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Eur. J. Cell Biol. 83 (2004); 691–700
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Telomerase activity is sufficient to bypass replicative senescence in human limbal and conjunctival but not corneal keratinocytes

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Keratinocyte; Senescence; Telomerase; Immortalization; Stem cells; Cornea

The human ocular surface is covered by the conjunctival, corneal and limbal stratified epithelia. While conjunctival stem cells are distributed in bulbar and forniceal conjunctiva, corneal stem cells are segregated in the basal layer of the limbus, which is the transitional zone between the cornea and the bulbar conjunctiva. Keratinocyte stem and transient amplifying (TA) cells when isolated in culture give rise to holoclones and paraclones, respectively. Keratinocyte replicative senescence ensues when all holoclones have generated paraclones which express high levels of $p16^{INK4a}$. In the present study, we show that enforced telomerase activity induces the bypass of replicative senescence in limbal and conjunctival keratinocytes, without the inactivation of the $p16^{INK4a}$/Rb pathway or the abrogation of p53 expression. hTERT-transduced limbal and conjunctival keratinocytes are capable to respond to both growth inhibitory and differentiation stimuli, since they undergo growth arrest in response to phorbol esters, and activate p53 upon DNA damage. Following a sustained PKC stimulation, occasional clones of $p16^{INK4a}$-negative cells emerge and resume ability to proliferate. Telomerase activity, however, is unable to induce the bypass of senescence in corneal TA keratinocytes cultured under the same conditions. These data support the notion that telomere-dependent replicative senescence is a general property of all human somatic cells, including keratinocytes, and suggest that telomerase activity is sufficient to extend the lifespan only of keratinocytes endowed with high proliferative potentials (which include stem cells), but not of TA keratinocytes.

Introduction

Human somatic cells age after a defined number of divisions in culture (Hayflick and Moorhead, 1961). Replicative senescence is triggered by the telomere shortening that occurs upon cell division (for reviews see (Wynford-Thomas, 1999; Greider, 1999; Blackburn, 2000; Sherr and DePinho, 2000; Shay and Wright, 2001; Blasco, 2003)). Telomerase, the enzyme responsible for maintaining telomere length (Greider and Blackburn, 1985), is expressed in human germinal, stem and neoplastic cells, but not in other somatic cells (Kolquist et al., 1998). Enforced expression of the catalytic subunit of telomerase (hTERT) is sufficient to induce a bypass of replicative senescence and thus to immortalize fibroblasts, mesothelial, endothelial and retinal pigment cells (Bodnar et al., 1998; Vaziri and Benchimol, 1998; Yang et al., 1999). Nevertheless, it has been shown that both $p16^{INK4a}$/Rb inactivation and telomerase activity are required for the immortalization of human keratinocytes (Kiyono et al., 1998; Jarrard et al., 1999; Dickson et al., 2000; Farwell et al., 2000; Jones et al., 2000; Stampfer et al., 2001; Rheinwald et al., 2002).

Keratinocyte stem cells govern the renewal of stratified epithelia by generating transient amplifying (TA) cells, which, after a limited number of cell divisions, stop dividing and undergo terminal differentiation (for review see (Watt and Hogan, 2000; Fuchs and Segre, 2000; Potten and Booth, 2002; Gambardella and Barrandon, 2003)). Human keratinocyte stem and TA cells when isolated in culture give rise to holoclones and paraclones, respectively (Barrandon and

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Green, 1987; Pellegrini et al., 1999a). Holoclones generate a mature epithelium when grafted onto athymic animals; holoclones, but not paraclones, possess telomerase activity and a tremendous proliferative potential such as that one holoclone can undergo an average of 160 cell divisions in culture. As the human contains only about 8×10^{10} epidermal keratinocytes, a single holoclone can in theory generate enough keratinocytes to cover 10^{30} humans. A paraclone instead is capable of a maximum of 15 divisions before undergoing growth arrest (Barrandon and Green, 1987; Rochat et al., 1994; Mathor et al., 1996; Pellegrini et al., 1999a, 2001; Dellambra et al., 2000). Holoclone-containing keratinocyte cultures are used to permanently restore life-threatening or disabling epithelial defects (Gallico et al., 1984; Pellegrini et al., 1997, 1999b; Ronfard et al., 2000; Rama et al., 2001).

