

# Location and Clonal Analysis of Stem Cells and Their Differentiated Progeny in the Human Ocular Surface

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**Abstract.** We have analyzed the proliferative and differentiation potential of human ocular keratinocytes. Holoclones, meroclones, and paraclones, previously identified in skin, constitute also the proliferative compartment of the ocular epithelium. Ocular holoclones have the expected properties of stem cells, while transient amplifying cells have variable proliferative potential. Corneal stem cells are segregated in the limbus, while conjunctival stem cells are uniformly distributed in bulbar and forniceal conjunctiva. Conjunctival keratinocytes and goblet cells derive from a common bipotent progenitor. Goblet cells were found in cultures of transient amplifying cells, suggesting that commitment for goblet cell differentiation can occur late in the life of a single conjunctival clone. We found that conjuncti-

val keratinocytes with high proliferative capacity give rise to goblet cells at least twice in their life and, more importantly, at rather precise times of their life history, namely at 45–50 cell doublings and at ~15 cell doublings before senescence. Thus, the decision of conjunctival keratinocytes to differentiate into goblet cells appears to be dependent upon an intrinsic "cell doubling clock." These data open new perspectives in the surgical treatment of severe defects of the anterior ocular surface with autologous cultured conjunctival epithelium.

**Key words:** keratinocyte • stem cells • differentiation • eye • goblet cells

**T**HE human ocular surface is covered with the highly specialized conjunctival and limbal-corneal epithelia (for review see Wagoner, 1997), which are formed by two genotypically different cell types (Schermer et al., 1986; Wei et al., 1996), hereafter referred to as conjunctival and limbal-corneal keratinocytes.

The conjunctival epithelium lies on a well-vascularized stroma, consists of several loosely organized cell layers and allows the movement of the eyelid over the cornea, the maintenance of the normal lid-globe apposition, and the limbal (see below) vascular supply (Friend and Kenyon, 1987; Nelson and Cameron, 1997). The conjunctival epithelium can be divided into three distinct regions: bulbar, which is contiguous with the corneal-limbal zone (see below) and covers the ocular globe; forniceal, which is located in the folding region; and palpebral, which is contiguous with the epidermis of the eyelid (Nelson and Cam-

eron, 1997). The conjunctival epithelium is populated by goblet cells (Friend and Kenyon, 1987), which are unicellular mucin-secreting glands representing the primary source of the mucin of the tear film (Dilly, 1994; Tiffany, 1994). Goblet cells are essential in maintaining the integrity of the ocular surface, since mucin deficiency has been implicated in several disabling diseases of the eye (Tseng et al., 1984). The corneal epithelium is a transparent and flat stratified squamous epithelium devoid of goblet cells with a cuboid basal layer lying on the avascular corneal stroma by Bowman's layer (Nishida, 1997). Its integrity is essential to visual acuity (Dua and Forrester, 1990).

The narrow transitional zone between the cornea and the bulbar conjunctiva is referred to as the limbus (Friend and Kenyon, 1987; Nishida, 1997). Limbal epithelium consists of several layers of epithelial cells organized in well-developed rete ridges, devoid of goblet cells and populated by Langerhans cells and melanocytes. Limbal cells are considered the progenitors of corneal (Schermer et al., 1986; Cotsarelis et al., 1989), but not of conjunctival keratinocytes (Wei et al., 1996).

Surface epithelia are renewed constantly during the lifetime of an organism. For instance, human epidermis is re-

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placed approximately every month (Green, 1980), while corneal epithelium is renewed in 9–12 mo (Wagoner, 1997). To accomplish their self-renewal process, these epithelia (as well as other self-renewing tissues) rely on the presence of stem and transient amplifying cells, which are the only proliferative cells in a normal tissue (for review see Barrandon, 1993; Lavker et al., 1993; Morrison et al., 1997). Stem cells can be defined as cells endowed with a high capacity for cell division and the ability to generate differentiated progeny (Lajtha, 1979; Barrandon, 1993; Morrison et al., 1997). The extensive proliferative capacity, maintained through the lifetime of an organism, is considered the basic and essential characteristic of a stem cell (Morrison et al., 1997). Transient amplifying cells, which arise from stem cells, have a low proliferative capacity and represent the largest group of dividing cells (Lajtha, 1979; Barrandon, 1993; Lavker et al., 1993).

Although some markers for the epithelial stem cell compartment have been proposed (Zieske et al., 1992; Jones and Watt, 1993; Li et al., 1998), their role in specifically identifying keratinocyte stem cells is still very controversial. Therefore, the identification of stem cells relies entirely on either the evaluation of their proliferative capacity *in vitro* (Barrandon and Green, 1987; Rochat et al., 1994; Mathor et al., 1996; Dellambra et al., 1998) or on the identification of slow-cycling, [<sup>3</sup>H]TdR- and BrdU-retaining cells *in vivo* (Cotsarelis et al., 1989, 1990; Lavker et al., 1993; Lehrer et al., 1998). For instance, [<sup>3</sup>H]TdR-retaining experiments have clearly shown that, in mice, putative stem cells for the corneal epithelium are located in the limbus (Schermer et al., 1986; Cotsarelis et al., 1989; Lehrer et al., 1998), whereas conjunctival stem cells appear to be concentrated in the fornix (Wei et al., 1995). However, not all epithelial stem cells are slow cycling (Morrison et al., 1997); the slow-cycling property of a cell does not necessarily indicate its proliferative potential; label-retaining experiments can be performed only on laboratory animals, hence differences between species should be taken into account. For instance, while in the mouse hair follicle clonogenic cells and label-retaining cells colocalize (Cotsarelis et al., 1990; Kobayashi et al., 1993), the location of most human hair follicle stem cells is different from the area where mouse label-retaining cells are located (Cotsarelis et al., 1990; Rochat et al., 1994). Finally, elegant studies have shown that putative stem cells and transient amplifying cells can colocalize (Lehrer et al., 1998).

These uncertainties can be clarified by analyzing, at a clonal level, the proliferative capacity of human lining epithelial stem cells *in vitro* (Rheinwald and Green, 1975; Barrandon and Green, 1985, 1987). Indeed, three types of keratinocytes with different capacities for multiplication have been identified and isolated in human epidermis and hair follicle (Barrandon and Green, 1987; Rochat et al., 1994), i.e., holoclones, meroclones, and paraclones.

The holoclone has the highest proliferative capacity (being able to undergo 120–160 divisions), and is considered the epidermal stem cell (Barrandon and Green, 1987; Barrandon, 1993; Mathor et al., 1996; Dellambra et al., 1998). In human skin, holoclones are uniformly distributed in the basal layer of interfollicular epidermis (Barrandon and Green, 1987), whereas, in the human hair follicle, they are segregated in a specific region of the outer root sheath

below the midpoint of the follicle and also in the matrix (Rochat et al., 1994).

The paraclone is a transient amplifying cell, which is committed to a maximum of 15 cell divisions and generates aborted colonies containing only terminally differentiated cells (Barrandon and Green, 1987). The meroclone is an intermediate type of cell and is a reservoir of transient amplifying cells (Barrandon and Green, 1987; Barrandon, 1993; Jones and Watt, 1993). The transition from holoclone to meroclone to paraclone is a unidirectional process that occurs during natural aging as well as during repeated keratinocyte sub-cultivation (Barrandon, 1993).

Here we evaluated, by clonal analysis, the proliferative capacity and the differentiation potential of different areas of the human ocular epithelium. We show that corneal stem cells are located exclusively in the limbus, fully confirming previous experiments performed by analyzing label-retaining cells in the mouse cornea (Cotsarelis et al., 1989; Lehrer et al., 1998).

We also show that in contrast with data obtained with [<sup>3</sup>H]TdR-retaining experiments in mice (Wei et al., 1995) and with cultivation of rabbit conjunctival cells (Wei et al., 1993), human conjunctival stem cells are uniformly distributed in the bulbar and forniceal conjunctiva; the conjunctival keratinocyte and the mucin-producing goblet cell are derived from a common bipotent progenitor (as suggested by Wei et al., 1997); commitment to differentiate into goblet cells occurs relatively late, so that goblet cells are generated by stem cell-derived transient amplifying cells; and the decision of a conjunctival keratinocyte to differentiate into a goblet cell appears to be dependent upon an intrinsic “cell doubling clock.”

## Materials and Methods

### Selection of Donors and Materials

Specimens were obtained in accordance with the tenets of the Declaration of Helsinki, and all donors provided informed consent for biopsy. Permission was obtained for specimens taken from an organ donor.

In preliminary experiments, ocular keratinocytes were cultivated from several biopsies taken from conjunctiva, cornea, and limbus of patients undergoing penetrating keratoplasty. Results of growth rate experiments and serial cultivation were inconsistent, probably because the epithelium was suffering from the original pathology.

Therefore, samples were obtained only from patients undergoing cataract, strabismus, and keratoconus surgery and presenting with undamaged anterior ocular epithelium. In one case, biopsies were taken (within 9 h from death) from the eye of a 54-yr-old organ donor woman with no history of ocular surface disorders. Biopsies (1–2 mm<sup>2</sup>) were taken from different areas of the eye, as indicated in Fig. 1.

3T3-J2 cells were a gift from Prof. Howard Green (Harvard Medical School, Boston, MA). The keratin 3-specific AE5 mAb was a gift from Dr. Tung-Tien Sun (New York University Medical Center, New York). The keratin 19-specific RCK108 mAb was purchased from Dako Corp.

