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Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation

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SUMMARY

Using double labeling techniques, we studied the replication of corneal epithelial stem cells that reside exclusively in the limbal zone, and their progeny transit amplifying cells. We show that corneal epithelial stem cells can be induced to enter DNA synthesis by wounding and by TPA. We demonstrate the existence of a hierarchy of TA cells; those of peripheral cornea undergo at least two rounds of DNA synthesis before they become post-mitotic, whereas those of central cornea are capable of only one round of division. However, the cell cycle time of these TA cells can be shortened and the number of times these TA cells can replicate is increased in response to wounding. These results thus demonstrate three strategies of epithelial repair: (i) stem cell replication, (ii) the unleashing of additional rounds of cell proliferation that remain as an untapped reserve under normal circumstances, and (iii) enhancement of TA cell proliferation via a shortening of the cycling time.

Key words: Proliferative capacity, Proliferative hierarchy, Corneal epithelium, Hyperproliferation

INTRODUCTION

Stem cells, postulated to exist in all self-renewing tissues, serve as the reserve for cell proliferation where they maintain the balance between cell production and cell loss (Lajtha, 1979; Leblond, 1981; Miller et al., 1993a; Potten and Morris, 1988). These cells are relatively undifferentiated, slowly-cycling and have a large capacity for proliferation (for review see Miller et al., 1993a). Replication of stem cells give rise to, on average, one stem cell that remains in the stem cell environment (or 'niche') and one transit amplifying (TA) cell, which replicates relatively rapidly but has only a limited proliferative potential (for review see Potten and Loeffler, 1990). Stem cells are relevant not only in tissue homeostasis and regeneration, but also in carcinogenesis as DNA mutations in critical genes can accumulate over time in these permanent residents of a tissue (Miller et al., 1993b; Morris, 1994). In addition, stem cells represent a key target for gene therapy, as alterations in stem cell DNA can result in permanent functional changes of the epithelial population (Greenhalgh et al., 1994).

Although much has been learned about stem cells, relatively little is known about TA cells. This is unfortunate considering that the TA cells are responsible for the majority of the routine proliferative activities and are capable of extensive expansion of the cell population (Lavker and Sun, 1983; Miller et al., 1993a; Reid, 1996). A small change in the number of cell divisions that a TA cell can undergo could have dramatic effects on the final cell population. This raises the question as to whether an increase in the number of TA replications may play a role in some hyperproliferative diseases such as psoriasis (Morrison et al., 1997). It is therefore important to understand how the replication of TA cells, in addition to stem cells, is regulated.

Corneal epithelium provides a particularly useful system for studying the in vivo attributes and regulation of stem and TA cells. We and others have shown earlier that corneal epithelial stem cells are not dispersed uniformly along the basal layer of corneal epithelium. Rather, they appear to reside exclusively in the limbus (the transitional zone between cornea and conjunctiva; Cotsarelis et al., 1989; Lavker et al., 1991; Schermer et al., 1986; Tseng and Tsai, 1991; Tseng, 1996; Wei et al., 1993). Evidence supporting the limbal location of corneal epithelial stem cells includes: (1) cells that are slow-cycling, a critical attribute of stem cells, that can be detected experimentally as 'label retaining cells' (LRCs), were found exclusively in the limbal basal layer (Cotsarelis et al., 1989); (2) K3 keratin, a major differentiation product of the corneal epithelium, is not expressed in limbal basal cells but is present in the entire thickness of central corneal epithelium, indicating that limbal basal cells are relatively undifferentiated (Schermer et al., 1986); (3) under identical in vitro growth conditions, K3 keratin is not expressed by bulbar conjunctival epithelial cells but is expressed in limbal and corneal cells (Schermer et al., 1986), thus refuting the long standing hypothesis that the neighboring conjunctival epithelium can serve

as a precursor of the corneal epithelium (the transdifferentiation theory; Friedenwald, 1951; Kinoshita et al., 1983a,b; Shapiro et al., 1981; Thoft and Friend, 1977; Tsai et al., 1990; Tseng et al., 1984); (4) epithelial cells of the limbal region have a higher in vitro life span than those of the central cornea (Ebato et al., 1987, 1988; Lindberg et al., 1993; Wei et al., 1993); (5) cells of the peripheral cornea normally undergo centripetal migration toward the central cornea (Auran et al., 1995; Buck, 1985); (6) destruction of limbal basal cells has catastrophic results on the integrity and wound healing capacity of central corneal epithelium (Chen and Tseng, 1990, 1991; Huang and Tseng, 1991); (7) transplanted limbal epithelium, but not conjunctival epithelium, can effectively replace damaged corneal epithelium, in many cases resulting in dramatic clinical outcome with the restoration of corneal transparency and sight (Kenyon and Tseng, 1989; Tsai et al., 1990; Tseng and Tsai, 1991). These results provide strong support to a model in which corneal epithelial stem cells reside in the basal layer of limbal epithelium. Such cells replicate, giving rise to progeny TA cells that divide while migrating centripetally. Stem cell proliferation can thus effectively replace the central corneal epithelial cells that are continuously shed and thus lost from the corneal epithelial surface.

The fact that corneal epithelial stem cells in the limbus are well separated from their progeny cells in the cornea provides unique opportunities for the identification and characterization of the several cellular populations in the scheme of 'Stem cell → TA cell → terminally differentiated cell'. Another advantage of the limbal/corneal epithelial system, in comparison with internal epithelia such as intestinal epithelium, is its physical accessibility allowing easy manipulation and topical drug treatments.