Keratinocyte replicative senescence ensues when all holoclones have generated paraclones which express high levels of $p16^{INK4a}$ (Mathor et al., 1996; Dellambra et al., 2000). The 14–3-3σ isoform (Aitken, 1996) promotes keratinocyte clonal evolution. When expression of 14–3-3σ is hampered, keratinocytes are forced into the holoclone compartment and immortalize. This process correlates with the decrease of $p16^{INK4a}$ expression and the maintenance of telomerase activity (Dellambra et al., 2000).

This said, the notion that both telomerase activity and inactivation of $p16^{INK4a}$ are required for the immortalization of human keratinocytes has been challenged by reports suggesting that the supposed telomere-independent replicative senescence is rather a stress-induced senescence due to inadequate culture conditions (Ramirez et al., 2001). Notably, keratinocyte clonal evolution is accelerated under inadequate cultivation procedures. For instance, preservation of holoclones has been demonstrated in the presence of fetal calf serum and of a feeder-layer of lethally irradiated fibroblasts (Rheinwald and Green, 1975; Barrandon and Green, 1987; Rochat et al., 1994; Mathor et al., 1996; Pellegrini et al., 1999a, 2001; Dellambra et al., 2000). This prompted us to investigate whether enforced expression of hTERT is able to extend the lifespan of keratinocytes endowed with different proliferative potential and cultivated under the same conditions used for the generation of epithelial sheets destined to clinical application.

For this purpose, we used human ocular keratinocytes that provide an ideal experimental system in which to distinguish keratinocytes intrinsically endowed with different proliferative