### Cell Culture

Ocular keratinocytes were cultivated on a lethally irradiated feeder layer of 3T3-J2 cells as described previously (Rheinwald and Green, 1975; Sun and Green, 1977; Zambruno et al., 1995; Pellegrini et al., 1997). In brief, samples were treated with trypsin (0.05% trypsin and 0.01% EDTA) at 37°C for ~80 min. Cells were collected every 20 min. We obtained an average of  $17.3 \times 10^3$  cells/mm<sup>2</sup>, a value lower than that obtained with skin biopsies, which yield an average of  $30 \times 10^3$  cells/mm<sup>2</sup> (our unpublished data). Cells were plated ( $1.5 \times 10^4$ /cm<sup>2</sup>) on lethally irradiated 3T3-J2 cells ( $2.4 \times 10^4$ /cm<sup>2</sup>) and cultured in 5% CO<sub>2</sub> and humidified atmosphere in:

DME and Ham's F12 media (2:1 mixture) containing FCS (10%), insulin (5 µg/ml), adenine (0.18 mM), hydrocortisone (0.4 µg/ml), cholera toxin (0.1 nM), triiodothyronine (2 nM), glutamine (4 mM), and penicillin-streptomycin (50 IU/ml). Epidermal growth factor (10 ng/ml) was added at 10 ng/ml beginning at the first feeding, 3 d after plating. Cultures were then fed every other day. Subconfluent primary cultures were passaged at a density of  $6 \times 10^3$  cells/cm<sup>2</sup> and cultured as above. For serial propagation, cells were passaged as above, always at the stage of subconfluence, until they reached senescence.

### Clonal Analysis

Single cells, isolated under the microscope, were inoculated onto multiwell plates already containing a feeder layer of 3T3 cells (Rochat et al., 1994; Mathor et al., 1996). After 7 d of cultivation, clones were identified under an inverted microscope, photographed and their area was measured. Each clone was then transferred to three dishes. Two dishes (3/4 of the clone) were used for serial propagation and further analysis. The third (indicator) dish (1/4 of the clone) was fixed 12 d later and stained with rhodamine B for the classification of clonal type. The clonal type was determined by the percentage of aborted colonies (scored as in Barrandon and Green, 1987) formed by the progeny of the founding cell. When 0–5% of colonies were terminal the clone was scored as holoclone. When all colonies formed were terminal (or when no colonies formed), the clone was classified as paraclone. When >5% but <100% of the colonies were terminal, the clone was classified as a meroclone (Barrandon and Green, 1987). Selected clones (see Results) were serially propagated to determine the number of cell generations. The entire procedure of cloning and subcultivation was done under strict timing conditions identical for each clone.

### Determination of the Colony-forming Efficiency

Cells (300–2,000) from each biopsy and from each cell passage of serially cultivated mass and clonal cultures were plated onto 3T3 feeder layers and cultivated as above. Colonies were fixed 12 d later, stained with rhodamine B and scored under a dissecting microscope. Values are expressed as the ratio of the number colonies on the number of inoculated cells. All colonies were scored whether progressively growing or aborted.

### Determination of the Number of Cell Generations

The number of cell generations was calculated using the following formula:  $x = 3.322 \log N/N_0$ , where N equals the total number of cells obtained at each passage and  $N_0$  equals the number of clonogenic cells. Clonogenic cells were calculated from the colony-forming efficiency data (see above), which were determined separately in parallel dishes at the time of cell passage.

### Histological Procedures

Confluent sheets of epithelium generated by either mass or clonal cultures, were detached from the vessels with Dispase II (Green et al., 1979). Specimens were fixed in paraformaldehyde (4% in PBS) overnight at 4°C and embedded in paraffin. Sections were either stained with hematoxylin-eosin or double-immunostained with K3-specific AE5 mAb and K19-specific RCK108 mAb (DAKO). AE5-immunoreaction was detected with the HRP-dextran-anti-mouse complex (EnVision Plus/HRP system; DAKO), using 3,3'-diaminobenzidine tetrahydrochloride (FAST DAB; Sigma Chemical Co.) as a chromogen. RCK108 immunoreaction was detected with the alkaline-phosphatase-dextran-anti-mouse complex (EnVision/AP system; DAKO), using Fast Red TR/Naphtol AS-MX (Fast Red; Sigma Chemical Co.) as a chromogen. Double-immunostained sections were washed, counterstained with hematoxylin, and mounted in an aqueous mounting media.

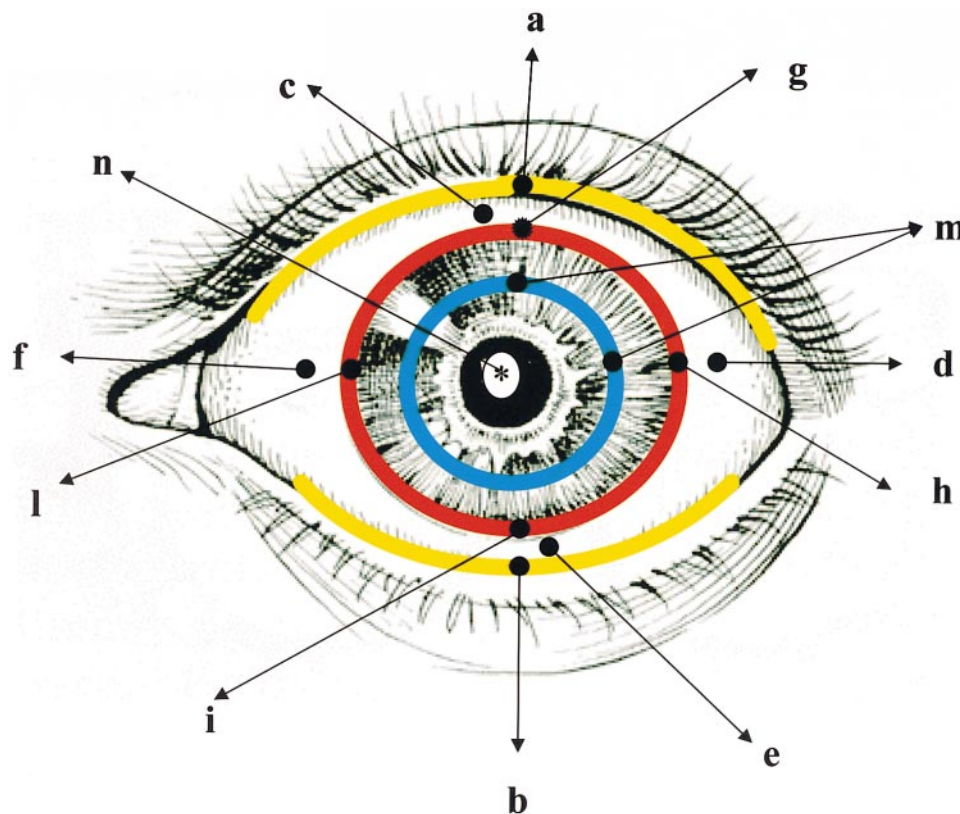
Goblet cells were stained with the Alcian blue-periodic acid-Schiff reaction as described (Pellegrini et al., 1997; Wei et al., 1997).

Dissociated cells obtained from either mass or clonal cultures were centrifuged on a coverslip (De Luca et al., 1988), fixed in methanol/acetone, and immunostained as above. Parallel coverslips were fixed in 4% paraformaldehyde and stained with Alcian blue-periodic acid-Schiff reaction for goblet cell quantification.

## Results

### Cultivation of Cells from the Human Ocular Surface

Ocular keratinocytes were isolated from 1-mm<sup>2</sup> biopsies



**Figure 1.** The ocular surface. This figure shows areas of the ocular surface from where biopsies were taken. Fornix, limbus, and paracentral cornea are colored in yellow, red, and blue, respectively. The central cornea is indicated by the asterisk. The bulbar region is indicated by the white color. Biopsies were taken from: superior (a) and inferior (b) fornix; superior (c), temporal (d), inferior (e), and nasal (f) bulbar conjunctiva; superior (g), temporal (h), inferior (i), and nasal (l) limbus; paracentral (m) and central (n) cornea.

(Fig. 1) taken only from donors with no history of ocular surface disorders and cultivated as described under Materials and Methods. In one case, biopsies were freshly harvested from a cadaver.

Mimicking the growth behavior of epidermal keratinocytes (Rheinwald and Green, 1975; Sun and Green, 1977; Lindberg et al., 1993; Mathor et al., 1996), limbal, bulbar, and forniceal keratinocytes founded colonies, each colony being the progeny of a single cell (Rheinwald and Green, 1975; Lindberg et al., 1993). The shape and overall appearance of colonies and of cells within the colonies were similar to those observed with epidermal keratinocytes (not shown). The doubling time of both limbal and conjunctival cells was  $\sim 20$  h. Colonies eventually fused and generated a stratified epithelium (Fig. 2, A and B). Corneal keratinocytes isolated from central and paracentral cornea usually did not form colonies. Occasionally, keratinocytes isolated from paracentral cornea formed scattered colonies which, however, could not be serially cultivated.