In the present study, we have investigated the in vivo growth dynamics of stem and TA cell populations in normal corneal epithelium using double-labeling techniques that permit the detection of two or more rounds of DNA synthesis in a given cell. We demonstrate that a large number of normally slow-cycling limbal stem cells can be induced to replicate in response to a single topical application of TPA or to the physical wounding of the central corneal epithelium. In addition, we show that corneal epithelial TA cells, located in unperturbed peripheral cornea, replicate at least twice and have a relatively long cell cycle time of about 72 hours. When induced to proliferate, however, these TA cells can reduce their cell cycle time and undergo additional cell divisions. In contrast, central corneal epithelial TA cells usually divide only once prior to becoming post-mitotic even after TPA stimulation, suggesting a reduced proliferative capacity. These results, in combination with data obtained from several other epithelia including those of epidermis, hair follicle, intestine and bladder, indicate that the in vivo proliferative activity of both the stem cell and TA cell populations of an epithelium can be modulated. This multifaceted response has important physiological ramifications.

MATERIALS AND METHODS

Animals

SENCAR mice (NCI) were utilized in all our experiments. Mice were housed in a climate-controlled facility with a 12-hour dark – 12-hour light cycle, with the light period from 6 am – 6 pm. The University of Pennsylvania Animal Care and Ethics Committee approved all experimental procedures.

Detection and stimulation of slow-cycling cells

To label slow-cycling cells, we anesthetized adult (5- to 6-week-old) mice with gamma hydroxybutyric acid and two Alzet osmotic minipumps (Model 2001; Alza Corp., Palo Alto, CA), each loaded with 200 μl of BrdU, 2 mg/ml in PBS (Boehringer Mannheim Biochemica, Amsterdam), were implanted intraperitoneally. Each animal received a total dose of approximately 960 μg of BrdU per day. After continuous labeling for 7 days, pumps were surgically removed and all animals were left untreated for 6 weeks. Following this chase period, only slowly-cycling cells retained their label and were considered label-retaining cells (LRCs).

The anterior surface ocular epithelium of one group of 5 LRC-containing mice received a single topical application of 0.5% phorbol myristate (TPA) in petrolatum in both eyes. This treatment is sufficient to produce a significant increase in proliferation within 24 hours (Lavker et al., 1998b). To another group of 5 anesthetized LRC-containing mice, a central corneal wound was created by applying a 1.0 mm diameter *n*-heptanol soaked filter paper disc to the central cornea for 90 seconds followed by irrigation with PBS. This technique has been shown to result in a marked induction of the limbal and peripheral corneal epithelial cells to proliferate between 12-24 hours after wounding (Cotsarelis et al., 1989). Twenty four hours later, each of these groups of mice, as well as a third group (n=5) receiving no ocular insult, were injected intraperitoneally with 10 μCi/g of (methyl-^{3}H) thymidine (specific activity 76 Ci/mmol; New England Nuclear, Boston, MA). One hour later, mice were killed by CO_2 asphyxiation. Whole globes with surrounding conjunctiva and eyelids were then excised and fixed in 70% ethyl alcohol in 50 mM glycine and processed for immunohistochemistry and autoradiography as described below.

Visualization of multiple TA cell divisions

To demonstrate at least two divisions by a TA cell, 15 adult mice were injected intraperitoneally with 50 μg/g of BrdU. To avoid potential confounding results due to circadian rhythms (Lavker et al., 1991), all mice received the initial intraperitoneal injection of BrdU at 10:00 am. After a 24 hour chase period to ensure exit from the current S phase, the same animals were injected with 10 μCi/g of ^{3}H-TdR In an attempt to label all cells entering S phase for the next three days, each animal was subsequently injected with 10 μCi/g of ^{3}H-TdR every 12 hours thereafter. Groups of mice (n=4) were killed at 24, 48, 72, and 96 hours following the initial injection. Eyes, small intestine, and dorsal lumbar skin were then excised, fixed, and processed for immunohistochemistry and autoradiography. To explore further the role of TA cells in a naturally hyperproliferative situation, the same protocol was then applied to 3 day old and 7 day old mice (n=4).

In the present study the sequence of administration of nucleotides (i.e. BrdU/^{3}H-TdR or ^{3}H-TdR /BrdU) did not alter either the number or pattern of distribution of double-labeled nuclei that were detected in any of the epithelia studied, whether resting or perturbed. Simultaneous administration of BrdU and ^{3}H-TdR has demonstrated that the two nucleotides are equally effective at detecting cells in S phase in vitro (Qin and Willems, 1993; Hauke et al., 1995). However, it has been observed that when employed in an in vivo situation, the high dose of BrdU used to detect cells in S can competitively interfere with the smaller dose of ^{3}H-TdR (Chwalinski et al., 1988). Thus, in estimating parameters of the cell cycle, when the time between administration of the two nucleotides is short (15 minutes – 2 hours), it has been cautioned that BrdU administration should follow that of ^{3}H-TDr. This caveat does not apply when the double-label method is used to follow TA cells over longer periods of time (i.e. 1-5 days) as was the case in the present study.

Stimulation of slowly proliferative tissues

To assess the response of corneal epithelium and epidermis to perturbation, a single topical application of 0.5% phorbol myristate

(TPA) in petrolatum was applied to the skin and anterior ocular surface of 12 adult mice. To experimentally recruit TA cells in the bladder epithelium, we injected an additional cohort of 6 adult mice intraperitoneally with 100 mg/g of cyclophosphamide in PBS. This chemotherapeutic agent is well known to induce hemorrhagic cystitis and a resultant hyperproliferation of the urothelium (Reitan, 1985). Twenty-four hours following TPA or cyclophosphamide stimulation, mice were injected intraperitoneally with 50 μg/g of BrdU. After another 24 hour chase, the mice were injected with 10 μCi/g of ^{3}H-TdR Following sacrifice 60 minutes later, whole globes as well as bladder and skin biopsies were removed and processed for immunohistochemistry and autoradiography.

Establishment of three rounds of division

We examined the hair follicle and intestinal epithelia from 6 day old mice, a time at which both tissues are rapidly proliferating (Potten and Morris, 1988; Wilson et al., 1994). All mice (n=21) were injected first with 50 μg/g of BrdU and 18 hours later with 10 μCi/g of ^{3}H-TdR. Groups of mice (n=7) were then killed at the following time intervals: (i) 60 minutes after injection of ^{3}H-TdR; (ii) 18 hours after injection of ^{3}H-TdR (1st chase period which detects double-labeled cells that have divided); and (iii) 36 hours after injection of ^{3}H-TdR (2nd chase period which detects double-labeled cells that have divided three times). Small intestinal and dorsal skin biopsies were then processed as previously described.

