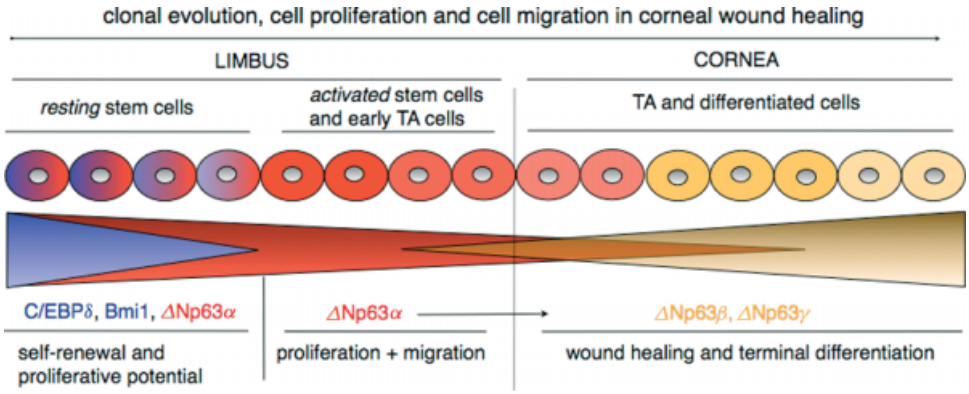

Figure 1. Schematic description of a model for human corneal regeneration (from Barbaro *et al* [35]). Under normal homeostasis, stem cells localized in the basal layer of the limbus co-express C/EBPδ and Bmi1 (blue), which are responsible for mitotic quiescence and self-renewal properties [35], and $\Delta Np63\alpha$ (red), which sustains the stem cell proliferative potential [26,64,65,69,74,77,78]. These cells give rise to holoclones in culture [35]. When a corneal wound occurs, a fraction of limbal stem cells switches off C/EBPδ (and Bmi1) but maintains $\Delta Np63\alpha$ (red). Activated $\Delta Np63\alpha^+$ stem cells proliferate and migrate to the central cornea to restore and regenerate the corneal epithelium [26,35]. Activated stem cells, however, lose their self-renewal properties, enter into the TA compartment and progressively lose $\Delta Np63\alpha$ expression. TA cells switch on $\Delta Np63\beta$ and $\Delta Np63\gamma$, which might regulate terminal differentiation and stratification during the regeneration of the damaged corneal epithelium [26]

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several stratified epithelia [64,65,69,77,78], including the limbus [26,35]. Instead, C/EBPδ but not ΔNp63α regulates mitotic quiescence and self-renewal of limbal stem cells [35], strengthening the notion that proliferation and self-renewal capabilities are two related, albeit distinct, processes. During an acute wound, ΔNp63α^+ limbal stem cells are released from C/EBPδ-dependent mitotic constraints, unchain their remarkable ΔNp63α-dependent proliferative capacity, multiply and migrate to repair the wound (Figure 1). This process causes the irreversible loss of self-renewal and leads to corneal terminal differentiation, which is associated with expression of ΔNp63β and ΔNp63γ [35] (Figure 1).

Thus, self-renewing human limbal stem cells can be identified by the co-expression of ΔNp63α, C/EBPδ and Bmi1, rather than ΔNp63α alone. ΔNp63α^+/C/EBPδ^- limbal cells maintain their regenerative capability but have lost their capacity for self-renewal, being already engaged in corneal regeneration. However, that ΔNp63α^+/C/EBPδ^- cells maintain stem cell features can be inferred from the notion that enforced expression of C/EBPδ indefinitely sustains the self-renewal of ΔNp63α^+ cells but cannot rescue the regenerative and self-renewal capability of clonogenic ΔNp63α^- cells (paraclones) [35].

Corneal regeneration by limbal stem cells

Allogeneic corneal transplantation (keratoplasty) is widely used to repair a damaged corneal stroma. Once an allogeneic corneal transplant has been grafted in a recipient, its outer surface will eventually be replaced by a host-derived corneal epithelium generated by undamaged autologous limbal stem cells. However, if the patient has an extensive destruction of the limbus, a functional corneal epithelium can no longer be formed, the keratoplasty is unsuccessful and the cornea re-acquires an epithelium by invasion of bulbar conjunctival cells originating beyond the destroyed limbus. This process worsens symptoms, neovascularization, inflammation, scarring and corneal opacification associated with the original injury. In the past, unsuccessful keratoplasty has been performed on patients with limbal deficiency because the role of the limbus in corneal regeneration was not fully understood. The recognition of the limbal location of corneal stem cells changed the therapeutic approach towards the management of massive limbal/corneal destruction. The only way to prevent the corneal conjunctivalization is indeed to restore the limbus. This was first carried out in the pioneering work of Kenyon and Tseng [17], by grafting rather large limbal fragments of the uninjured eye onto the diseased eye of patients with unilateral limbal–corneal destruction. The possibility of reducing the loss of normal limbus by growing small limbal fragments in culture was soon recognized [79] and cultured limbal cells were found to include stem cells detectable as holoclones [10].

This made possible the therapeutic use of limbal cultures (Figure 2A) for the permanent regeneration of a corneal epithelium in patients with limbal stem cell deficiency due to chemical or thermal burns [40,43]. If the injury detroys the limbal–corneal epithelium but not the corneal stroma (Figure 2B), limbal cultures are sufficient to restore corneal integrity and visual acuity (Figure 2C). But if there had been scarring of the corneal stroma (Figure 2D), the grafted eye — although covered by a normal epithelium — might recover only 10–30% of visual acuity. In order to improve the recovery of vision, a keratoplasty to remove the stromal scar is needed (Figure 2E). The engrafted limbal stem cells are able, a second time, to generate the corneal epithelium necessary to resurface the underlying stroma, and this time the grafted eye recovers its visual acuity (Figure 2E). The possibility of covering the entire corneal surface using a 1 mm^2 limbal biopsy avoids potential damage to the healthy eye. Limbal cells can be cryopreserved and used in the event that the first graft fails. More importantly, cultivation of limbal cells offers a therapeutic chance to patients with bilateral corneal damage.