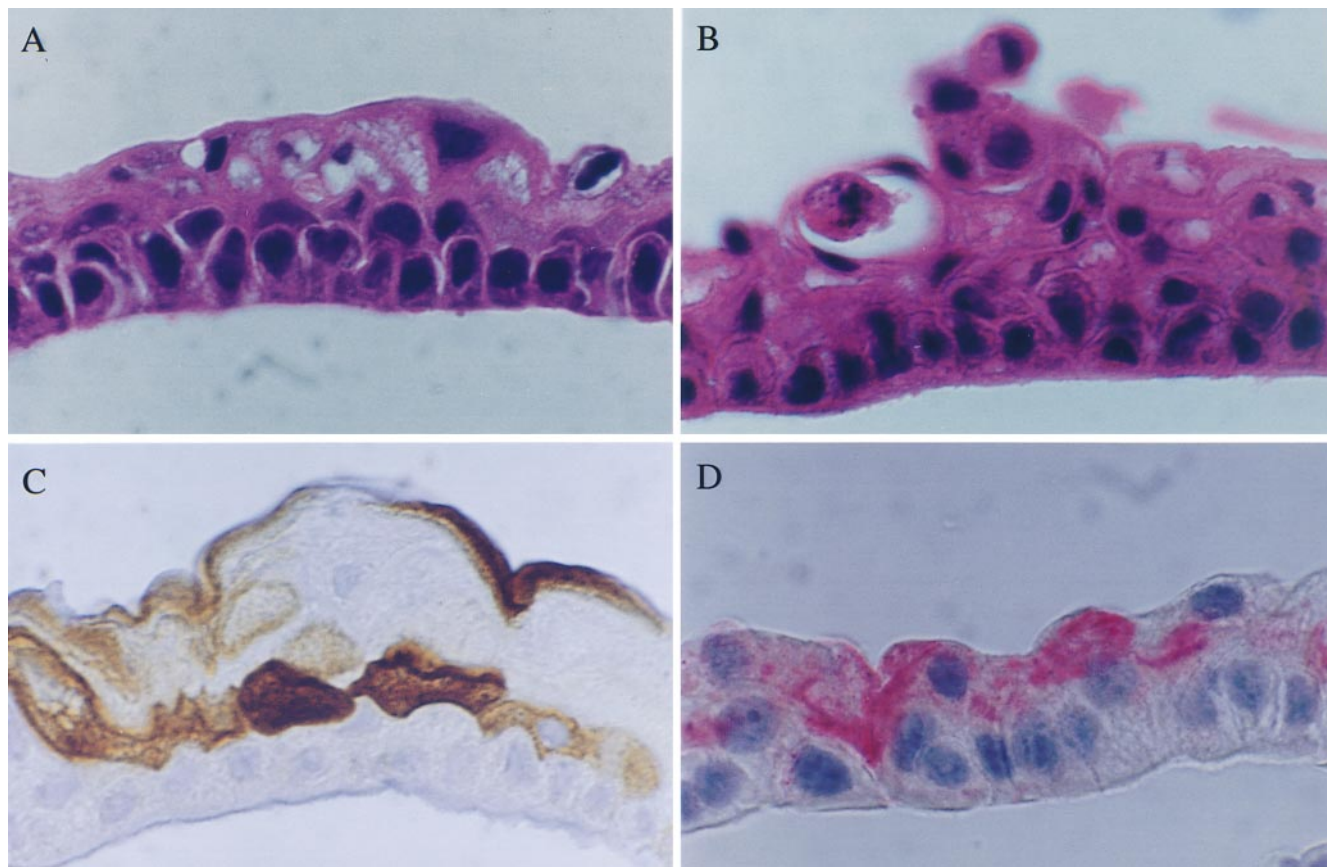
Epithelia from different parts of the body express keratin pairs that are unique for each location (Franke et al., 1981; Moll et al., 1982). For instance, the epidermis expresses the K1/K10 keratin pair whereas the corneal epithelium expresses the K3/K12 keratin pair (Schermer et al., 1986). The conjunctival epithelium expresses K19 but not

K3/K12 (Schermer et al., 1986). Fig. 2 shows that cultured corneal-limbal epithelium is K3<sup>+</sup> and K19<sup>-</sup> (C), while cultured conjunctival epithelium is K3<sup>-</sup> and K19<sup>+</sup> (D), suggesting that the site-specific differentiation program is maintained under these culture conditions.

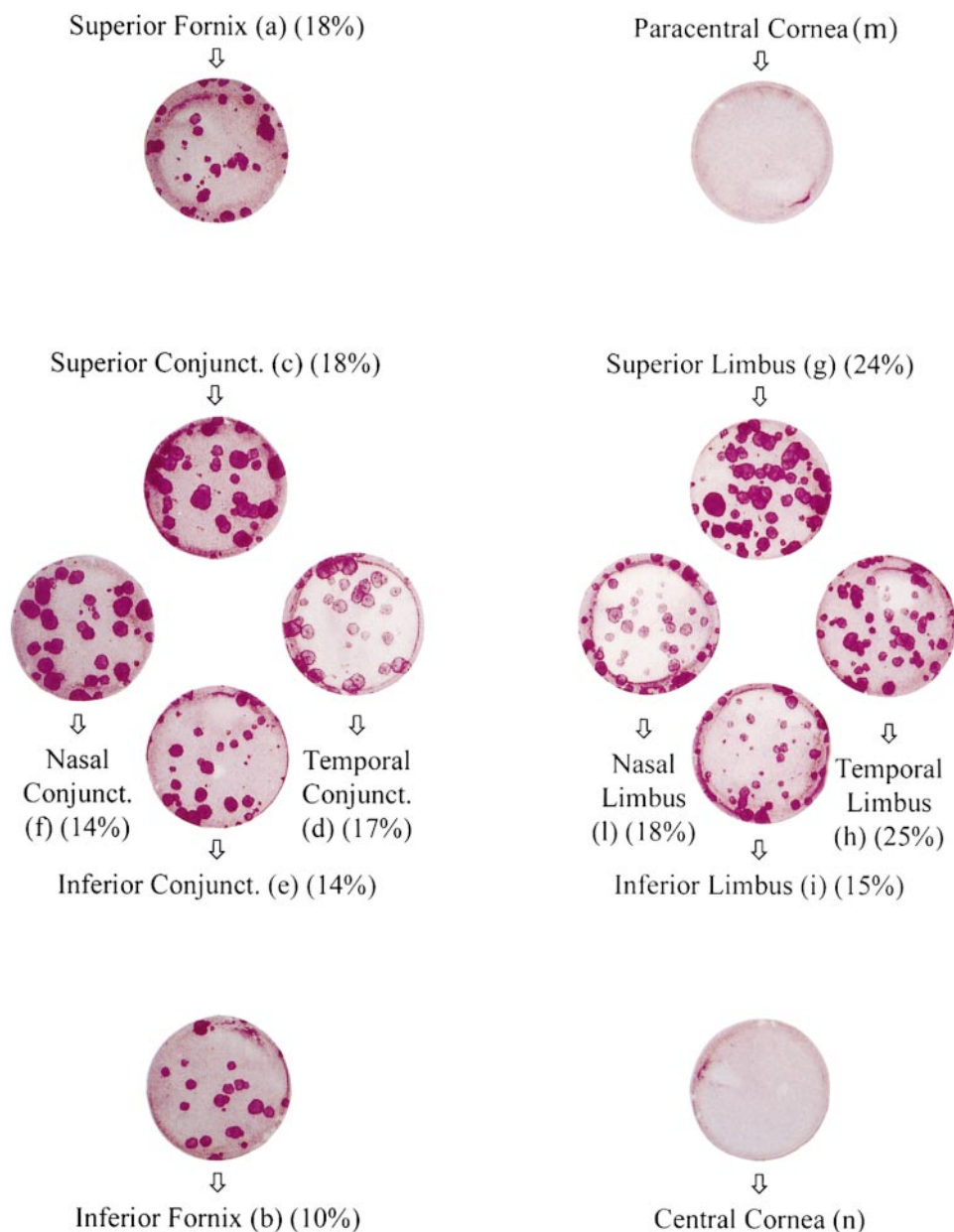
### Evaluation of Clonogenic Ability and Proliferative Potential

12 1–2-mm<sup>2</sup> biopsies were taken from the eye of a female, 54-yr-old organ donor. Biopsies were taken from different areas of the eye, as indicated in Fig. 1. Four limbal biopsies (from the four quadrants of the eye: g, h, i, and l), four bulbar conjunctival biopsies (from the four quadrants of the eye: c, d, e, and f), and biopsies from paracentral cornea (m), central cornea (n), superior fornix (a), and inferior fornix (b) were processed simultaneously within 24 h from death.

To evaluate the clonogenic ability of the different areas of the ocular surface, 300 cells from each area were plated onto lethally irradiated 3T3-J2 cells and stained 12 d later with rhodamine B. As shown in Fig. 3, the limbus was the only area of the corneal-limbal epithelium able to form large and smooth colonies, while cells from paracentral and central cornea were not clonogenic. In contrast, keratinocytes isolated from the superior and inferior fornix, as



**Figure 2.** Histology. Sheets of epithelial cells cultivated from superior limbus (A and C) and superior fornix (B and D) were detached from the culture vessel with the neutral protease Dispase II. Epithelial sheets were either stained with hematoxylin-eosin (A and B) or double-immunostained with K3-specific AE5 mAb and K19-specific RCK108 mAb (C and D). Note that cultured corneal-limbal epithelium is K3<sup>+</sup> and K19<sup>-</sup> (C), whereas cultured conjunctival epithelium is K3<sup>-</sup> and K19<sup>+</sup> (D).