Immunohistochemistry and autoradiography

Tissue sections (5 μm thick) were immunochemically stained with anti-BrdU antibody and alkaline phosphatase-labeled secondary IgG (both from Boehringer Manheim Corp., Indianapolis, IN), followed by visualization with red substrate (Vector Laboratories, Burlingame, CA; substrate #1). Slides were then air dried overnight.

For autoradiography, these slides were dipped in a nuclear track emulsion (Ilford K-2, Ilford, London) diluted 1:3 in distilled water at 40°C. Coated slides were air dried for 2 hours and then exposed for 14 days at 4°C in light-tight boxes containing anhydrous $CaSO_4$ (Hamond Drierite, Xenia, OH). Autoradiographs were developed as described previously (Wei et al., 1995). Slides were lightly counterstained with Gill's 2 hematoxylin.

All tissues were examined under oil immersion (×63) with a Zeiss Axiophot light microscope. A cell was considered to be double-labeled if it contained greater than 5 silver grains per nucleus over a bright nuclear pattern of red staining.

RESULTS

Corneal epithelial stem cells can be recruited to proliferate

Although it is frequently said that epithelial stem cells can proliferate following injury based on an increase in ^{3}H-TdR-incorporating cells following perturbation (Cotsarelis et al., 1989; Lavker et al., 1998b; Morris et al., 1985; Potten, 1974; Wilson et al., 1994), it is unclear whether this increase in replicating cells results from an active recruitment of stem cells or is due solely to an increase in TA cell activity. To address this issue, we tagged the slow-cycling corneal epithelial stem cells of a group of mice by first labeling the entire corneal epithelium via BrdU profusion for 7 days, followed by a chase period of 6 weeks after which only the slow cycling cells of the limbal area were found to retain the BrdU-label (Fig. 1A,B; red stain), which confirmed our earlier ^{3}H-TdR data (Cotsarelis et al., 1989). The rapidly cycling TA cells were then tagged by a single intraperitoneal injection of ^{3}H-TdR (Fig. 1A,C; dark grains). These ^{3}H-TdR-labeled TA cells

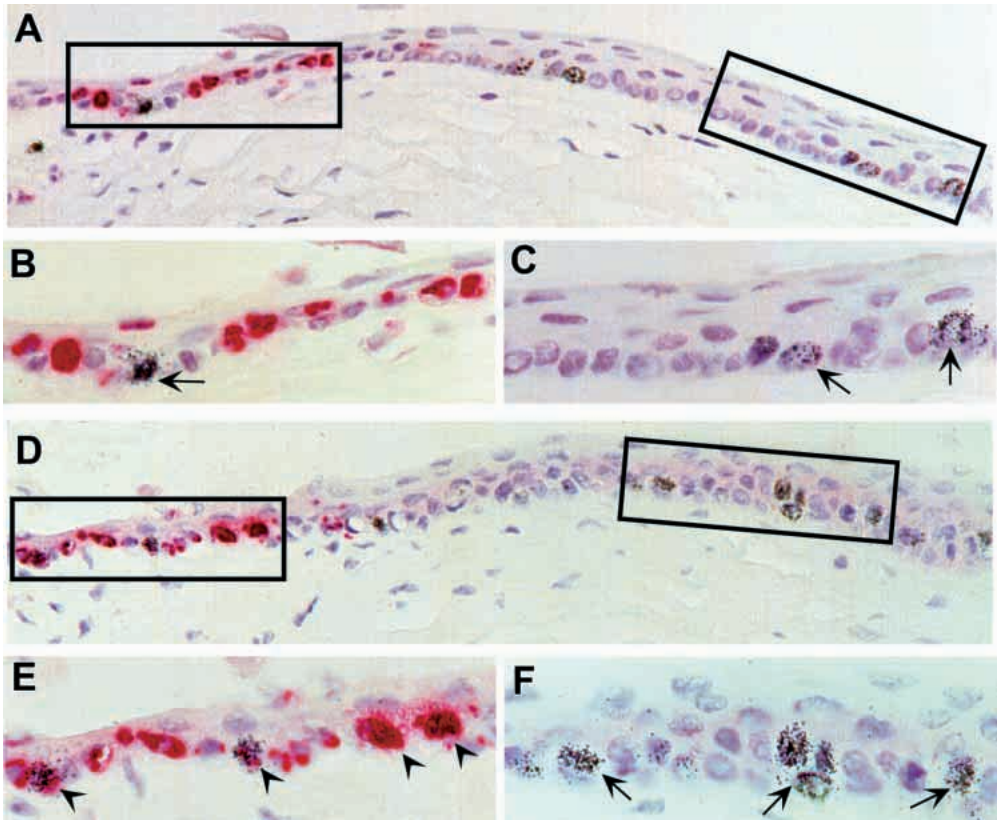

Fig. 1. Stem cells located in limbal epithelium can be rapidly induced to enter the proliferative population. Long term labeling with BrdU to detect slow cycling stem cells (LRCs; red stained nuclei) followed by a single pulse of ^{3}H-TdR to detect rapidly cycling TA cells (arrows) demonstrates that under resting conditions (A,B) all slow-cycling cells are preferentially located in the limbus, while most TA cells are located in the peripheral corneal epithelium (A,C). An occasional TA cell can also be observed among the limbal epithelial stem cells (arrow, B). Twenty-four hours following a *n*-heptanol-induced central corneal wound (D,E,F), a single pulse of ^{3}H-TdR was administered to mice that had populations of LRCs (red stained nuclei). Many of the LRCs were now double-labeled (arrowheads, E) indicating that they had incorporated ^{3}H-TdR and thus were undergoing a round of DNA synthesis. In addition, there was an increase in TA cells in the peripheral corneal epithelium (F; arrows) suggestive that this population also was induced to proliferate in response to wounding.