During the last 10 years, in collaboration with 26 ophthalmological departments, we have treated over 240 patients with limbal stem cell deficiency resulting from chemical burns. Many patients had previously received an unsuccessful keratoplasty. Grafting of cultured limbal cells was successful in 78% of the patients, as indicated by disappearance of symptoms and the regeneration of a normal corneal epithelium. Up to 9 years of subsequent observation has confirmed the continuing integrity of that epithelium. This entire treatment is now reimbursed as an approved therapy by the National Health System of Italy.

The clinical use of cultured limbal stem cells was first described by Pellegrini *et al* in 1997 [40]. Since then, many reports of the clinical use of this technology have been published, and the clinical outcome of these studies has been reviewed recently [8,22,80,81]. Different culture methods and substrates adopted for limbal cell cultivation has also been reviewed [22] but not in relation to the clinical performance of the cultures. We therefore address some of the issues that have profound implications for the regenerative potential of limbal stem cell cultures.

The culture method and the preservation of functional stem cells

The clinical success of keratinocyte-mediated cell therapy depends first on the quality of the cultures used to prepare the grafts. This does not mean that the cultures should contain a well-organized stratified epithelium, but rather that they must contain a sufficient number of stem cells essential for long-term epithelial renewal. Only when this criterion is met does success then depend exclusively on the clinician. In the absence of an adequate number of limbal stem cells

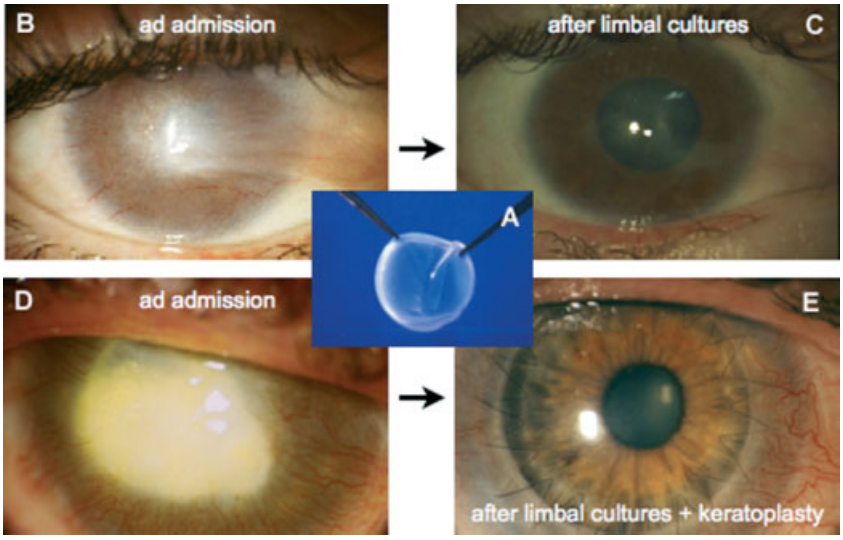

Figure 2. Restoration of the corneal epithelium in patients with complete unilateral limbal stem cell deficiency. (A) Secondary culture of limbal cells on a fibrin matrix approximately 3 cm in diameter. The fibrin is completely digested within 24 h after grafting, leaving no intervening matrix between the limbal cells and the wound bed [43]. (B) A 20 year-old woman had a thermal burn of her right eye in 2001. At admission, the corneal surface was covered by a vascularized conjunctival epithelium. Her visual acuity was reduced to hand movement. (C) In July 2005, a 2 mm biopsy taken from her left limbus was used to make secondary limbal cultures for grafting to her injured eye. Within 1 week after grafting, the corneal surface was covered by a transparent epithelium. Since the patient did not have severe stromal scarring, limbal cultures were sufficient to restore corneal integrity and visual acuity. The appearance of the eye is shown 1.5 years after the grafting of the limbal culture. (D) A 43 year-old male had a chemical burn of his right eye in 1998 and was treated unsuccessfully by pannectomy and amniotic membrane. At admission, his corneal surface was covered with a fibrovascular tissue with overlying pannus of abnormal conjunctival epithelium and his visual acuity was reduced to hand movement. (E) In May 2004, a 2 mm biopsy taken from his left limbus was used to make secondary limbal cultures for grafting to his injured eye. Within 1 week after grafting, the corneal surface was covered by a transparent epithelium. Due to scarring of the corneal stroma resulting from the initial injury, the visual acuity was only 0.1. A penetrating keratoplasty was therefore performed in September 2005. The appearance of the eye is shown 4 years after the grafting of the limbal culture and nearly 3 years after the keratoplasty. The cornea appears virtually normal. The patient recovered a visual acuity of 0.7 (because of a subcapsular cataract)

detectable as holoclones, failures of corneal regeneration are inevitable and will entail not only patient suffering but also general confusion as to what results are to be expected [8]. The same concept holds true for epidermal regeneration in full-thickness burns [41]. Evaluation of the number of holoclones within a cultured epithelial graft is considered the best available quality control assuring the clinical performance of keratinocyte cultures [8,41–43].

Preservation of holoclones requires both a properly prepared feeder-layer of lethally irradiated 3T3 cells and an appropriately selected fetal calf serum [8,41,42,81]. Keratinocytes cultured using this method, originally developed for epidermal keratinocytes [82], have been used: (a) since the 1980s on thousands of patients for the life-saving treatment of massive full-thickness burns (reviewed in [8]); (b) to restore pigmentation of stable vitiligo and piebaldism [83,84]; (c) to regenerate a functional urethral epithelium in patients with posterior hypospadias [39,85]; and (d) since the late 1990s for the restoration of the corneal surface on hundreds of patients with limbal stem cell deficiency (reviewed in [8]). During these nearly three decades, no adverse effect has been reported and this culture method has been approved by the Food and Drug Administration (FDA) in both the USA and Japan.

Alternative culture methods have nevertheless been proposed, envisaging the removal of the feeder-layer and/or the fetal calf serum, on the assumption of potential (theoretical) risks from the use of these reagents. In none of these studies, however, has a careful evaluation of stem cell preservation been performed.