*Figure 3.* Determination of the colony-forming efficiency. 12 biopsies were taken from different areas (also indicated by lower case letters in parentheses, see Fig. 1) of the ocular surface of a 54-yr-old organ donor woman. 300 cells from each biopsy were plated onto lethally irradiated 3T3-J2 cells. Dishes were stained 12 d later with rhodamine B. Values are expressed as the ratio of the number of colonies over the number of inoculated cells and are indicated in parentheses. Note that conjunctival colony-forming cells are uniformly distributed in fornical and bulbar conjunctiva, whereas clonogenic cells of the limbus-cornea are segregated in the limbus.

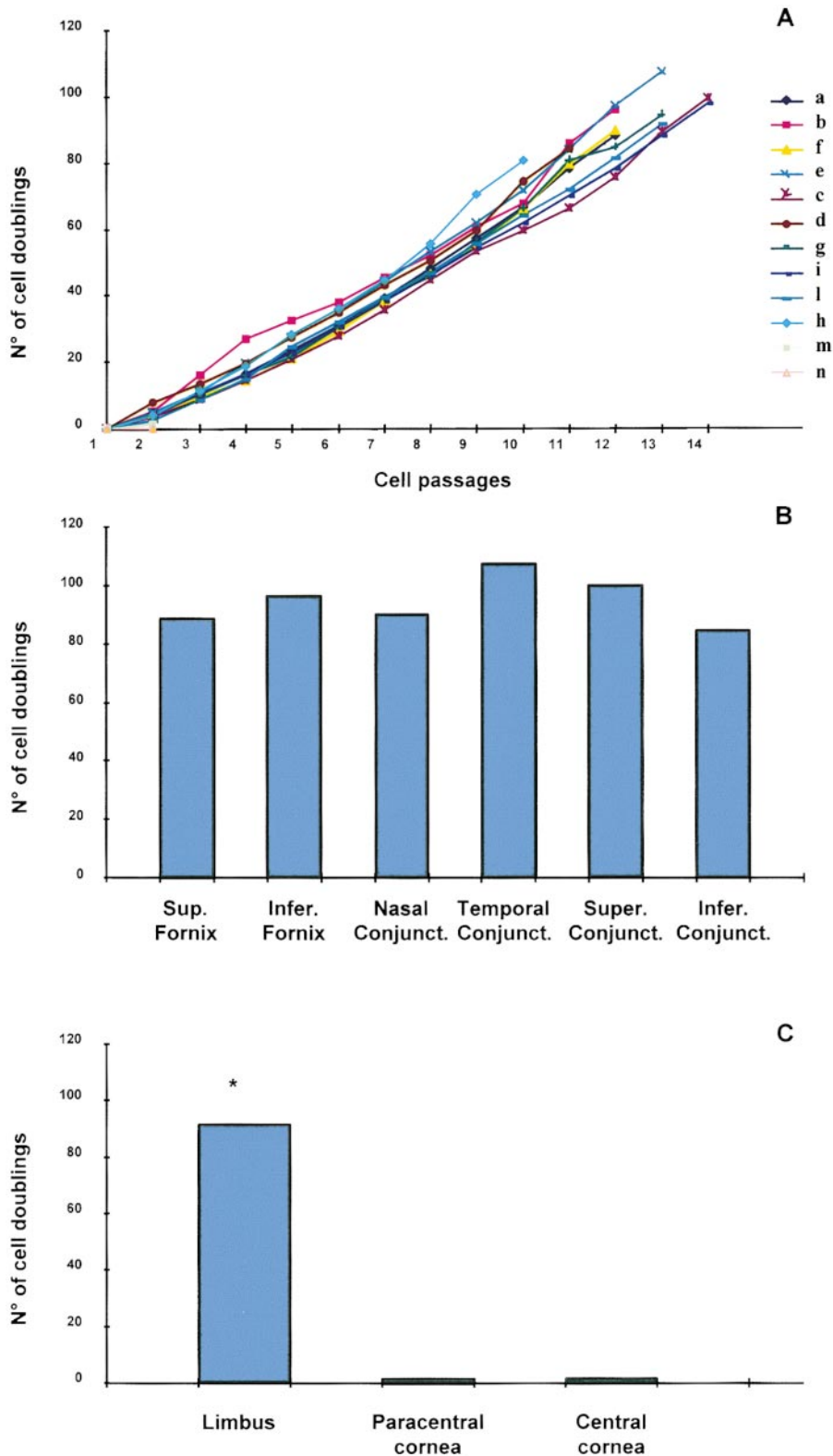
well as from the four quadrants of the bulbar conjunctiva, displayed a comparable colony forming ability (values for each area are indicated in Fig. 3). It is worth noting that values obtained from the limbus and from the different areas of the conjunctiva were comparable.

Clonogenic ability and growth potential of epithelial cells are two very different concepts. The former indicates the capacity of a basal cell to found a colony, the latter deals with its capacity of producing cell generations, hence it deals with its self-renewal potential. Therefore, the proliferative capacity of cells isolated from different areas of the eye was evaluated by serial cultivation. As shown in Fig. 4 A, keratinocytes from the limbus (four quadrants: g, h, i, and l), from either the superior and inferior fornix (a and b) and from bulbar conjunctiva (four quadrants: c, d, e, and f) could be cultivated up to 14 passages (2–3 mo) and underwent 80–100 divisions before senescence. As

clearly shown by Fig. 4, B and C, conjunctival cells with very high capacity for cell division were uniformly distributed on the ocular surface, whereas corneal cells with high proliferative capacity were segregated in the limbus. Again, it is worth noting that the values obtained from the limbus and from different areas of the conjunctiva were similar.

These results were confirmed by serially cultivating ocular keratinocytes obtained from 42 biopsies of unrelated donors of different ages. As shown in Table I, cells from 12 fornical biopsies underwent an average of 79 doublings; cells from 21 bulbar biopsies underwent an average of 82 doublings; cells from 9 limbal biopsies underwent 85 doublings before senescence. Cells from central cornea could not be serially cultivated.

These data demonstrate that clonogenic cells endowed with high capacity for cell division (typical of stem cells)



**Figure 4.** Determination of the number of cell generations. (A) Ocular keratinocytes isolated from the same biopsies analyzed in Fig. 3 (a–n), were serially cultivated until they reached senescence and the number of cell generations was calculated as described in Materials and Methods. (B) Cells obtained from different areas of fornical and bulbar conjunctiva underwent a comparable number of cell divisions (80–100) before senescence. (C) Corneal-limbal cells endowed with high proliferative capacity were segregated in the limbus. \*This value ( $90.5 \pm 7$ ) is the average of cell generations produced by keratinocytes cultivated from superior, temporal, inferior, and nasal limbus.

are segregated in the limbal region of the corneal-limbal epithelium and are evenly distributed in the conjunctival epithelium covering the eye bulb and the fornix.

#### **Clonal Analysis of Stem and Transient Amplifying Cells**

To investigate whether holoclones, meroclones and para-

clones, previously identified in human skin (Barrandon and Green, 1987; Rochat et al., 1994; Mathor et al., 1996), were also present in the ocular epithelium, single cells were isolated from 15 different sub-confluent primary cultures obtained from 7 different donors. After 7 d of cultivation, each single clone was photographed and its area was measured. Each clone was then transferred to three

Table I. Proliferative Potential

Origin of biopsies	Quadrant	Number of samples	Age of donors	No. of cell doublings
Fornix	Superior (a)	12	6-77	79 ± 10
	Inferior (b)			
	Superior (c)			
Bulbar conjunctiva	Nasal (f)	21	5-85	82 ± 21
	Temporal (d)			
	Inferior (e)			
	Superior (g)			
Limbus	Nasal (l)	9	5-65	85 ± 9
	Temporal (h)			
	Inferior (i)			

Ocular keratinocytes isolated from 42 biopsies taken from different areas of the ocular surface (indicated by letters in parentheses, see Fig. 1) of different donors were serially cultivated as described in Materials and Methods. The number of cell generations was calculated using the following formula:  $x = 3,322 \log N/N_0$ , where N equals the total number of cells obtained at each passage and  $N_0$  equals the number of clonogenic cells (see Materials and Methods).

dishes. Two dishes (3/4 of the clone) were used for serial propagation and further analysis. The third dish (1/4 of the clone) was fixed 12 d later and stained with rhodamine B for the classification of clonal type, which is based on the relative number of aborted colonies (Barrandon and Green, 1987; see Materials and Methods). As shown in Table II, we analyzed 339 clones (129 from the inferior and superior fornix, 152 from the bulbar conjunctiva and 58 from the superior limbus). The majority of clones were classified as meroclones. Holoclones were identified, in similar percentage, in limbus, fornix, and bulbar conjunctiva (Table II). Confluent sheets of epithelium or dissociated cells, both obtained from randomly chosen clones, were analyzed using anti-K3 or anti-K19 antibodies. Immunohistochemistry confirmed that all clones from fornical and bulbar conjunctiva were K19<sup>+</sup> and K3<sup>-</sup>. Although conjunctival cells are usually present in a limbal biopsy, the majority of clones from limbal epithelium was K3<sup>+</sup> and K19<sup>-</sup> (not shown). Only clones expressing the proper differentiation markers were selected for further experiments.