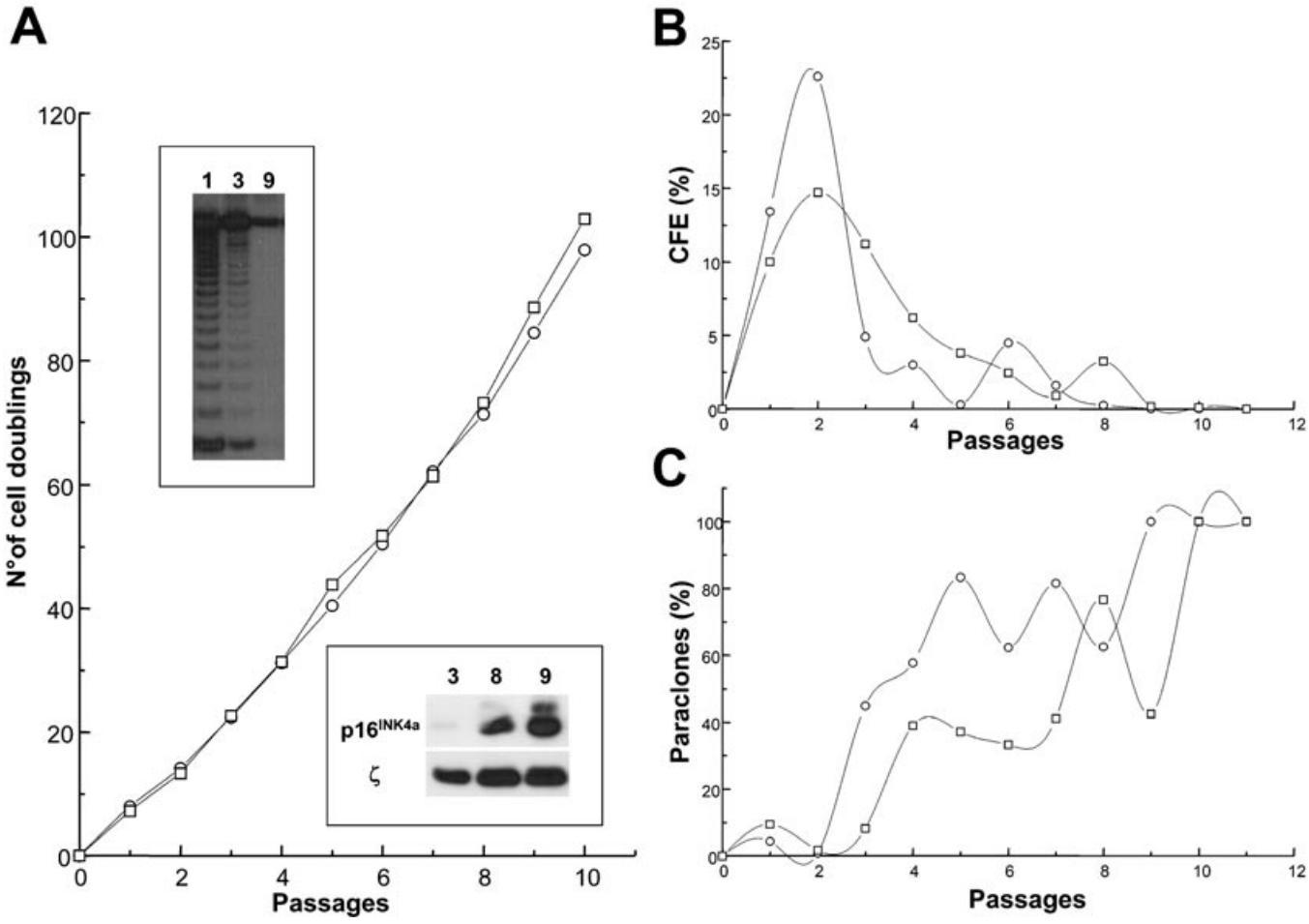

Fig. 1. Proliferative capacity of limbal keratinocytes. A: Primary limbal keratinocytes (strains L-20, circles and L29, squares) were serially cultivated and the number of cell doublings was calculated. Telomerase activity (upper inset) and $p16^{INK4A}$ expression (lower inset) were evaluated by TRAP assays and Western blot analysis, respectively, on L-20 cells at cell passages indicated by the numbers. 14–3-3ξ (ξ) was used as a loading control. Identical results were obtained in L-29 cells. B and C: Clonogenic ability (CFE, B) and percentage of aborted terminal TA cells (paraclones, C) were evaluated at each cell passages during serial cultivation of L-20 (circles) and L-29 (squares) limbal keratinocytes.

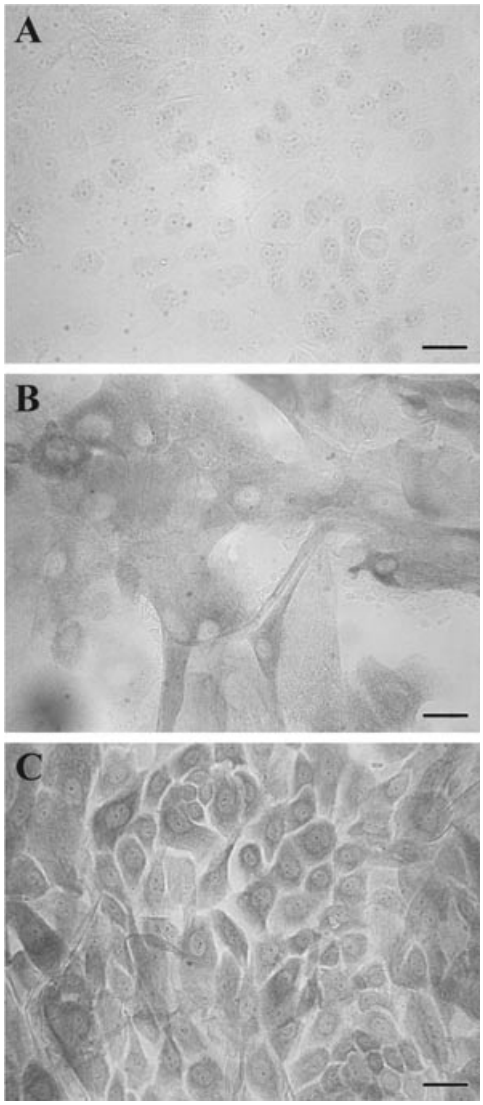

Fig. 2. Expression of $p16^{INK4a}$ in keratinocyte colonies. Colonies generated by control non-transduced limbal keratinocytes at early (A) and late (B) passages, and by hTERT-transduced limbal keratinocytes after bypass of replicative senescence (C) were stained with $p16^{INK4a}$-specific antiserum. Bars equal 30 μm.

potentials. The human ocular surface is covered by the conjunctival, corneal and limbal epithelia. All of these are stratified squamous epithelia and are formed by two phenotypically different cell types (Wei et al., 1996), referred to as conjunctival and limbal-corneal keratinocytes. Conjunctival stem cells are uniformly distributed in bulbar and forniceal conjunctiva. Corneal stem cells are segregated in the basal layer of the limbus, which is the transitional zone between the cornea and the bulbar conjunctiva. Exclusively TA cells that continuously migrate from the limbus form the corneal epithelium, and limbal stem cells can be located millimeters away from their corneal TA progeny (Schermer et al., 1986; Cotsarelis et al., 1989; Lehrer et al., 1998; Pellegrini et al., 1999a, 2001). In this context we show that the enforced expression of hTERT is capable to extend the lifespan only of keratinocyte cultures with the highest proliferative potentials.