The colony-forming efficiency assay is not sufficient to evaluate the number of stem cells. It indicates the capacity of a cell to found a colony, but it does not inform about its capacity to produce cell generations. Aborted colonies (paraclones) can be distinguished from the other clonal types, but it is not possible to distinguish holoclones from meroclones solely based on a colony forming efficiency. Evaluation of stem cell content requires a clonal analysis, in which single cell-derived colonies are analysed and scored as holoclones after sub-cultivation [10,31]. The presence of holoclones in limbal grafts has been evaluated in only one study [43].

This said, the determination of the number of holoclones would be cumbersome [31] as a standard test for the quality of cultured grafts. The number of aborted colonies, which is inversely related to the number of holoclones, is easier to score [35,42]. The proportion of colonies aborting rises slightly during the two stages of cultivation but the mean value should not reach 10% of the total. Autologous cultures of this

quality are acceptable for grafting [8]. The identification of holoclones by immunodetection of ΔNp63α is another important and simple method for determining the presence of an adequate number of stem cells in a cultured limbal graft [8]. The abundance of ΔNp63α — but not that of the ΔNp63β and ΔNp63γ isoforms — strongly declines during clonal transition from holoclone to meroclone, and the protein is virtually absent from paraclones [26,35]. The immunodetection of ΔNp63α should first be validated and based on the level of expression of the protein. The frequency of $p63^+$ cells can be evaluated by computerized analysis of the intensity of staining of single cells, as assessed by automated quantitative immunocytochemistry, using as a reference a limbal strain that contains a known percentage of holoclones [8,86]. Holoclone content, long-term proliferative capacity and expression of high levels of ΔNp63α correlated well in both mass and clonal cultures [8,86]. Analyses of clinical data indicated that grafts containing <3% of cells expressing a high level of ΔNp63α had a high risk of failure (manuscript in preparation). Based on these data, primary cultures possessing <3% of cells with high levels of p63 are currently not used to prepare grafts.

The first clinical application of cultured human limbal cells could not be reproduced on a large scale because of the fragility of the epithelium [40]. A significant improvement was the use of supporting materials for cell culture, transportation and transplantation onto patients, such as fibrin glue [41–43], amniotic membrane [87–90], polymers [91], collagen sponges or strips, devitalized membranes or polymers (reviewed in [92]). As with culture media, however, the issue of holoclone preservation arises when different substrates are proposed for limbal cell cultivation.

The fibrin matrix is an ideal mechanical support, since does not alter the cultured cells, yet it is highly manageable [41–43]. Fibrin glue has adhesive properties, therefore no sutures are needed to tie the cultured epithelium to the underlying corneal stroma; it is able to protect the proliferative compartment of the epithelium during transportation and surgery and it is degraded within 24 h after transplantation [41–43]. Of paramount importance, fibrin is the only substrate where maintenance of holoclone-forming cells, preservation of a proper amount of p63-positive cells and limbal cell long-term proliferation have so far been formally proved [41–43].

The most widely used support is human amniotic membrane. When the amnion is to be used as a substrate for cultured cells, the amniotic epithelium is usually removed in order to allow their attachment. Amniotic membrane has been used for cell cultivation by numerous investigators, either with or without a 3T3 feeder layer [87–90]. When limbal keratinocytes cultured on such support were applied to the eye affected by different diseases, the treatment resulted in notable improvement of the ocular surface [93,94]. However, neither holoclone preservation, nor clonogenic ability and proliferative potential, nor p63 immunodetection have been tested in these culture conditions.

Poly(*N*-isopropylacrylammide) polymer is a newly developed temperature-responsive support [91]. Such a polymer allows cells to be detached from the culture dish because it reverses its hydration properties with temperature: reducing the temperature of the culture below 30 °C causes swelling and complete detachment of adherent cells. It would be interesting to determine whether this support affects cell behaviour. Indeed, as with the amniotic membrane, neither holoclone preservation nor proliferative potential have been tested on cells grown onto this temperature-sensitive support.

Furthermore, in many of these studies the number of cases treated is small and includes a variety of causes of the ocular diseases treated. It will be difficult to obtain clearly interpretable results until studies are carried out on a larger group of patients with disease of common aetiology. The follow-up periods were occasionally longer, but often 1 year or less. Information on the long-term durability of the improvement is important for understanding both the value of the treatment and the mechanism by which the improvement takes place.

In summary, during the last few years, new culture technologies have been proposed for limbal stem cell culture, envisaging new culture media and/or cultivation of limbal cells onto different carriers, even with cells in suspension. Maintenance of holoclones has never been demonstrated in these conditions, with the exception of limbal cells grown on the fibrin matrix and in the presence of lethally irradiated 3T3 cells and fetal calf serum [43]. It should be considered that irreversible clonal evolution occurs during serial keratinocyte cultivation *in vitro* [10,35]. Incorrect (or non-validated) culture conditions can irreversibly accelerate the clonal conversion and hence can cause a rapid disappearance of stem cells (our unpublished data), rendering the cultured autograft useless. In our opinion, the proposal of a new culture system and/or of a new carrier for autologous limbal cells destined for the permanent restoration of massive corneal destruction should be preceded by: (a) direct demonstration of the presence of holoclones in the culture; (b) periodical clonal analysis of a reference strain of limbal cells (in terms of both clonogenic and growth potential); (c) evaluation of the percentage of aborted colonies during cultivation; (d) evaluation of the percentage of cells expressing appropriate molecular markers. These basic quality controls eliminate one important hitherto uncontrolled variable in the evaluation of the clinical performance of autologous limbal cultures, and should represent a starting point for improving this emerging stem cell-mediated cell therapy.

Gene therapy of genetic skin disease

About 30 years have passed since the discovery of a method of producing a great number of human

epidermal keratinocytes by cultivation from a small skin biopsy [82], about 25 years since the first application of cultured epidermal grafts to regenerate epidermis of humans suffering from third-degree burns [95], and over 20 years since large-scale grafting of such cultures proved to be life-saving [6]. This pluriennial experience (reviewed in [8]) paved the way to the use of keratinocyte stem cells in gene therapy of genetic skin diseases.