Cultures from 60 conjunctival (33 bulbar and 27 fornical) clones (4 holoclones, 43 meroclones, and 13 paraclones) obtained from the same donor were then serially cultivated to evaluate their proliferative capacity. In agreement with the number of doublings of the original mass cultures (97 doublings for the bulbar mass culture and 96 doublings for the fornical mass culture), holoclones isolated from both bulbar (Fig. 5 H, blue bars) and fornical (Fig. 5 H, yellow bars) conjunctiva were able to produce 91.7 and 84.5 (mean values) cell generations before senescence, respectively. Instead, meroclones from both areas displayed a high heterogeneity in their growth potential, with clones able to undergo as many as 60 and as few as 25 cell doublings (Fig. 5 M). As expected, paraclones underwent a very limited number of cell divisions (Fig. 5 P). The three types of clones were similarly represented in bulbar (Fig. 5, blue bars) and fornical (Fig. 5, yellow bars) epithelium. We also serially cultivated five holoclones isolated from the superior limbus of a different donor. As with conjunctival holoclones, limbal holoclones

Table II. Clonal Analysis

Total number of clones	Origin Number of donors	Quadrants Number of Samples	Types of clones* Number of clones			Age of donors
339	Fornix	Superior (a)	Holo	Mero	Para	6-77
			3	6	41	30
		6				
	Bulbar conjunctiva	Inferior (b)	Holo	Mero	Para	27-76
			3	4 <sup>‡</sup>	41 <sup>‡</sup>	7 <sup>‡</sup>
		7				
	Limbus	Temporal (d)	Holo	Mero	Para	5-77
			4	7	47	37
		7				
	Limbus	Inferior (e)	Holo	Mero	Para	27-76
		3	5 <sup>‡</sup>	41 <sup>‡</sup>	15 <sup>‡</sup>	
2						
	Superior (g)	Holo	Mero	Para	5-54	
	2	11 <sup>§</sup>	39	8		

Single cells were isolated from 15 sub-confluent primary cultures originated from different areas of the ocular surface (indicated by letters in parentheses, see Fig. 1) of seven donors. After 7 d of cultivation, clones were identified under an inverted microscope and photographed. Each clone was transferred to three dishes. Two dishes (3/4 of the clone) were used for serial propagation and/or further analysis. The third dish (1/4 of the clone) was fixed 12 d later and stained with Rhodamine B for the classification of clonal type. The clonal type was determined by the percentage of aborted colonies formed by the progeny of the founding cell (see Materials and Methods). Holoclones (Holo), Meroclones (Mero), and Paraclones (Para) were identified in the limbus and both in the fornical and bulbar conjunctiva.

\* One dish obtained from each clone was PAS-stained for goblet cell detection (see Results).

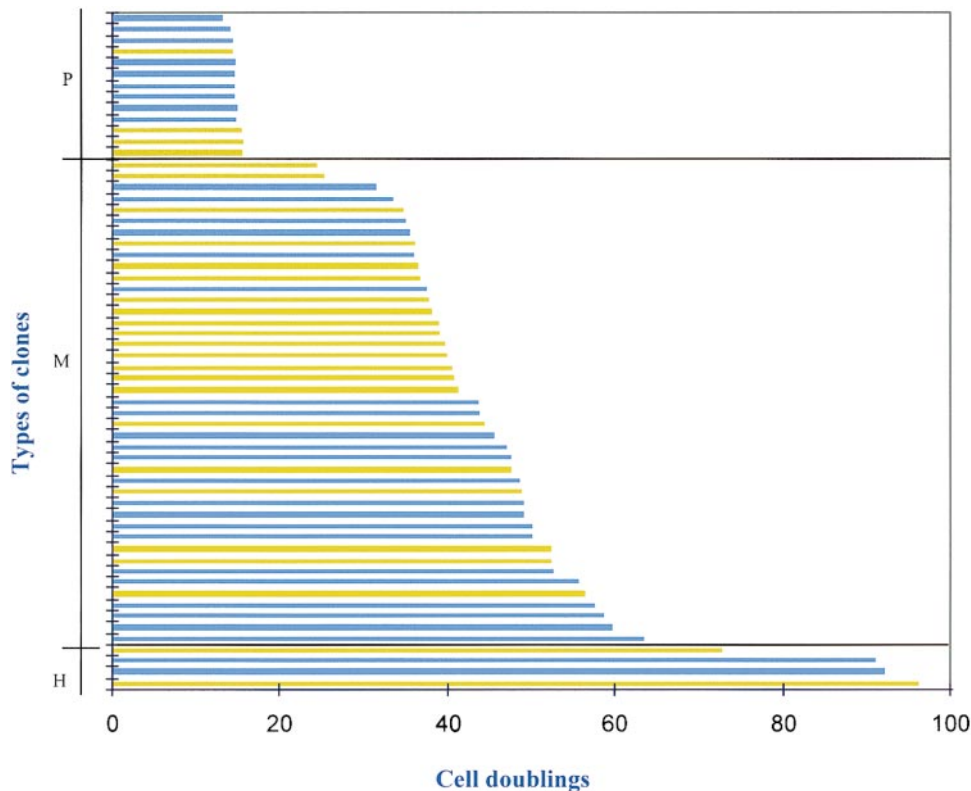
<sup>‡</sup> These clones were serially cultivated until they reached senescence.

<sup>§</sup> 5 limbal holoclones were serially cultivated until they reached senescence.

underwent 95 ± 8 doublings before senescence, accounting for the entire proliferative potential of the original mass culture.

Previous studies have demonstrated that the relative percentage of epidermal holoclones declines in vivo during aging (Barrandon and Green, 1987; Barrandon, 1993), suggesting that newborns start their life with a high content of keratinocyte stem cells which are programmed to undergo a fixed number of cell divisions (also referred to as the Hayflick limit; Hayflick, 1965) before senescence. Since holoclones are found also in the adulthood (Tables I and II and Fig. 5), it is conceivable that recruitment of stem cells, to generate transient amplifying and differentiated cells, occurs in "waves" during life and that this recruitment is somehow accelerated by emergency situations as wound healing or cultivation (Barrandon, 1993; Morrison et al., 1997). Accordingly, stem cell-derived transient amplifying cells are expected to have a quite variable proliferative potential. As shown in Fig. 5, conjunctival meroclones are highly heterogeneous in their capacity for cell division, hence we consider them as transient amplifying cells endowed with a higher proliferative potential than paraclones.

Taken together, these data fulfill the criteria for the existence of ocular holoclones arising from stem cells and demonstrate that holoclones, meroclones, and paraclones, previously identified only in skin, constitute the proliferative cell compartment also of the human anterior ocular surface; corneal stem cells are located exclusively in the limbus; conjunctival stem cells are uniformly distributed in the clonogenic layer of both bulbar and fornical epithelium; the proliferative potential of both corneal and conjunctival stem cells is of 80-100 doublings, a value considerably lower than that characterizing the epidermal (and hair follicle) stem cell compartment (120-160 doublings);



**Figure 5.** Determination of the number of progeny generated by conjunctival clones. Clonal analysis and classification of clonal types were performed as described in Materials and Methods. Cultures from 60 conjunctival clones obtained from the same donor were serially cultivated until they reached senescence. The number of cell generations was calculated as described in Materials and Methods. 4 holoclones (H), 43 meroclones (M), and 13 paraclones (P) were analyzed. Blue bars indicate clones from fornical conjunctiva. Yellow bars indicate clones from bulbar conjunctiva.

and the transient amplifying cell compartment is formed by cells with highly variable proliferative potential.

### **Origin of Goblet Cells**

The conjunctival epithelium is populated by goblet cells, which are essential for the maintenance of ocular surface integrity. PAS staining of confluent sheets prepared from secondary cultures of bulbar conjunctival keratinocytes showed that several goblet cells were present in suprabasal layers (Fig. 6 A, arrows), while, as expected, goblet cells were absent in the epithelium cultured from limbal biopsies (Fig. 6 B). PAS-staining performed during serial cultivation of fornical (Fig. 6 C, blue lines) and bulbar (Fig. 6 C, yellow lines) keratinocytes (growing colonies) from 11 different donors indicated that goblet cells were present during the entire life span of the cultures. The relative content of goblet cells was investigated during the exponential phase of conjunctival cell growth (growing colonies) and after generation of cohesive epithelial sheets (2–3 d after confluence). As shown in Fig. 6 D, an increasing number of goblet cells was present during serial cultivation. However, while growing colonies displayed a goblet cell content of 200–500 cells per cm<sup>2</sup>, confluent conjunctival sheets had >5,000 goblet cells/cm<sup>2</sup> (Fig. 6 D). We have calculated that this value corresponds to a goblet/keratinocyte ratio of ~1/30, a ratio not far from that found in vivo in resting bulbar conjunctiva (Nelson and Cameron, 1997).

These data could be explained by either proliferation of differentiated goblet cells in culture; existence of an undifferentiated goblet cell precursor, able to proliferate and differentiate in vitro (as suggested by Tsai et al., 1997); differentiation of bipotent conjunctival stem cells able to give

rise to two different cell types (as strongly suggested by Wei et al., 1997; see also Discussion).

Therefore, we investigated whether the progeny of conjunctival clones was populated by goblet cells. As shown in Fig. 6, E–H goblet cells were present in cultures of both holoclones (E and F) and meroclones (G and H) isolated from both fornical (E–G) and bulbar (F–H) conjunctiva. PAS-staining of mass cultures from the same 339 clones shown in Table II, revealed that 100% and 93% of holoclone- and meroclone-derived cultures contained goblet cells, respectively. Paraclones were usually goblet-negative, but a few paraclones, notably those able to be passaged once (hence generating only aborted daughter colonies; Barrandon and Green, 1987), were also able to produce scattered goblet cells. Cultures from limbal holoclones and meroclones were invariably goblet-negative.

This set of data proves that conjunctival keratinocytes are bipotent since they can generate also goblet cells. Moreover, goblet cells were found in cultures from meroclones, suggesting that commitment for goblet cell differentiation can occur late in the life of a single conjunctival clone.