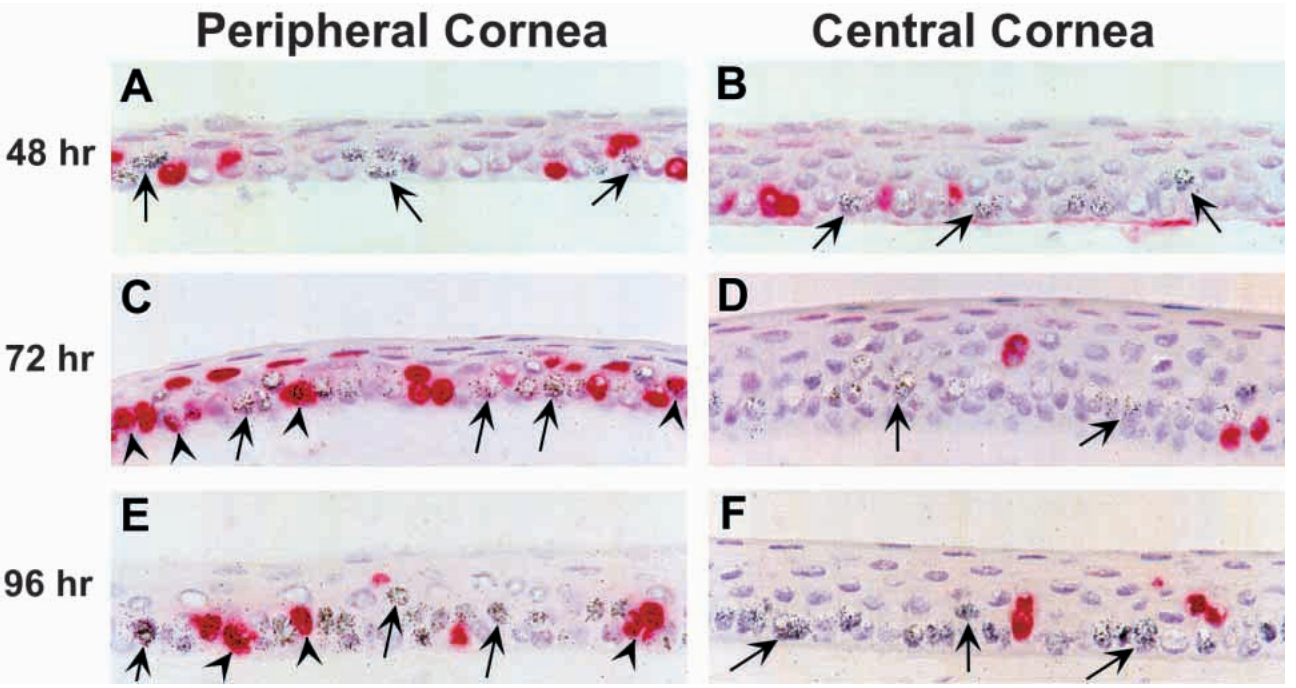

Fig. 2. TA cells capable of multiple divisions are preferentially located in the peripheral corneal epithelium. Mice received a single injection of BrdU followed 24 hours later by an injection of ^{3}H-TdR Each animal was subsequently injected with 10 μCi/g of ^{3}H-TdR every 12 hours thereafter in order to detect whether TA cells in the corneal epithelium would undergo multiple rounds of DNA synthesis. Forty-eight hours after the initial BrdU injection, only BrdU-labeled (red nuclei) or ^{3}H-TdR-labeled nuclei (arrows) were detected in peripheral (A) and central (B) corneal epithelia. Double-labeled cells were not detected in either the peripheral or central corneal epithelia at this time. Double-labeled cells (arrowheads) were observed in the peripheral corneal epithelium after 72 (C) and 96 (E) hours of continuous exposure to ^{3}H-TdR No double-labeled cells were detected in the central corneal epithelium (D,F) at these later times. This indicates that TA cells in the peripheral corneal epithelium were capable of at least two rounds of division, with a cell cycle time of 60-72 hours.

were mainly found in corneal epithelium (Fig. 1A-C) although some were interspersed with the BrdU-labeled stem cells in limbal zones (Fig. 1A,B). This provides the first direct demonstration that limbal basal epithelium is a heterogeneous population containing a mixture of stem and TA cells.

To ascertain that the long-term BrdU-labeled limbal cells are healthy and can proliferate, we created a central corneal epithelial wound using n-heptanol (Cintron et al., 1979). A ^{3}H-TdR-pulse labeling of the cells 24 hours later revealed, as expected, a large number of ^{3}H-TdR-incorporating cells