The first successful attempt to introduce and express foreign genes into transplantable human keratinocytes by retroviral vectors dates back to 1987 [96]. Since then, several groups have shown that genetically modified keratinocytes can express and secrete transgene products, including apolipoprotein-E, clotting factors IX and VIII and interleukin-6, both *in vitro* and *in vivo* in animal models [97–100]. A persistent problem that investigators have faced is the apparent loss of expression of retrovirally-encoded transgenes after transplantation *in vivo* [101,102]. In most cases, this was ascribed to 'shut-off' of the murine retroviral vector promoter, although failure to transduce stem cells is a just as likely cause of 'unstable' transgene expression, at least in the earliest studies. It soon became clear that the two most important factors to assure persistent expression of retroviral transgenes are the vector design and the use of transduction protocols allowing gene transfer into self-renewing epidermal stem cells. The use of retroviral vectors derived from the MFG prototype, in which the protein of interest is translated from an efficiently spliced genomic RNA under the control of the viral *env* translation initiation sequences, allowed expression of *lacZ* or factor IX transgenes for >1 year in human epidermal xenografts [99,103]. Transduction of epidermal stem cells was achieved either by co-culture with packaging cell lines [98,99,104] or by the use of high-titre vector preparations [103].

Pre-clinical research carried out in the last decade provided evidence that transduction by retroviral vectors may restore a normal phenotype in keratinocytes obtained from patients affected by inherited skin adhesion defects, in culture or upon transplantation on immunodeficient mice [105–107]. These studies targeted different forms of epidermolysis bullosa (EB), a family of severe skin adhesion defects due to disruption of the epidermal–dermal junction. EB is classified into simplex (EBS), junctional (JEB) or dystrophic (DEB) forms, depending on the level at which the junction is compromised (above, within or below the basement membrane) [108]. A subclass of JEB is due to autosomal recessive mutations in one of the three chains of laminin-5 (LAM5), a key component of the epidermal–dermal junction linking the keratinocyte-specific $\alpha 6\beta 4$ integrin to the type-VII collagen dermal fibrils. LAM5 is a heterotrimeric protein made of $\alpha 3$, $\beta 3$ and $\gamma 2$ chains, encoded by the *LAMA3, LAMB3* and *LAMC2* genes, respectively [109]. The severity of LAM5-deficient JEB can vary from early lethality in the so-called Herlitz variant to much milder conditions in variants characterized by residual gene function [108]. Most of the JEB mutations occur in 'hot spots' within the *LAMB3* gene. In the non-Herlitz forms, the mutations allow the formation of residual, partially functional LAM5, leading to a phenotype characterized by severe, disfiguring, blistering, recurrent infections, visual impairment and an increased risk of skin cancer (reviewed in [110]). There is no cure for JEB, and current therapeutic approaches are essentially aimed at controlling infections and maintaining an acceptable quality of life.

LAMB3-deficient JEB became the first disease to be targeted by a therapeutic approach based on autologous transplantation of cultured epidermis derived from genetically corrected epidermal stem cells. In 1998, Dellambra *et al* achieved a high percentage of transduction in epidermal stem cells by co-culturing *LAMB3*-deficient keratinocytes onto a feeder layer composed of lethally irradiated 3T3-J2 cells, and packaging cells for a Moloney murine leukaemia (MLV)-derived retroviral vector carrying the *LAMB3* cDNA under the control of the viral long terminal repeat (LTR) promoter. Gene correction fully restored the keratinocyte adhesion properties and prevented the loss of colony-forming ability, suggesting a direct link between cell adhesion to the basal lamina and keratinocyte proliferative capacity [105]. Functional proof of correction of the adhesion defect was later also provided *in vivo*, by transplantation of genetically corrected skin grafts onto immunodeficient mice [106].

Clinical translation of gene therapy of JEB started in Italy in 1999, with the generation of clinical-grade packaging cells and full validation of the gene transfer technology under GMP/GLP standards. A phase I clinical trial was authorized in June 2002. The trial was aimed at assessing the overall safety of the transduction/transplantation procedure, analysing long-term survival of transduced stem cells and persistence of transgene expression, and monitoring humoral and/or cytotoxic immune responses against the genetically modified cells. The first patient was a 36 year-old male affected by non-lethal JEB, caused by double heterozygosity for a *LAMB3* null mutation and a single point mutation (E210K), resulting in residual levels of LAM5 at the level of the basal lamina. Keratinocytes obtained from palm skin biopsies were transduced at $>95\%$ efficiency by a MLV-derived retroviral vector carrying the full-length *LAMB3* cDNA under the control of the viral LTR. Transduced epithelial sheets were grafted onto both upper legs [44]. On clinical examination, no blisters were observed in the transplanted area throughout almost 3 years of follow-up, while the surrounding skin was characterized by the usual chronic blistering lesions. There was no evidence of inflammation, and specific tests carried out 3 and 6 months after transplantation indicated the absence of both humoral and T cell-mediated cytotoxic immune responses against the transgene product. Skin biopsies taken 1–12 months after grafting showed secretion of LAM5 heterotrimers, formation of hemidesmosomes

and restoration of the epidermal–dermal junction [44]. A genome-wide analysis of the retroviral integration sites was carried out on DNA extracted from skin biopsies 1 and 4 months after transplantation. Taking the integrated proviruses as a marker of cell clonality, it was estimated that an almost normal repertoire of genetically corrected, long-lasting epidermal stem cells was present in the regenerated epidermis. This study showed that transplantation of autologous cultured epidermis derived from genetically corrected epidermal stem cells is feasible, well tolerated, and leads to long-term functional correction of a skin adhesion defect [44].

The pilot clinical trial was based on the use of a MLV-derived retroviral vector. This type of vector has been used in hundreds of clinical trial since 1991. It was considered safe until lymphoproliferative disorders were reported in two patients treated with retrovirally-transduced haematopoietic progenitor cells for X-linked severe combined immunodeficiency (SCID) [111]. In both cases, the retroviral vector inserted into, and activated, a T cell proto-oncogene (reviewed in [112]). Three more patients in two different trials of gene therapy of X-linked SCID developed a similar pathology, indicating that the use of retroviral vectors can be associated to a concrete risk of insertional oncogenesis. However, no such adverse event was reported in other clinical trials based on MLV-derived retroviral vectors, such as that for adenosine deaminase (ADA)-deficient SCID [113], suggesting that specific risk factors may have contributed to the malignant progression observed in X-SCID patients. Nevertheless, the results of the X-SCID trials led most regulatory authorities in Europe and the USA to consider the use of MLV LTR-based vectors as no longer acceptable for transduction of stem cells.