### **Conjunctival Cell Differentiation Is Regulated by a Cell Doubling Clock**

To investigate whether the generation of a goblet cell by a conjunctival keratinocyte was related to a specific time of its life, we selected (from the same donor) 14 conjunctival clones endowed with significant proliferative potential and analyzed the formation of goblet cells during their serial cultivation (PAS reactions were carried out at each cell passage on exponentially growing colonies).



Based on data shown in Fig. 5, we arbitrarily defined as “young” transient amplifying cells those meroclonal cells still able to undergo 35–60 doublings, and “old” meroclonal cells those cells undergoing 20–35 cell divisions before senescence. We found that conjunctival keratinocytes with high proliferative capacity give rise to goblet cells at least twice in their life and, more importantly, at a specific time of their cycles of cell duplication. As shown in Fig. 7 A (first peak), a first generation of goblet cells occurred at 45–50 cell doublings. A second, and more substantial, bulk of goblet cells was generated very late in the life of the clones, at 10–20 doublings before senescence (Fig. 7 A, second peak).

As shown in Table II and Fig. 5, holoclones (and young meroclonal cells) are usually present in low abundance. Also, it is not possible to determine a priori whether a cell will generate a holoclone, a young meroclone or an old meroclone. This explains the low number of cells with high proliferative potential, hence able to generate two peaks of goblet cells, that we were able to analyze in a single experiment (Fig. 7 A). Therefore, to substantiate the observation of a cell doubling-dependent mechanism for goblet cell generation, we decided to perform clonal analysis of sub-confluent primary cultures initiated from new conjunctival biopsies taken from a different donor.

53 new clones were analyzed by serial cultivation. 12 clones had a significant proliferative capacity and 4 clones were classified as holoclones (Fig. 7 B). Serial cultivation of these clones confirmed that conjunctival keratinocytes with high proliferative capacity give origin to a bulk of goblet cells at precise times of their life history. As with clones analyzed in Fig. 7 A, a first generation of goblet cells occurred at 45–50 cell doublings (Fig. 7 B, first peak). A second generation of goblet cells occurred very late in the life of the clones, at 10–20 doublings before senescence (second peak). Holoclones in Fig. 7 A produced ~90 cell generations, while holoclones in Fig. 7 B produced an average of 104 cell generations before senescence. In both cases, however, goblet cells were generated at 45–50 cell doublings and at ~15 cell doublings before senescence. This suggests that generation of goblet cells occurs at times precisely set for the number of cell doublings and explains the longest interval observed between the two peaks in Fig. 7 B as compared with Fig. 7 A.

The contemporary presence of transient amplifying cells with very different residual proliferative potential (see Fig. 5) explains why the overall goblet cell content of the conjunctival epithelium tends to remain constant in growing colonies of mass cultures (Fig. 6), even though the bulk of goblet cells generated by a single clone occurs only at precise times in its life history (Fig. 7, A and B; see Discussion). It is worth noting that conjunctival keratinocytes consistently generate a higher overall number of goblet cells late in their life (Fig. 7, A and B). In vivo, this is the time when clones are approaching the end of their life, are close to their postmitotic state and are therefore preparing themselves for migrating in the suprabasal layers. This might explain the strong and sudden increase in the number of goblet cells observed when growing colonies reach confluence and stratify (Fig. 6 C), and fits with the suprabasal location of goblet cells.

This set of data is summarized in Fig. 7 C and demon-

strate that forniceal and bulbar conjunctival stem cells are bipotent, since they can give rise to conjunctival keratinocytes and goblet cells; both young and old transient amplifying conjunctival keratinocytes are able to produce goblet cells; transient amplifying cells generate goblet cells at precise times of their life related to an intrinsic cell doubling clock; and the total amount of goblet cells generated by old transient amplifying cells is consistently higher than that generated by young transient amplifying cells.

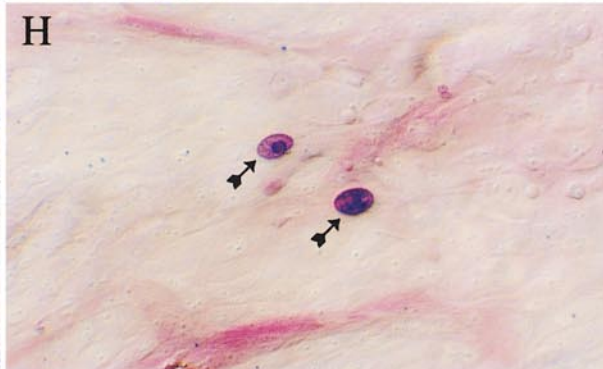
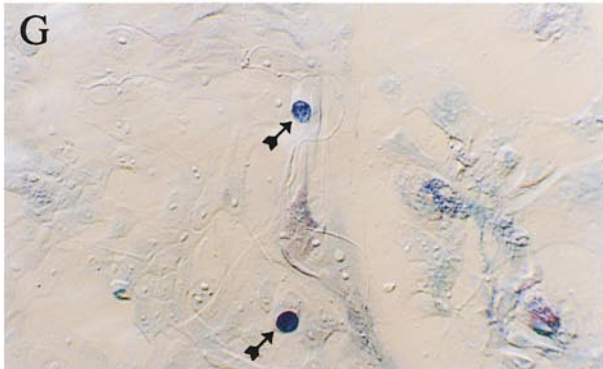
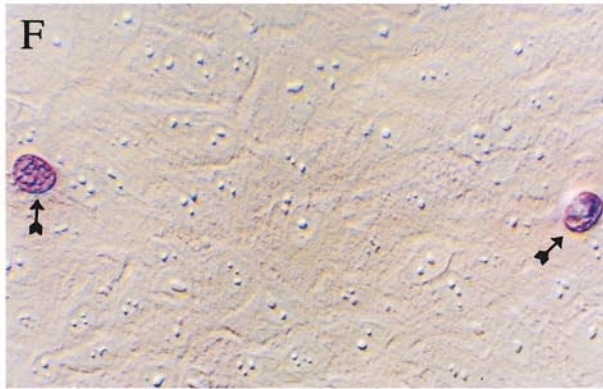
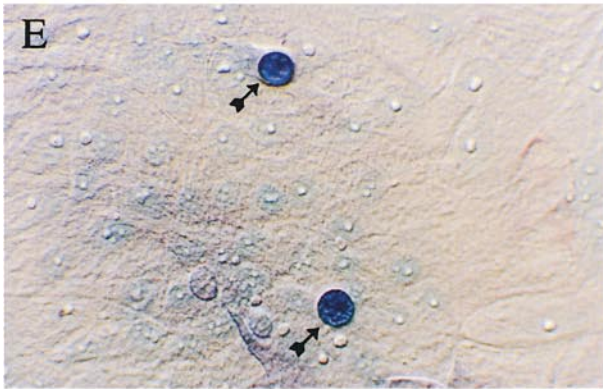
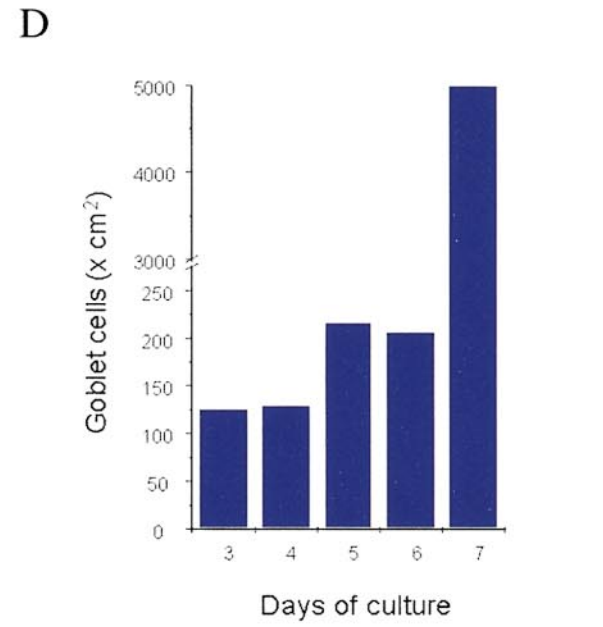
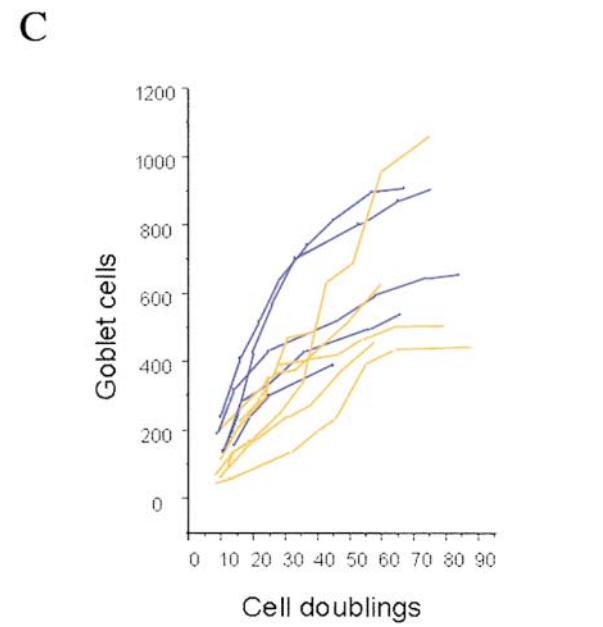
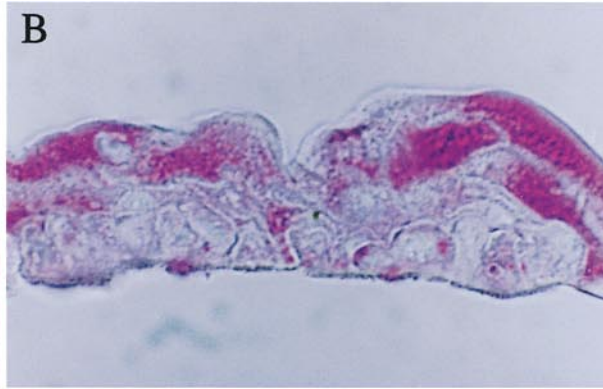
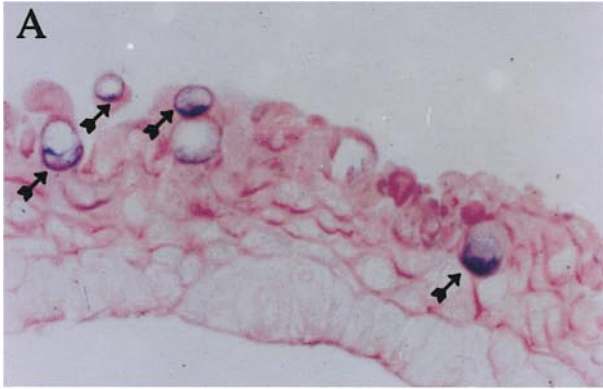
Analysis of goblet cells in selected clones began after 20–30 doublings in culture (Fig. 7, A and B), the preceding interval being devoted to the processing of the biopsy, the isolation of the clones and their growth to suitable large populations. Therefore, we cannot exclude generation of goblet cells also during the first 20–30 doublings. Also, scattered production of goblet cells can be observed between the two peaks. It has been reported that goblet cells can duplicate in vivo (Wei et al., 1995, 1997). Thus, we cannot exclude that goblet cells can undergo additional cycles of duplications (Fig. 7 C, yellow arrows and question marks).