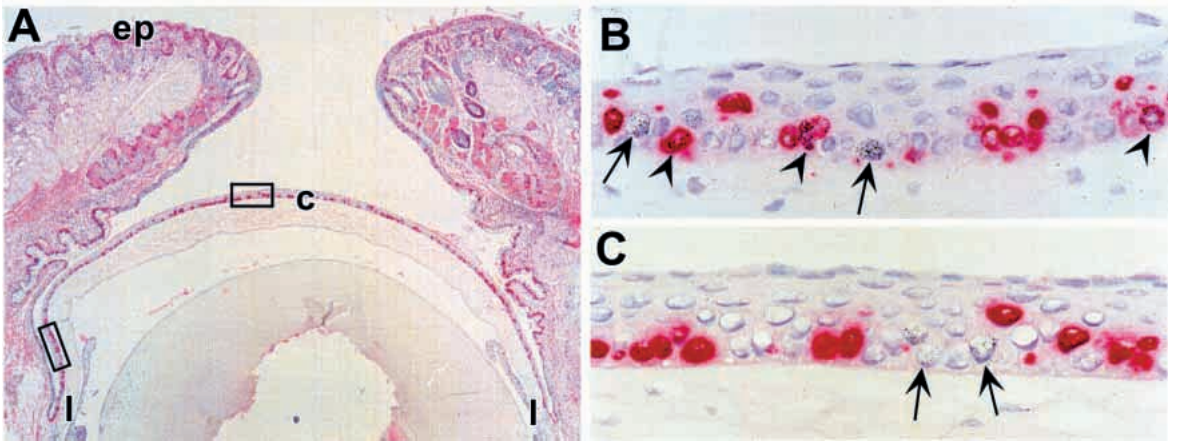

Fig. 3. Perturbation upregulates the number of TA cells capable of multiple divisions in the peripheral corneal epithelium. A single application of TPA to the ocular anterior segmental epithelia prior to double-labeling results in a marked increase in TA cells (A). Peripheral corneal epithelium (B) and central corneal epithelium (C) are higher magnification micrographs of the areas within the boxes in A. Numerous double (pulse)-labeled TA cells (arrowheads) are detected in the peripheral corneal epithelium, whereas few if any double-labeled cells are observed in the central corneal epithelium (C). In addition, the interval between nucleotides needed to detect double (pulse)-labeled cells was reduced to 24 hours indicative of a shortening of the cell cycle time. c, corneal epithelium; l, limbal epithelium; ep, epidermis.

Table 1. The proliferative response of transit amplifying (TA) cell populations within the murine corneal epithelium

	Peripheral corneal TA cells			Central corneal TA cells		
		Double-label‡			Double-label‡	
Corneal status	Single-label*	24 hours§	72 hours¶	Single-label*	24 hours§	72 hours¶
Resting	7.2±2.0	0.0	7.8±3.8	5.9±2.6	0.0	0.0
24 hours post-stimulation‖	23.5±7.2	15.0±4.7	n.d	24.7±5.0	0.0	0.0
48 hours post-stimulation‖	17.4±5.7	17.5±4.6	n.d	7.8±2.5	0.0	0.0

*Values represent the percentage of labeled nuclei per 1,000 basal nuclei and are presented as mean ± standard deviation.
‡Values represent percentage of BrdU labeled TA cells that were additionally labeled with ^{3}H-TdR.
§Detected with a 24 hour interval between nucleotides.
¶Detected with a 72 hour interval between nucleotides.
‖Topical application of 0.5% TPA in petrolatum.
n.d., not determined.

throughout the entire corneal epithelium. In addition, we found that >50% of the BrdU-labeled limbal stem cells were stimulated to incorporate ^{3}H-TdR, thus proving that these tagged stem cells were not only healthy, but also could be recruited to proliferate by wounding (Fig. 1D-F), as well as by topical application of TPA (data not shown).

Peripheral corneal epithelial TA cells can divide multiple times while those of central cornea can probably divide only once

To assess the proliferative cell cycle time of corneal epithelial TA cells, we performed two experiments. In the first, we labeled the proliferating cells by 2 consecutive pulse labels, first with BrdU and second with ^{3}H-TdR, that were 24 hours apart. Both the BrdU and ^{3}H-TdR pulses labeled 6-8% of corneal basal cells (Table 1). We did not observe any double (pulse)-labeled cells, however, indicating that cells that were tagged during the first labeling were not making DNA 24 hours later (Fig. 2A,B). In the second experiment, we pulse-labeled the cells with BrdU, followed by continuous ^{3}H-TdR labeling (starting 24 hours later through 96 hours). About 8% of the BrdU-labeled cells were additionally labeled by ^{3}H-TdR after 72 hours (Fig. 2C,D; Table 1) indicating that these cells underwent two consecutive rounds of DNA synthesis with a cell cycle time of approximately 72 hours. Almost all these double-labeled cells were located in peripheral cornea near the stem cell zone (Fig. 2C,E). In contrast, double-labeled cells were rarely detected in the central cornea, even after 96 hours of continuous ^{3}H-TdR (Fig. 2F). These results indicate that corneal epithelial TA cells comprise a proliferative hierarchy; young TA cells in the peripheral cornea normally undergo at least 2 rounds of DNA synthesis, but as cells migrate toward the central cornea (mature TA cells), their replicative capacity decreases.

Table 2. Double-labeled matrix keratinocytes increase with time in growing murine hair follicles

Time after ^{3}H-TdR Injection* (hours)	Double-labeled cells‡ (mean ± s.d.)	Silver grains per cell§ (mean ± s.d.)
1	11.6±1.5¶	53.5±11.0
18	20.4±2.8	28.6±6.8
36	36.1±4.2	15.1±2.7

*Mice were injected first with BrdU and 18 hours later with ^{3}H-TdR.
‡The matrix region from at least 50 follicles in the anagen phase of the hair growth cycle was counted for each time interval. Only those follicular matrices that were longitudinally sectioned through the center of the follicular papilla (Fig. 4H-J) were counted.
§Only those double-labeled keratinocytes located below the widest diameter of the matrix (the critical line of Auber) were counted because this is the region of the majority of proliferative activity in the follicle (Auber, 1950-1951).
¶All double-labeled matrix keratinocytes were below the critical line of Auber.

Peripheral and central TA cells are differentially regulated

To determine whether the central corneal TA cells had a proliferative reserve that we were unable to detect in the normal cornea, we stimulated mouse corneal epithelium by two topical applications of TPA and then analyzed the distribution of double (pulse)-labeled cells. Twenty-four hours after the first application of TPA, a uniform 3-fold increase in BrdU-labeled basal cells was detected over the entire corneal epithelium (Table 1), indicative of a shortening of the cell cycle time of the peripheral and central corneal TA cells. Following a second TPA treatment, 24 hours later, a continued elevation in labeling index in the peripheral corneal epithelial cells was observed (Table 1). In contrast, this second TPA treatment resulted in a decline in central corneal labeling index (Table 1), suggestive that these TA cells had little proliferative reserve. This was confirmed upon analysis of the distribution of double (pulse)-labeled cells. Few if any double (pulse)-labeled cells were observed in the central cornea (Fig. 3A,C), whereas a marked increase in double (pulse)-labeled cells was seen in the peripheral cornea (from 8% to 15%, Table 1; Fig. 3A,B). This inability to detect double (pulse)-labeled cells in the central cornea, even after stimulation, indicates that TA cells of the central corneal epithelium have little proliferative reserve and can divide only once before they become terminally differentiated.