It can be argued that extrapolating safety concerns from one disease context to another is a rather weak rationale for putting a promising treatment on hold. Considering that ADA-deficient SCID patients never suffered from the side-effects observed in X-linked SCID patients, extrapolating risks to patients suffering from a completely different disease, treated with different stem cells and with a vector carrying a different gene seems a rather odd choice. This is particularly true for JEB patients, who have very high chances of developing skin cancer anyway. Gene transfer vectors can and will be improved, but in the meantime patients with no therapeutic alternatives are prevented form accessing a potentially beneficial treatment in the absence of any evidence of potential risks.

Self-inactivating (SIN), γ-retroviral or HIV-derived lentiviral vectors [114] appear to have a potentially more favourable safety profile, based on their integration preferences [115], the lack of strong viral enhancers in the LTR, and the lower propensity to generate tumours in pre-clinical animal models [116]. Pre-clinical development of SIN γ-retroviral or SIN lentiviral vectors specifically targeted to basal layer keratinocytes is in progress, and should provide the next generation of clinical-grade vectors for gene therapy of JEB. Unfortunately, developing clinical-grade vectors and assessing their full safety and efficacy profile is going to take several years.

Despite their flexibility and high efficiency in transducing epidermal stem cells, retroviral vectors, of either γ-retroviral or lentiviral origin, might not provide a solution for all JEB disorders. In particular, developing a gene transfer strategy for DEB, which is due to the deficiency of type-VII collagen, appears to be a formidable challenge. The COL7A1 cDNA exceeds 9 kb in length, a size that is hardly accommodated by a retroviral genome. Although there have been successful attempts to produce full-length type-VII collagen in human keratinocytes by lentiviral vectors [107], titre and genetic stability, due to both the size and the highly repeated nature of the cDNA sequence, are persistent problems that hamper a real clinical development of this type of vector. To overcome this problem, COL7A1 'minigenes' have been developed, which maintain proper biochemical functions *in vitro* and could potentially fit into a retrovirus-based gene transfer vector [117]. However, the efficiency of proteins of reduced size in correcting a collagen deficiency has yet to be demonstrated. Potential alternatives include the use of DNA-based integrating systems, such as those derived by adeno-associated viral integrases, bacteriophage integrases and retrotransposons, or gene correction based on homologous recombination (reviewed in [118]). Although some of these strategies have shown some potential in the correction of collagen deficiencies [119], none of them appears to have the efficiency in transducing epidermal stem cells which would be necessary for a real clinical translation. Last but not least, a significant proportion of DEB variants is inherited with a dominant pattern [108], a situation in which gene replacement is not going to provide a solution, regardless of the efficiency of the gene transfer system. In these cases, gene correction by homologous recombination seems to be the only potential alternative. Homologous recombination has undergone spectacular progress in the recent past, essentially due to the introduction of zinc-finger nuclease technology for targeting specific alleles in the mammalian genome (reviewed in [120]). However, the clinical translation of such technology is still in its infancy, and it will take many years before it will become available to patients.

Conclusions

Clinical data on patients with disabling diseases have contributed greatly to our understanding of stem cells. This is a time of great enthusiasm for the prospects of regenerative medicine with cultured adult stem cells or somatic derivatives of human embryonic stem cells. It therefore seems a fitting time to reflect on the history of therapy with cultured stem cells

of the category for which there exists a history (see also [8] for a review on epidermal stem cell-mediated cell therapy). The difficulties encountered in keratinocyte therapy have been great but much progress has been made even if this progress has not yet been assimilated and used by the large number of scientists and physicians who have an interest in the subject. Therapy with cultured keratinocytes is ready for more widespread use because the necessary criteria for graftable cultures and for their surgical use are now quite well understood. We have laid major emphasis on the importance of a discipline for defining the suitability and the quality of cultured epithelial grafts, which is relevant to future use of any cultured cell type for therapeutic purposes.

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Teaching Materials

Power Point slides of the figures from this Review may be found in the supporting information.