## Discussion

### *Identification and Location of Ocular Stem and Transient Amplifying Cells*

We have identified cells with extensive capacity for cell division (holoclones) in cultured limbal, forniceal, and bulbar human ocular epithelia. One might argue that, since holoclones have been taken from their natural “niche” (Cotsarelis et al., 1989; Potten and Loeffler, 1990; Lavker et al., 1993; Rochat et al., 1994) and forced to undergo rapid proliferation, they have irreversibly lost their “stemness,” hence they should not be considered as representative of the in vivo stem cell compartment. However, permanent epithelial regeneration obtained with cultured keratinocytes in massive full-thickness burns (O’Connor et al., 1981; Gallico et al., 1984; Compton et al., 1989; De Luca et al., 1989) and in severe lining epithelial defects (Pellegrini et al., 1997) is the best available proof that stem cells are indeed preserved in culture. Recently, this was further confirmed by long-term analysis of cultured retrovirus-transduced porcine and human keratinocytes after grafting onto syngeneic or athymic animals (Deng et al., 1997; Ng et al., 1997; Kolodka et al., 1998; Levy et al., 1998). Since holoclones are endowed with the highest proliferative potential in vitro and account for the entire proliferative capacity of the original mass culture destined to transplantation, we feel quite confident in considering them as keratinocyte stem cells. Interestingly, ocular holoclones have a lower proliferative potential (80–100 doublings) than epidermal holoclones (120–160 doublings; Barrandon and Green, 1987; Rochat et al., 1994; Mathor et al., 1996). This might reflect the fact that human epidermis is renewed monthly, while the ocular epithelium is renewed every year, and suggests that holoclones can adjust their proliferative potential according to the needs of the tissue of origin.

We have shown here that corneal stem cells are segregated in the limbus, whereas conjunctival stem cells are evenly distributed in the epithelium covering the eye bulb



and the fornix. Our data fully confirm the location of corneal stem cells suggested by the slow-cycling properties (quiescence) of mouse limbal cells (Cotsarelis et al., 1989), but are in contrast with data (also based on [<sup>3</sup>H]TdR-retaining experiments) suggesting that murine conjunctival stem cells were concentrated in the fornix (Wei et al., 1995). Usually, in vivo, stem cells are slow-cycling, hence they stay in the G<sub>0</sub> phase of the cell cycle and enter in the S phase very rarely. However, at variance with the extensive capacity for cell division, quiescence is not an obligatory property of stem cells (Morrison et al., 1997). For instance, stem cells inhabiting the mouse limbus are induced to rapid division under wound healing stimuli (Lehrer et al., 1998), while stem cells of the human intestinal crypts and of the *Drosophila* ovary have been estimated to divide every 24 h, even during normal homeostasis (Potten and Loeffler, 1990; Margolis and Sprading, 1995). However, it is worth noting that in rabbits, forniceal keratinocytes have a much higher proliferative capacity in vitro than bulbar keratinocytes (Wei et al., 1993), further suggesting a segregation of stem cells in the fornix of some animals. Whether these differences between species reflect divergent mechanisms of normal tissue homeostasis or a different behavior of the epithelium in wound healing remains to be determined.

The discrete location of corneal stem cells in the limbus and the absence of cells with proliferative capacity in the central cornea, suggests that corneal epithelium is formed mostly by transient amplifying cells. This gradient of distribution of cells with different capacity for multiplication fits well with the hypothesis of a continuous centripetal migration of limbal stem cell-derived transient amplifying cells, which is governed by a circadian rhythm (Lavker et al., 1991) and is strongly increased in wound healing (Lehrer et al., 1998). It is worth noting that murine corneal cells are still able to divide (at least twice) in vivo (Lehrer et al., 1998). Therefore, it is conceivable to speculate that in order to keep the integrity of the ocular surface, human corneal cells must also undergo some rounds of division in vivo in the central region of the cornea. These transient amplifying cells are not clonogenic under our culture conditions. This strongly resembles a similar situation in the human hair follicle, where a second population of non-clonogenic transient amplifying cells has been postulated to exist in the hair bulb (Rochat et al., 1994).

### **Conjunctival Keratinocytes Give Rise to Goblet Cells**

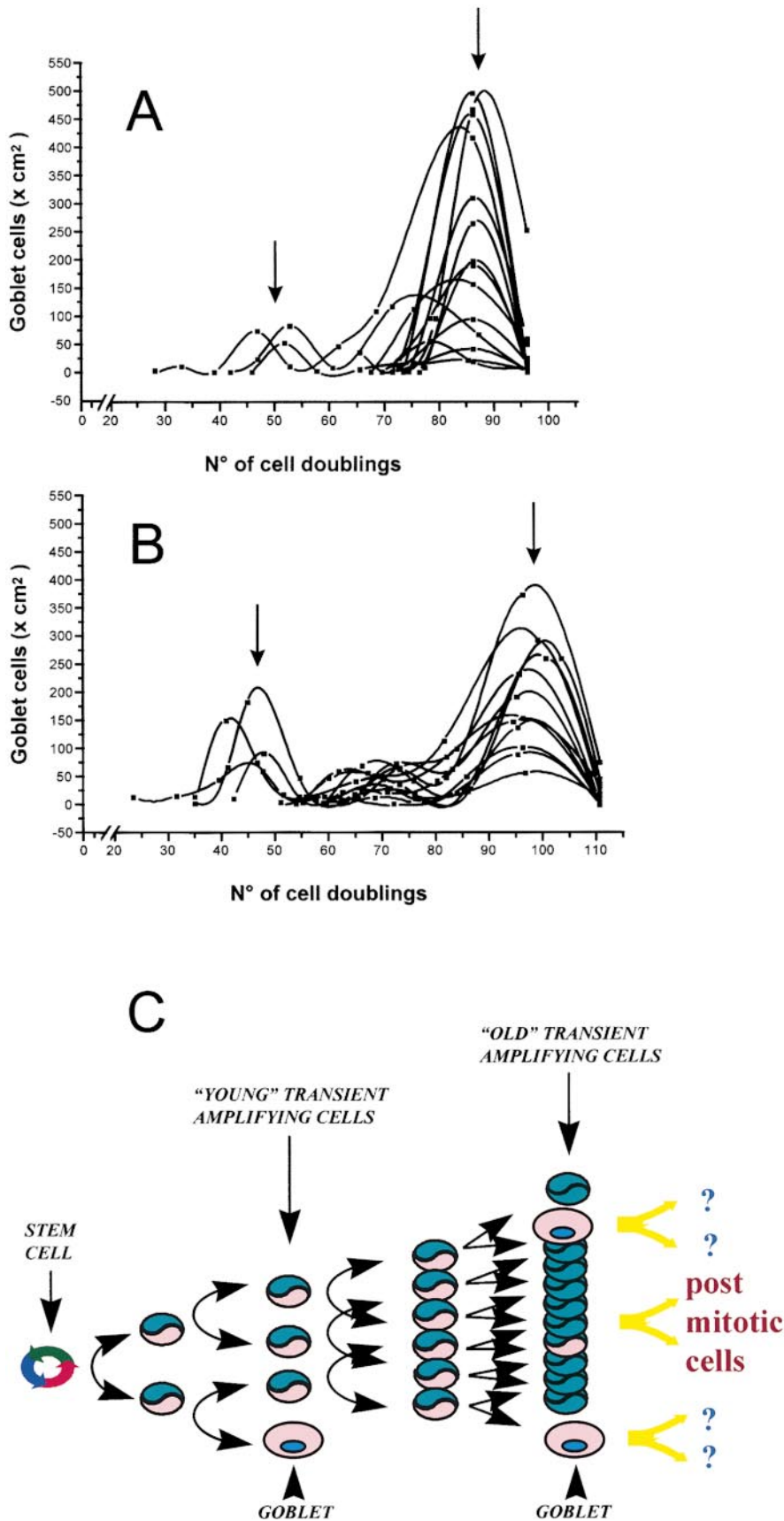
The differentiated progeny of a stem cell can be represented by a single cell type or by distinct cell types (Morrison et al., 1997). For instance, epidermal stem cells give rise to basal and subbasal keratinocytes at different level of differentiation whereas, in the hemopoietic tissue, a pluripotent stem cell can generate committed lymphoid or myeloid progenitors which, in turn, give rise to several distinct blood cell types.