In contrast, the increase in double (pulse)-labeled cells seen in the peripheral corneal epithelium using only a 24 hour interval between BrdU- and ^{3}H-TdR-tagging suggests that the young TA cells may be upregulated in two ways: (i) by shortening their cell cycle time from 72 hours to 24 hours; and (ii) by recruiting additional divisions in some TA cells that under normal circumstances would not utilize their full capacity to divide.

Various epithelia display proliferative reserve

To demonstrate whether the TA cells of other epithelia were capable of multiple rounds of DNA synthesis, which could be

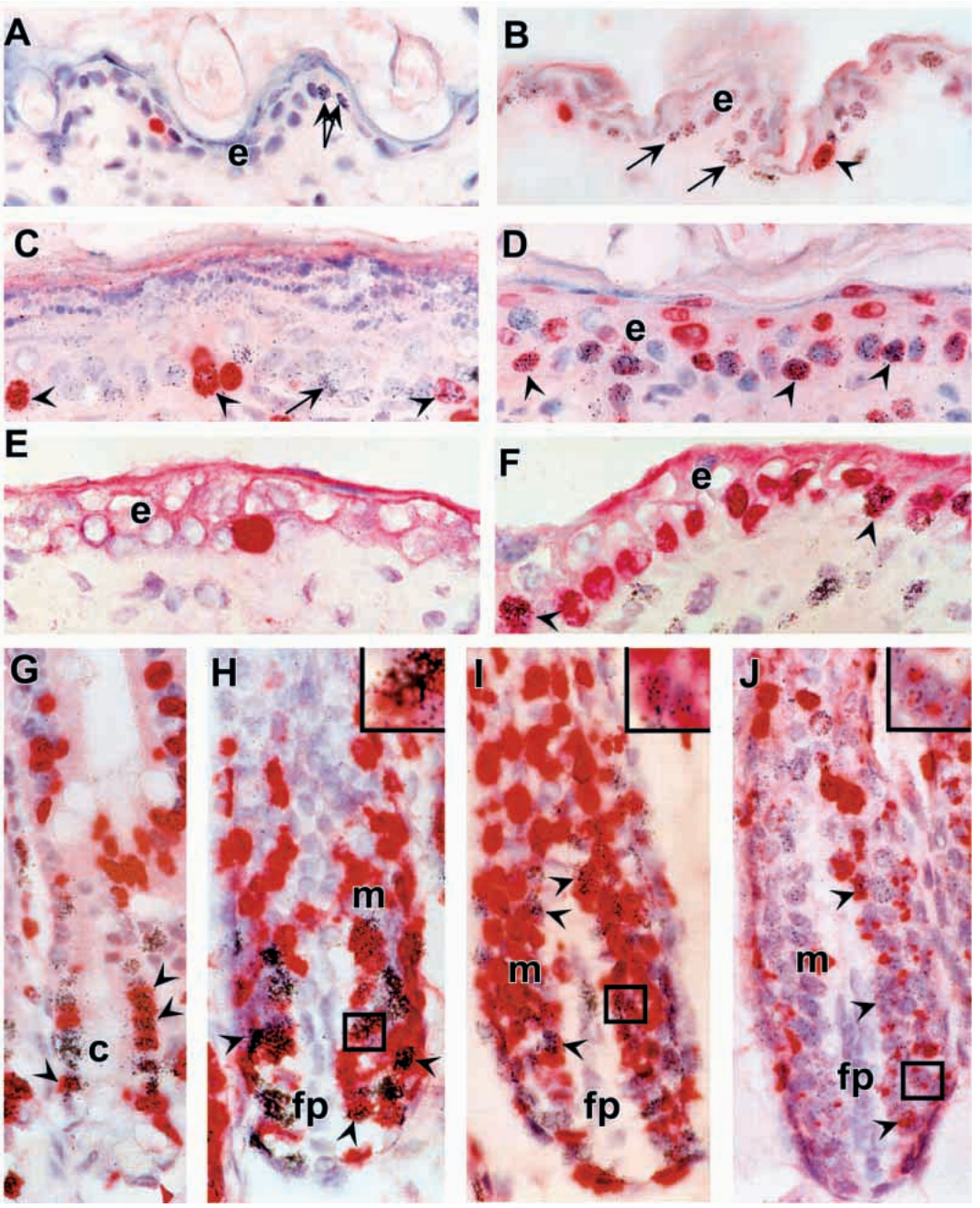

Fig. 4. TA cells of various epithelia are capable of multiple rounds of division. Double (pulse)-labeled keratinocytes are not detected in adult mouse epidermis when an intraperitoneal injection of BrdU is followed 24 hours later by a similar injection of ^{3}H-TdR (A). However, BrdU-labeled cells become double-labeled (arrowheads) after 72 hours of continuous ^{3}H-TdR (B). Arrows indicate ^{3}H-TdR-labeled nuclei. In spontaneous epidermal hyperproliferation typical for the 3 day old neonatal mouse (C), as well as experimentally induced hyperproliferation following a single application of TPA to adult skin (D), numerous double (pulse)-labeled cells (arrowheads) are seen when an intraperitoneal injection of BrdU is followed 24 hours later by a similar injection of ^{3}H-TdR. Adult bladder epithelium is normally very slowly cycling with only an occasional cell labeled after an intraperitoneal injection of BrdU (E). Twenty-four hours after cyclophosphamide treatment many TA cells underwent 2 rounds of DNA replication (arrowheads) in 24 hours (F). Intestinal epithelium (G), and hair follicles in the anagen phase of the hair growth cycle (H), are normally rapidly cycling and numerous double (pulse)-labeled epithelial cells (arrowheads) are detected with a cell cycle time of only 18 hours. Double-labeled cells are restricted to the intestinal crypt (c) and those matrix keratinocytes (m) located beneath the widest diameter of the matrix. Following an 18 hour chase (I) an increase in double-labeled cells are noted (arrowheads) throughout the matrix portion of the follicle. The decrease in silver grains per nucleus (see Table 2) indicates that these cells are the progeny of a second division (arrowheads). After a subsequent 18 hour chase (J) the double-labeled cells in the lower portion of the matrix had red-speckled nuclei containing few silver grains (arrowheads) and represent cells that had undergone a third division. Changes in the density of silver grains and BrdU stain in double-labeled cells due to multiple divisions are shown in high magnification in upper right portion of H-J. fp, follicular papilla.