References

1. Niemann C, Watt FM. Designer skin: lineage commitment in postnatal epidermis. *Trends Cell Biol* 2002;**12:**185–192.
2. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niche. *Cell* 2004;**116:**769–778.
3. Blanpain C, Horsley V, Fuchs E. Epithelial stem cells: turning over new leaves. *Cell* 2007;**128:**445–458.
4. Lajtha LG. Stem cell concepts. *Differentiation* 1979;**14:**23–34.
5. Potten CS, Booth C. Keratinocyte stem cells: a commentary. *J Invest Dermatol* 2002;**119:**888–899.
6. Gallico GG III, O'Connor NE, Compton CC, Kehinde O, Green H. Permanent coverage of large burn wounds with autologous cultured human epithelium. *N Engl J Med* 1984;**311:**448–451.
7. Green H. The birth of therapy with cultured cells. *BioEssays* 2008;(in press).
8. De Luca M, Pellegrini G, Green H. Regeneration of squamous epithelia from stem cells of cultured grafts. *Regen Med* 2006;**1:**45–57.
9. Wei ZG, Lin T, Sun TT, Lavker RM. Clonal analysis of the *in vivo* differentiation potential of keratinocytes. *Invest Ophthalmol Vis Sci* 1997;**38:**753–761.
10. Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama 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.
11. Pepose J, Ubels JL. The cornea. In *Adler's Physiology of the Eye*, 9th edn, Hart WM Jr (ed.). Mosby Year Book Inc., St. Louis, MO 1992; 29–70.
12. 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.
13. 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.
14. Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci* 1998;**111**(19):2867–2875.
15. Nagasaki T, Zhao J. Centripetal movement of corneal epithelial cells in the normal adult mouse. *Invest Ophthalmol Vis Sci* 2003;**44:**558–566.
16. Dua HS, Azuara-Blanco A. Limbal stem cells of the corneal epithelium. *Surv Ophthalmol* 2000;**44:**415–425.
17. Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989;**96:**709–722; discussion, 722–703.
18. Tsai RJ, Sun TT, Tseng SC. Comparison of limbal and conjunctival autograft transplantation in corneal surface reconstruction in rabbits. *Ophthalmology* 1990;**97:**446–455.
19. Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res* 2005;**81:**247–264.
20. Chee KY, Kicic A, Wiffen SJ. Limbal stem cells: the search for a marker. *Clin Exp Ophthalmol* 2006;**34:**64–73.
21. Dua HS, Kulkarni B, Singh R. Quest for limbal stem cells. *Clin Exp Ophthalmol* 2006;**34:**1–2.
22. Shortt AJ, Secker GA, Notara MD, Limb GA, Khaw PT, Tuft SJ, *et al*. Transplantation of *ex vivo* cultured limbal epithelial stem cells: a review of techniques and clinical results. *Surv Ophthalmol* 2007;**52:**483–502.
23. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature* 1971;**229:**560–561.
24. Kinoshita S, Friend J, Thoft RA. Sex chromatin of donor corneal epithelium in rabbits. *Invest Ophthalmol Vis Sci* 1981;**21:**434–441.
25. Lemp MA, Mathers WD. Corneal epithelial cell movement in humans. *Eye* 1989;**3**(4):438–445.
26. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of ΔNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci USA* 2005;**102:**9523–9528.
27. Sharma A, Coles WH. Kinetics of corneal epithelial maintenance and graft loss. A population balance model. *Invest Ophthalmol Vis Sci* 1989;**30:**1962–1971.
28. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci* 1983;**24:**1442–1443.
29. Huang AJ, Tseng SC. Corneal epithelial wound healing in the absence of limbal epithelium. *Invest Ophthalmol Vis Sci* 1991;**32:**96–105.
30. 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.
31. Barrandon Y, Green H. Three clonal types of keratinocyte with different capacities for multiplication. *Proc Natl Acad Sci USA* 1987;**84:**2302–2306.
32. Rochat A, Kobayashi K, Barrandon Y. Location of stem cells of human hair follicles by clonal analysis. *Cell* 1994;**76:**1063–1073.
33. Gambardella L, Barrandon Y. The multifaceted adult epidermal stem cell. *Curr Opin Cell Biol* 2003;**15:**771–777.
34. Claudinot S, Nicolas M, Oshima H, Rochat A, Barrandon Y. Long-term renewal of hair follicles from clonogenic multipotent stem cells. *Proc Natl Acad Sci USA* 2005;**102:**14677–14682.
35. Barbaro V, Testa A, Di Iorio E, Mavilio F, Pellegrini G, De Luca M. C/EBPδ regulates cell cycle and self-renewal of human limbal stem cells. *J Cell Biol* 2007;**177:**1037–1049.
36. Dellambra E, Golisano O, Bondanza S, Siviero E, Lacal P, Molinari M, *et al*. Downregulation of 14–3–3σ prevents clonal evolution and leads to immortalization of primary human keratinocytes. *J Cell Biol* 2000;**149:**1117–1130.

37. Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 2004;**118:**635–648.
38. Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell* 2001;**104:**233–245.
39. Romagnoli G, De Luca M, Faranda F, Bandelloni R, Franzi AT, Cataliotti F, *et al*. Treatment of posterior hypospadias by the autologous graft of cultured urethral epithelium. *N Engl J Med* 1990;**323:**527–530.
40. Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 1997;**349:**990–993.
41. Pellegrini G, Ranno R, Stracuzzi G, Bondanza S, Guerra L, Zambruno G, *et al*. The control of epidermal stem cells (holoclones) in the treatment of massive full-thickness burns with autologous keratinocytes cultured on fibrin. *Transplantation* 1999;**68:**868–879.
42. Ronfard V, Rives JM, Neveux Y, Carsin H, Barrandon Y. Long-term regeneration of human epidermis on third degree burns transplanted with autologous cultured epithelium grown on a fibrin matrix. *Transplantation* 2000;**70:**1588–1598.
43. Rama P, Bonini S, Lambiase A, Golisano O, Paterna P, De Luca M, *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.
44. Mavilio F, Pellegrini G, Ferrari S, Di Nunzio F, Di Iorio E, Recchia A, *et al*. Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nat Med* 2006;**12:**1397–1402.
45. Majo F, Rochat A, Nicolas M, Abou Jaoudé G, Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. *Nature* 2008;(in press).
46. Ito M, Liu Y, Yang Z, Nguyen J, Liang F, Morris RJ, *et al*. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 2005;**11:**1351–1354.
47. Lavker RM, Sun TT. Epithelial stem cells: the eye provides a vision. *Eye* 2003;**17:**937–942.
48. Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. *Cell* 1997;**88:**287–298.
49. Potten CS, Loeffler M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* 1990;**110:**1001–1020.
50. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, *et al*. Identification of stem cells in small intestine and colon by marker gene *Lgr5*. *Nature* 2007;**449:**1003–1007.
51. Kiel MJ, He S, Ashkenazi R, Gentry SN, Teta M, Kushner JA, *et al*. Haematopoietic stem cells do not asymmetrically segregate chromosomes or retain BrdU. *Nature* 2007;**449:**238–242.
52. Clayton E, Doupe DP, Klein AM, Winton DJ, Simons BD, Jones PH. A single type of progenitor cell maintains normal epidermis. *Nature* 2007;**446:**185–189.
53. Jones PH, Simons BD, Watt FM. *Sic transit gloria*: farewell to the epidermal transit amplifying cell? *Cell Stem Cell* 2007;**1:**371–381.
54. Jensen KB, Watt FM. Single-cell expression profiling of human epidermal stem and transit-amplifying cells: Lrig1 is a regulator of stem cell quiescence. *Proc Natl Acad Sci USA* 2006;**103:**11958–11963.
55. Larcher F, Dellambra E, Rico L, Bondanza S, Murillas R, Cattoglio C, *et al*. Long-term engraftment of single genetically modified human epidermal holoclones enables safety pre-assessment of cutaneous gene therapy. *Mol Ther* 2007;**15:**1670–1676.
56. Nowak JA, Polak L, Pasolli HA, Fuchs E. Hair follicle stem cells are specified and function in early skin morphogenesis. *Cell Stem Cell* 2008;**3:**33–43.
57. Durbeej M, Campbell KP. Muscular dystrophies involving the dystrophin–glycoprotein complex: an overview of current mouse models. *Curr Opin Genet Dev* 2002;**12:**349–361.
58. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells* 2004;**22:**355–366.
59. Lambiase A, Bonini S, Micera A, Rama P, Aloe L. Expression of nerve growth factor receptors on the ocular surface in healthy subjects and during manifestation of inflammatory diseases. *Invest Ophthalmol Vis Sci* 1998;**39:**1272–1275.
60. Pajoohesh-Ganji A, Ghosh SP, Stepp MA. Regional distribution of $\alpha 9\beta 1$ integrin within the limbus of the mouse ocular surface. *Dev Dyn* 2004;**230:**518–528.
61. Dua HS, Joseph A, Shanmuganathan VA, Jones RE. Stem cell differentiation and the effects of deficiency. *Eye* 2003;**17:**877–885.
62. Cotsarelis G. Epithelial stem cells: a folliculocentric view. *J Invest Dermatol* 2006;**126:**1459–1468.
63. Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dotsch V, *et al*. *p63*, a *p53* homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 1998;**2:**305–316.
64. Senoo M, Pinto F, Crum CP, McKeon F. p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* 2007;**129:**523–536.
65. Parsa R, Yang A, McKeon F, Green H. Association of p63 with proliferative potential in normal and neoplastic human keratinocytes. *J Invest Dermatol* 1999;**113:**1099–1105.
66. Mills AA, Zheng B, Wang XJ, Vogel H, Roop DR, Bradley A. *p63* is a *p53* homologue required for limb and epidermal morphogenesis. *Nature* 1999;**398:**708–713.
67. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, *et al*. *p63* is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* 1999;**398:**714–718.
68. Celli J, Duijf P, Hamel BC, Bamshad M, Kramer B, Smits AP, *et al*. Heterozygous germline mutations in the *p53* homolog *p63* are the cause of EEC syndrome. *Cell* 1999;**99:**143–153.
69. McKeon F. p63 and the epithelial stem cell: more than status quo? *Genes Dev* 2004;**18:**465–469.
70. Koster MI, Kim S, Roop DR. p63 deficiency: a failure of lineage commitment or stem cell maintenance? *J Invest Dermatol Symp Proc* 2005;**10:**118–123.
71. Koster MI, Roop DR. p63 and epithelial appendage development. *Differentiation* 2004;**72:**364–370.
72. Yang A, Kaghad M, Caput D, McKeon F. On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet* 2002;**18:**90–95.
73. Blanpain C, Fuchs E. p63: revving up epithelial stem-cell potential. *Nat Cell Biol* 2007;**9:**731–733.
74. Pellegrini G, Dellambra E, Golisano O, Martinelli E, Fantozzi I, Bondanza S, *et al*. p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 2001;**98:**3156–3161.
75. Kim HS, Jun Song X, de Paiva CS, Chen Z, Pflugfelder SC, Li DQ. Phenotypic characterization of human corneal epithelial cells expanded *ex vivo* from limbal explant and single cell cultures. *Exp Eye Res* 2004;**79:**41–49.
76. Hernandez Galindo EE, Theiss C, Steuhl KP, Meller D. Expression of ΔNp63 in response to phorbol ester in human limbal epithelial cells expanded on intact human amniotic membrane. *Invest Ophthalmol Vis Sci* 2003;**44:**2959–2965.
77. Koster MI, Kim S, Mills AA, DeMayo FJ, Roop DR. p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev* 2004;**18:**126–131.
78. Nguyen BC, Lefort K, Mandinova A, Antonini D, Devgan V, Della Gatta G, *et al*. Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. *Genes Dev* 2006;**20:**1028–1042.
79. 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.
80. Daniels JT, Notara M, Shortt AJ, Secker G, Harris A, Tuft SJ. Limbal epithelial stem cell therapy. *Expert Opin Biol Ther* 2007;**7:**1–3.