The origin of conjunctival goblet cells has been controversial. Experiments by Tsai and colleagues (1997) suggested that conjunctival keratinocytes and goblet cells derive from different precursors. A first indication on the possibility of a bipotent common progenitor came from experiments by Wei et al. (1997). These authors isolated epithelial cells from the fornix of rabbits and plated them at low density onto a 3T3 feeder-layer. When they implanted primary cultures into the flanks of BALB/c mice they observed the development of epithelial cysts bearing variable amounts of goblet cells.

Our data clearly settle this controversy and confirms data by Wei et al. (1997) by showing that, indeed, clones of conjunctival keratinocytes give rise to the mucin-producing goblet cells and that both are therefore derived from a common bipotent progenitor (Fig. 7 C). It is worth noting that the differentiation of a keratinocyte into a goblet cell is more drastic than the differentiation of a basal into a suprabasal keratinocyte. It amounts to a thorough revision of cytoplasmic structure and function from a filament-rich cell whose function is to confer strength and resistance into a cell secreting proteoglycan (mucin), which conditions the surface formed by the keratinocytes. Commitment to differentiate into goblet cells occurs relatively late, so that goblet cells are preferentially generated by old transient amplifying cells. This is consistent with the suprabasal location of goblet cells in vivo, the low but reproducible number of goblet cells found in growing conjunctival cell colonies, and the spectacular increase of the number of goblet cells at a specific time of cell culture (when colonies fuse and generate a stratified epithelium). Indeed, goblet cells elicit their function in the suprabasal layers, where they secrete the mucin responsible for the formation of the tear film (Nelson and Cameron, 1997).

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*Figure 6.* Histology and PAS reaction. (A and B) PAS-staining of confluent sheets prepared from secondary cultures of bulbar (A) and limbal (B) keratinocytes. Note that several goblet cells were present in suprabasal layers of cultured conjunctival epithelium (A, at arrows), while none were present in the epithelium generated by limbal-corneal cells (B). (C) Bulbar (yellow lines) and forniceal (blue lines) keratinocytes were serially cultivated and goblet cell content was determined at each cell passage on exponentially growing colonies. Goblet cells were present during the entire life span of the cultures. (D) Forniceal keratinocytes (from a sub-confluent primary culture) were plated ( $6 \times 10^3$  cells/cm<sup>2</sup>) in parallel dishes in triplicates. EGF was added after 2 d of cultivation. Cells were trypsinized and counted every 24 h (starting from the third day after plating). Aliquots of the cell suspension were centrifuged on a coverslip and goblet cells were identified and counted (see Materials and Methods). Cells reached confluence 5–6 d after plating. Note that the number of goblet cells increased 25-fold by 1–2 d after confluence. Similar data were obtained with two forniceal and two bulbar cultures. (E–H) Clonal analysis and classification of clonal types were performed as described in Materials and Methods. An aliquot of each of the 339 clones shown in Table II was transferred to a dish and cell were cultivated as described. 3–4 d after plating cultures were fixed and PAS-stained as described. Goblet cells (arrows) are present in cultures generated from a forniceal (E) and a bulbar (F) holoclone. Goblet cells are also present in cultures generated from a forniceal (G) and a bulbar (H) meroclone. PAS-staining of the other clones revealed that 100% and 93% of holoclone- and meroclone-derived cultures, respectively, contained goblet cells. Paraclones were usually goblet negative.



*Figure 7.* Cell doubling-dependent generation of goblet cells. Quantification of goblet cells during serial cultivation of 26 conjunctival clones. 14 clones, isolated from one donor, are shown in A, and 12 clones generated from a different donor are shown in B. 7 clones were classified as holoclones and 19 clones were classified as meroclones. The x-axis indicates the number of doublings made by each clone. PAS reactions were carried out at each cell passage on exponentially growing colonies. Note that holoclones generated two peaks (arrows in A and B) of goblet cells, at 45–50 cell generations and at 10–20 doublings before senescence. Meroclones generated only one peak of goblet cells at ~15 doublings before senescence. Analysis of goblet cells began after 20–30 doublings, the preceding interval being devoted to the processing of the biopsy, the isolation of the clones and their growth to suitable large cell population. (C) Schematic description of a model for cell doubling-dependent generation of goblet cells from bipotent conjunctival stem cells. In this model we arbitrarily defined as young transient amplifying cells those cells still able to undergo 35–60 doublings, and old transient amplifying cells those cells undergoing 20–35 cell divisions before senescence. Bipotent conjunctival cells are colored in green and pink. Unipotent epithelial cells are indicated by the uniform green color. Differentiated goblet cells are colored in pink. Both young and old transient amplifying cells can generate goblet cells, at precise times of their life history.

## The Cell Doubling Clock

We show here that the rate of formation of goblet cells from conjunctival keratinocytes depends upon the number of cell doublings. In particular, we show that conjunctival keratinocytes give origin to a bulk of goblet cells at least twice in their life. A first generation of goblet cells occurs at 45–50 cell doublings, while a second generation of goblet cells occurs at ~15 doublings before senescence. The second peak is usually greater than the first peak (Fig. 7), suggesting that a young transient amplifying cell tends to generate less goblet cells than an old meroclone. Because of the heterogeneity in the clonal composition of the epithelium, this cell doubling-dependent form of differentiation would not be observable in the intact epithelium. The precision of the conjunctival cell doubling clock argues in favor of a deterministic way of generating a differentiated goblet progeny. However, as shown in Fig. 7, clones generate a highly variable number of goblet cells (sometimes the second peak is very low), even when they reach the right number of doublings, suggesting a role for probabilistic events. Whether this reflects a flexibility of fate decisions within the same cell, or the presence of distinct predetermined progenitor cells remains to be determined.

In a recent review, Morrison et al. (1997) addressed the question concerning control of stem cell differentiation and regulation of the repertoire of stem cell fate. Usually, instructive or selective actions of external factors are evoked to explain the decision of a multipotent cell to enter a particular differentiation pathway. A nice example of an instructive mechanism has been reported for the neural crest stem cell, whose differentiation is promoted by members of the TGF- $\beta$  superfamily (Shah et al., 1996). A selective action of environmental factors implies that the initial choice of differentiated fate by a multipotent cell is controlled by a cell autonomous mechanism, which, however, has never been shown in mammalian stem cells (Morrison et al., 1997).

Generation of goblet cells by conjunctival keratinocytes can also occur in serum free medium, in the absence of 3T3 feeder-layer and in the absence of EGF, cholera toxin and insulin (not shown), suggesting a cell-autonomous mechanism. Alternatively, it might be argued that instructive (non-cell-autonomous) mechanisms might arise through secretion of growth factors that induce a specific set of sister cells to differentiate into goblet cells. Future experiments will clarify these issues and will hopefully shed light on the molecular mechanisms responsible for the conjunctival cell fate decision.

## Implications for Clinical Application of Cultured Ocular Epithelia

The unambiguous identification and characterization of stem and transient amplifying cells in lining epithelia is of paramount importance for cell therapy and gene therapy (De Luca and Pellegrini, 1997). Indeed, improvements of cell culture techniques (Rheinwald and Green, 1975) allow the preparation of cohesive sheets of stratified epithelia (Green et al., 1979) suitable for autologous transplantation in patients suffering from life-threatening or highly disabling epithelial defects (O'Connor et al., 1981; Gallico et al., 1984; De Luca et al., 1989; Romagnoli et al., 1990;

Pellegrini et al., 1997, 1998 [for a comprehensive review]). It is evident that the long-term persistence of the regenerated epithelia requires engraftment of stem cells. Moreover, any attempt at using keratinocytes for gene therapy of genetic disorders (Christiano and Uitto, 1996), requires identification and stable transduction of stem cells (Mather et al., 1996; De Luca and Pellegrini, 1997; Dellambra et al., 1998).

Cultured limbal cells can generate cohesive sheets of authentic corneal epithelium, which have already been used to restore the corneal surface of patients with complete loss of the corneal-limbus epithelium (Pellegrini et al., 1997). The data presented in this paper suggest that severe bilateral destruction of the conjunctival epithelium from alkaline burns could be cured by the engraftment of cultures of autologous conjunctival cells initiated from a tiny biopsy which can be taken not only from the fornix, but from any spared area of the conjunctival surface. Goblet cell-dependent mucin deficiency has been implicated in various disorders, including ocular cicatricial pemphigoid, Stevens-Johnson syndrome, xerophthalmia, and certain sicca syndromes as a result of chronic keratoconjunctivitis. The engraftment of cultured sheets of conjunctival epithelium bearing goblet cells may accomplish for these diseases what has already been accomplished for corneal epithelium by the engraftment of limbal cultures.

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