regulated, we studied the cell kinetic properties of epithelia which are normally slowly proliferating (e.g. epidermis and bladder epithelium) and those which rapidly proliferate (e.g. intestinal and hair follicle epithelia). Epidermal TA cells behave very similarly to the corneal cells in that their TA cells were rarely seen to undergo consecutive DNA replication in 24 hours (Fig. 4A); some cells were double-labeled with a cycle time of 60-72 hours (Fig. 4B). However, under hyperproliferative conditions such as in neonatal skin (Fig. 4C) and after topical TPA stimulation (Fig. 4D), numerous cells can be double (pulse)-labeled with a 24 hour cycling time. Similar results were obtained with bladder epithelium that had a low labeling index of 0.01% (Fig. 4E). However, many cells underwent 2 rounds of DNA replication in 24 hours (Fig. 4F) once the epithelium was stimulated to proliferate by cyclophosphamide (Reitan, 1985).

Cells of the intestinal crypt and hair follicular matrix area are, even under normal unstimulated conditions, known to be

among the most rapidly proliferating (Van Scott et al., 1963; Potten and Loeffler, 1990). These cells frequently undergo DNA replication within an 18 hour span (Fig. 4G,H). We found that after a chase of an additional 36 hours (2 cell cycles), the number of double-labeled cells quadrupled with approximately a quarter of the silver grains per nucleus (Table 2; Fig. 4H-J), suggesting that these cells have undergone a third round of DNA replication.

DISCUSSION

Corneal epithelium as a paradigm for studying various stages of TA cells

The corneal epithelium can be divided into three compartments: limbus, peripheral cornea, and central cornea. In order to maintain ocular health by providing proper barrier function, basal keratinocytes in each of these zones proliferate, migrate, undergo stratification and terminally differentiate. While these cellular process are similar for a variety of stratified squamous epithelia, the corneal epithelium provides an excellent model to examine the regulation of cell proliferation because, in contrast to other epithelia, the stem and TA populations are compartmentalized and can be millimeters apart.

In the present study we examined further the dynamics of the stem and TA cell populations and demonstrated that the limbal epithelium contains both slow-cycling stem cells, as well as TA cells. We show for the first time that large numbers of slow-cycling stem cells can be induced to proliferate. Our data also indicate that corneal epithelium consists of a hierarchy of TA cells, with cells in the periphery capable of multiple divisions and central corneal TA cells having only one remaining division. The idea that central corneal TA cells may have very little replicative capacity is consistent with recent findings showing that cells derived from the central part of human corneas generated mostly terminal colonies and could never be subcultivated more than twice (Pellegrini et al., 1997). These colonies were similar to paraclone colonies, which represent TA cells with little proliferative reserve (Barrandon and Green, 1985, 1987). In contrast, limbal cells generated large colonies, were easily serially cultivated and behaved similarly to holoclone colonies (Pellegrini et al., 1997), which are believed to be derived from the stem cells (Barrandon and Green, 1985, 1987). These data indicate that as a cell migrates from its point of origin in a stem cell-enriched region it becomes closer to a post-mitotic state.

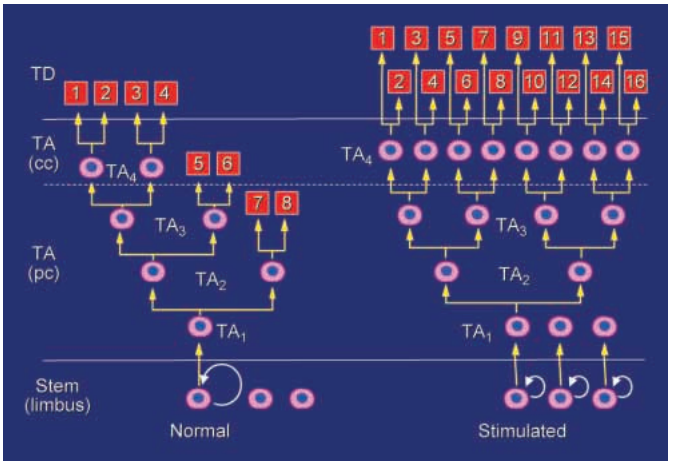

Fig. 5. Three strategies of epithelial proliferation. Using the corneal epithelium as a paradigm, this schematic summarizes the ways in which epithelial tissues can meet various proliferative demands. In the normal situation, stem cells (S) located in the limbus, cycle infrequently with a relatively long cell cycle time (large curved arrow). Upon division, stem cells give rise to regularly cycling TA cells (vertical arrows) located in the peripheral (pc) and central (cc) corneal epithelium. Young TA cells (TA1,2,3) with multiple division capacity (shown here as 3 divisions) are preferentially located in the peripheral cornea, whereas the more mature TA cells (TA4) having little proliferative reserve reside in the central cornea and may divide only once prior to becoming terminally differentiated (TD; squares). Our data suggest that under normal circumstances not every TA cell would utilize its full capacity to divide, represented by those TA cells that give rise to TD cells 5-8. The decision to become a terminally differentiated cell is stochastically determined. Upon stimulation, a self-renewing epithelial tissue can adopt three strategies to expand its cell population. It may recruit more stem cells to divide with a more rapid cell cycle time (small curved arrows) producing more TA cells. It may induce the young TA cells (TA2,3) in the peripheral cornea to exercise their full replicative potential thereby generating more (mature) TA cells (TA4). Finally, it may increase the efficiency of TA cell replication by shortening the cell cycle time (short vertical arrows). Taken together these three strategies result in the production of a large number of post-mitotic terminally differentiated cells. This model depicts an ideal case where all TA cells exercise their full potential and thus 16 terminally differentiated cells are generated per stem cell division.