81. Pellegrini G, De Luca M, Arsenijevic Y. Towards therapeutic application of ocular stem cells. *Semin Cell Dev Biol* 2007;**18:**805–818.
82. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975;**6:**331–343.
83. Guerra L, Capurro S, Melchi F, Primavera G, Bondanza S, Cancedda R, *et al*. Treatment of 'stable' vitiligo by timed surgery and transplantation of cultured epidermal autografts. *Arch Dermatol* 2000;**136:**1380–1389.
84. Guerra L, Primavera G, Raskovic D, Pellegrini G, Golisano O, Bondanza S, *et al*. Permanent repigmentation of piebaldism by erbium: YAG laser and autologous cultured epidermis. *Br J Dermatol* 2004;**150:**715–721.
85. Romagnoli G, De Luca M, Faranda F, Franzi AT, Cancedda R. One-step treatment of proximal hypospadias by the autologous graft of cultured urethral epithelium. *J Urol* 1993;**150:**1204–1207.
86. Di Iorio E, Barbaro V, Ferrari S, Ortolani C, De Luca M, Pellegrini G. Q-FIHC: quantification of fluorescence immunohistochemistry to analyse p63 isoforms and cell cycle phases in human limbal stem cells. *Microsc Res Tech* 2006;**69:**983–991.
87. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease(1). *Am J Ophthalmol* 2000;**130:**543–544.
88. 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.
89. Koizumi N, Inatomi T, Suzuki T, Sotozono C, Kinoshita S. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens–Johnson syndrome. *Arch Ophthalmol* 2001;**119:**298–300.
90. Grueterich M, Espana EM, Touhami A, Ti SE, Tseng SC. Phenotypic study of a case with successful transplantation of *ex vivo* expanded human limbal epithelium for unilateral total limbal stem cell deficiency. *Ophthalmology* 2002;**109:**1547–1552.
91. Nishida K, Yamato M, Hayashida Y, Watanabe K, Yamamoto K, Adachi E, *et al*. Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N Engl J Med* 2004;**351:**1187–1196.
92. Schwab IR, Johnson NT, Harkin DG. Inherent risks associated with manufacture of bioengineered ocular surface tissue. *Arch Ophthalmol* 2006;**124:**1734–1740.
93. Fernandes M, Sridhar MS, Sangwan VS, Rao GN. Amniotic membrane transplantation for ocular surface reconstruction. *Cornea* 2005;**24:**643–653.
94. Kenyon KR. Amniotic membrane: mother's own remedy for ocular surface disease. *Cornea* 2005;**24:**639–642.
95. O'Connor NE, Mulliken JB, Banks-Schlegel S, Kehinde O, Green H. Grafting of burns with cultured epithelium prepared from autologous epidermal cells. *Lancet* 1981;**1:**75–78.
96. Morgan JR, Barrandon Y, Green H, Mulligan RC. Expression of an exogenous growth hormone gene by transplantable human epidermal cells. *Science* 1987;**237:**1476–1479.
97. Garlick JA, Katz AB, Fenjves ES, Taichman LB. Retrovirus-mediated transduction of cultured epidermal keratinocytes. *J Invest Dermatol* 1991;**97:**824–829.
98. Mathor MB, Ferrari G, Dellambra E, Cilli M, Mavilio F, Cancedda R, *et al*. Clonal analysis of stably transduced human epidermal stem cells in culture. *Proc Natl Acad Sci USA* 1996;**93:**10371–10376.
99. White SJ, Page SM, Margaritis P, Brownlee GG. Long-term expression of human clotting factor IX from retrovirally transduced primary human keratinocytes *in vivo*. *Hum Gene Ther* 1998;**9:**1187–1195.
100. Fakharzadeh SS, Zhang Y, Sarkar R, Kazazian HH Jr. Correction of the coagulation defect in hemophilia A mice through factor VIII expression in skin. *Blood* 2000;**95:**2799–2805.
101. Gerrard AJ, Hudson DL, Brownlee GG, Watt FM. Towards gene therapy for haemophilia B using primary human keratinocytes. *Nat Genet* 1993;**3:**180–183.
102. Fenjves ES, Yao SN, Kurachi K, Taichman LB. Loss of expression of a retrovirus-transduced gene in human keratinocytes. *J Invest Dermatol* 1996;**106:**576–578.
103. Kolodka TM, Garlick JA, Taichman LB. Evidence for keratinocyte stem cells *in vitro*: long term engraftment and persistence of transgene expression from retrovirus-transduced keratinocytes. *Proc Natl Acad Sci USA* 1998;**95:**4356–4361.
104. Levy L, Broad S, Zhu AJ, Carroll JM, Khazaal I, Peault B, *et al*. Optimised retroviral infection of human epidermal keratinocytes: long-term expression of transduced integrin gene following grafting on to SCID mice. *Gene Ther* 1998;**5:**913–922.
105. Dellambra E, Vailly J, Pellegrini G, Bondanza S, Golisano O, Macchia C, *et al*. Corrective transduction of human epidermal stem cells in laminin-5-dependent junctional epidermolysis bullosa. *Hum Gene Ther* 1998;**9:**1359–1370.
106. Robbins PB, Lin Q, Goodnough JB, Tian H, Chen X, Khavari PA. *In vivo* restoration of laminin 5 $\beta 3$ expression and function in junctional epidermolysis bullosa. *Proc Natl Acad Sci USA* 2001;**98:**5193–5198.
107. Chen M, Kasahara N, Keene DR, Chan L, Hoeffler WK, Finlay D, *et al*. Restoration of type VII collagen expression and function in dystrophic epidermolysis bullosa. *Nat Genet* 2002;**32:**670–675.
108. Epstein EH Jr. The genetics of human skin diseases. *Curr Opin Genet Dev* 1996;**6:**295–300.
109. Aumailley M, Krieg T. Laminins: a family of diverse multifunctional molecules of basement membranes. *J Invest Dermatol* 1996;**106:**209–214.
110. Uitto J, Richard G. Progress in epidermolysis bullosa: from eponyms to molecular genetic classification. *Clin Dermatol* 2005;**23:**33–40.
111. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, *et al*. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. *Science* 2003;**302:**415–419.
112. McCormack MP, Rabbitts TH. Activation of the T-cell oncogene *LMO2* after gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2004;**350:**913–922.
113. Aiuti A, Cassani B, Andolfi G, Mirolo M, Biasco L, Recchia A, *et al*. Multilineage hematopoietic reconstitution without clonal selection in ADA–SCID patients treated with stem cell gene therapy. *J Clin Invest* 2007;**117:**2233–2240.
114. Zufferey R, Dull T, Mandel RJ, Bukovsky A, Quiroz D, Naldini L, *et al*. Self-inactivating lentivirus vector for safe and efficient *in vivo* gene delivery. *J Virol* 1998;**72:**9873–9880.
115. Bushman F, Lewinski M, Ciuffi A, Barr S, Leipzig J, Hannenhalli S, *et al*. Genome-wide analysis of retroviral DNA integration. *Nat Rev Microbiol* 2005;**3:**848–858.
116. Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, *et al*. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nat Biotechnol* 2006;**24:**687–696.
117. Chen M, O'Toole EA, Muellenhoff M, Medina E, Kasahara N, Woodley DT. Development and characterization of a recombinant truncated type VII collagen 'minigene'. Implication for gene therapy of dystrophic epidermolysis bullosa. *J Biol Chem* 2000;**275:**24429–24435.
118. Porteus MH, Connelly JP, Pruett SM. A look to future directions in gene therapy research for monogenic diseases. *PLoS Genet* 2006;**2:**e133.
119. Ortiz-Urda S, Thyagarajan B, Keene DR, Lin Q, Fang M, Calos MP, *et al*. Stable nonviral genetic correction of inherited human skin disease. *Nat Med* 2002;**8:**1166–1170.
120. Porteus MH. Mammalian gene targeting with designed zinc finger nucleases. *Mol Ther* 2006;**13:**438–446.