A large number of epithelial stem cells can be induced to proliferate

We showed here that over 50% of the BrdU-tagged, slow-cycling stem cells could be induced to synthesize DNA after the removal of a portion of the central corneal epithelium or following topical TPA stimulation (Fig. 1D,E). Similar results were obtained with the LRCs of the epidermis as well as those in the bulge region of the follicular epithelium following TPA stimulation (data not shown). Our data therefore contradict an earlier study showing that only a small percentage (16%) of the slow-cycling murine epidermal stem cells divided 24 hours following a single application of TPA (Morris et al., 1985). This low percentage may be due to the fact that the earlier investigators counted mitotic label-retaining cells, which were present in very small numbers and thus were difficult to quantitate. Our demonstration that a large fraction of stem cells can be induced to proliferate upon environmental stimulation suggests that stem cell proliferation plays an important role in tissue regeneration.

The ability of a single stimulation to recruit a large number of stem cells to proliferate may also have a bearing on carcinogenesis. For example, in the mouse skin model of multistage carcinogenesis, covalent binding of carcinogen to DNA occurs readily, even after a single subcarcinogenic dose. However, DNA replication is required to ‘fix’ a mutation in the DNA (for review see Bowden et al., 1995). As stem cells are important targets of carcinogenic agents (Loehrke et al., 1983; Miller et al., 1993b; Morris et al., 1986; Potten and Loeffler, 1990; Stenback et al., 1981), our finding that 50% of the stem cell population can be induced to proliferate by a single

stimulus suggests that following a wound or other proliferative stimulation, large percentages of stem cells could become 'initiated' or fixed, which is a critical first step towards neoplasia.

TA cells are capable of multiple rounds of division

The double (pulse)-label technique has been employed previously in cell culture experiments and in vivo to measure parameters of the cell cycle, particularly to estimate the rate at which cells enter and exit the S phase of the cell cycle (Hyatt and Beebe, 1992) and the length of the S phase (Chwalinski et al., 1988). In the present study, we have adapted the double (pulse)-label technique to detect two or more rounds of DNA synthesis in a given cell, and have provided direct evidence that the TA cells of cornea and several other epithelia can divide at least 2-3 times. This result confirmed the prediction made by Potten and Morris (1988) who, based on mathematical modeling, postulated that a murine epidermal TA cell can divide one to three times. It should be noted that under the present experimental conditions the actual proliferative capacity of a peripheral corneal TA cell cannot be determined precisely, and estimates of 2-3 divisions are likely an underestimation.

The cell cycle time of the TA cells varies greatly from less than 18 hours to over 60 hours, depending on the normal proliferative rate of the tissue, and the physiological state of the epithelium. Thus corneal epithelial and epidermal TA cells have an average cell cycle time of over 60 hours consistent with earlier cell kinetic measurements (Brockwell et al., 1972; Potten et al., 1982). This can be shortened, however, to less than 24 hours upon TPA-stimulation (Figs 3, 4). The cell cycle time for the TA cells of the hair matrix and intestinal epithelium are less than 18 hours (Fig. 4G-J), and it seems unlikely that these times can be shortened greatly.

The number of times a TA cell can replicate can be modulated

In addition to stem cell division and a shortening of the TA cell cycle, another important mechanism by which an epithelial tissue can rapidly expand is to increase the number of times its TA cells can divide. This strategy is clearly illustrated in the behavior of the peripheral corneal epithelial TA cells. Normally only 8% of the peripheral corneal epithelial TA cells are double (pulse)-labeled indicative of two rounds of cell division. Wounding results in the detection of an additional 8% of double (pulse)-labeled TA cells (with a greatly shortened cell cycle time). This increase in double (pulse)-labeled TA cells represents the recruitment of TA cells that under normal circumstances would not utilize their full capacity to divide. This result is consistent with, and confirms a model for epidermal TA cell behavior postulated by Potten and coworkers (Loeffler et al., 1987; Potten and Loeffler, 1987). These investigators mathematically modeled the changes in proportions of single, paired and clustered cells labeled by a single pulse of ^{3}H-TdR in epidermal sheets, and proposed that from any transit generation cells could become post-mitotic.

The hair follicle matrix provides another example of the plasticity of TA cells. We have proposed earlier that cells of the bulge area of the hair follicle proliferate during early anagen, giving rise to cells of the hair bulb or matrix area (Cotsarelis et al., 1990; Wilson et al., 1994). The length of the first and second anagen in mice is approximately 12-14 days (Wilson et al., 1994), after which time cells cease proliferating and the lower two-thirds of the follicle involutes and dies, a stage in the hair growth cycle known as catagen (Lavker et al., 1998a). One possible explanation for the onset of catagen is that the matrix TA cells exhaust their proliferative capacity (Sun et al., 1991). However, this explanation may be oversimplified, since the abrogation of FGF5 gene resulted in a prolonged anagen (Hebert et al., 1994). These data show clearly that the removal of signals induced by FGF5 can unleash the unrealized proliferative potential of some of the matrix cells.

Comparing with the upregulation of stem cell growth, increasing the proliferative capacity of TA cells has the advantages of: (1) amplifying each stem cell division and minimizing the need for stem cell proliferation, (2) minimizing the chance of introducing replicative DNA errors into the stem cell population, and (3) providing new cells that are much closer to the terminally differentiated, functional cellular compartment-that covers the central cornea in the case of the limbal/corneal epithelium.

A major conclusion of this work is that a self-renewing epithelium can adopt three strategies to expand its cell population (Fig. 5), namely: (i) recruitment of the stem cells to produce more TA cells; (ii) increasing the number of times a TA cell can replicate; and/or (iii) increasing the efficiency of TA cell replication by shortening the cell cycle time.

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