Materials and methods

Cell culture, colony-forming assay and cell generations

Ocular biopsies presenting undamaged anterior ocular epithelium were obtained from both patients undergoing cataract and keratoconus surgery and organ donors (informed consent or permission were obtained). Keratinocytes were cultivated and serially propagated on a lethally irradiated feeder layer of 3T3-J2 cells (a gift from Prof. Howard Green), as described (Pellegrini et al., 1999a). To evaluate the clonogenic potential, 1000–2000 cells obtained from each biopsy and from each cell passage were plated, and the colonies obtained after 12 days in culture were stained with rhodamine B, scored and classified under a dissecting microscope (Barrandon and Green, 1987). Values were expressed as the ratio between the number of colonies generated and the number of inoculated cells. Cell generations were calculated using the formula: $x = 3.322 \log N/No$, where N equals the total number of cells obtained at each passage and No equals the number of clonogenic cells.

Retrovirus-mediated gene transfer and TRAP assay

LTERTSN was constructed by cloning a 3.4-kb fragment containing the full-length human TERT cDNA into the Eco RI/Eco RI sites of LXSN retroviral vector. The Am12/LTERTSN and Am12/LXSN producer cell lines were generated as previously described (Mathor et al., 1996) and showed a viral titer of 5×10^5 cfu/ml. Infections were carried out as described (Mathor et al., 1996). Briefly, sub-confluent primary keratinocytes were seeded (5×10^3 cells/cm^2) onto a feeder-layer (2.3×10^4 cells/cm^2) composed of lethally irradiated 3T3-J2 cells and producer cells (a 1:2 mixture). After 3 days of cultivation cells were collected and plated onto a regular 3T3-J2 feeder-layer. Sub-confluent cultures were used for further analysis and serial cultivation. Southern analysis was performed as described (Mathor et al., 1996). Telomerase activity was detected using the PCR-based TRAP assay (Kim and Wu, 1997).

In situ hybridization, immunocytochemistry and Western analysis

For in situ hybridization, keratinocyte colonies were fixed and permeabilized. Slides were pre-treated with pepsin (0.001%) for 4 minutes, post fixed in paraformaldehyde (PFA) 1% for 10 minutes and acetylated. Hybridization mix (60% formamide, 300 mM NaCl, 30 mM NaCitrate, 10 mM EDTA, 5% dextransulphate, 250 ng/μl salmon sperm DNA in 25 mM PBS, pH 7.4) was used for prehybridization at 52 °C for 2 hours, and for hybridization with digoxigenin-labeled hTERT antisense-riboprobe (1 ng/μl), overnight at 52 °C. Detection of labelled probe was performed using the DIG Nucleic Acid Detection kit (Boehringer).

Monoclonal antibodies against keratin type 3 (AE5) and $p14^{ARF}$ were a gift from Drs. Tung-Tien Sun and Susana Llanos, respectively. The keratin 19-specific RCK108 monoclonal antibody was from Dako Corporation. Antibodies to $p16^{INK4a}$, p53 (FL-393) and 14–3-3ξ were from Santa Cruz.

For immunocytochemistry, keratinocyte colonies were fixed for 5 minutes in 3% PFA, 2% sucrose in PBS, and permeabilized in 0.5% Triton X-100 for 5 minutes at 4 °C. Slides were incubated with anti-p16 antibody (1:100) for 30 minutes and stained with diaminobenzidine (DAB) after incubation with peroxidase-conjugated secondary antibodies (DAKO, K4000).

For immunoblots, equal amounts of protein from sub-confluent keratinocytes lysed in RIPA buffer, were electrophoresed on 12.5% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride filters (Immobilon-P, Millipore). Immunoreactions were carried as described (Dellambra et al., 2000). Immunoblots were developed with the ECL chemiluminescent reagent (Amersham).

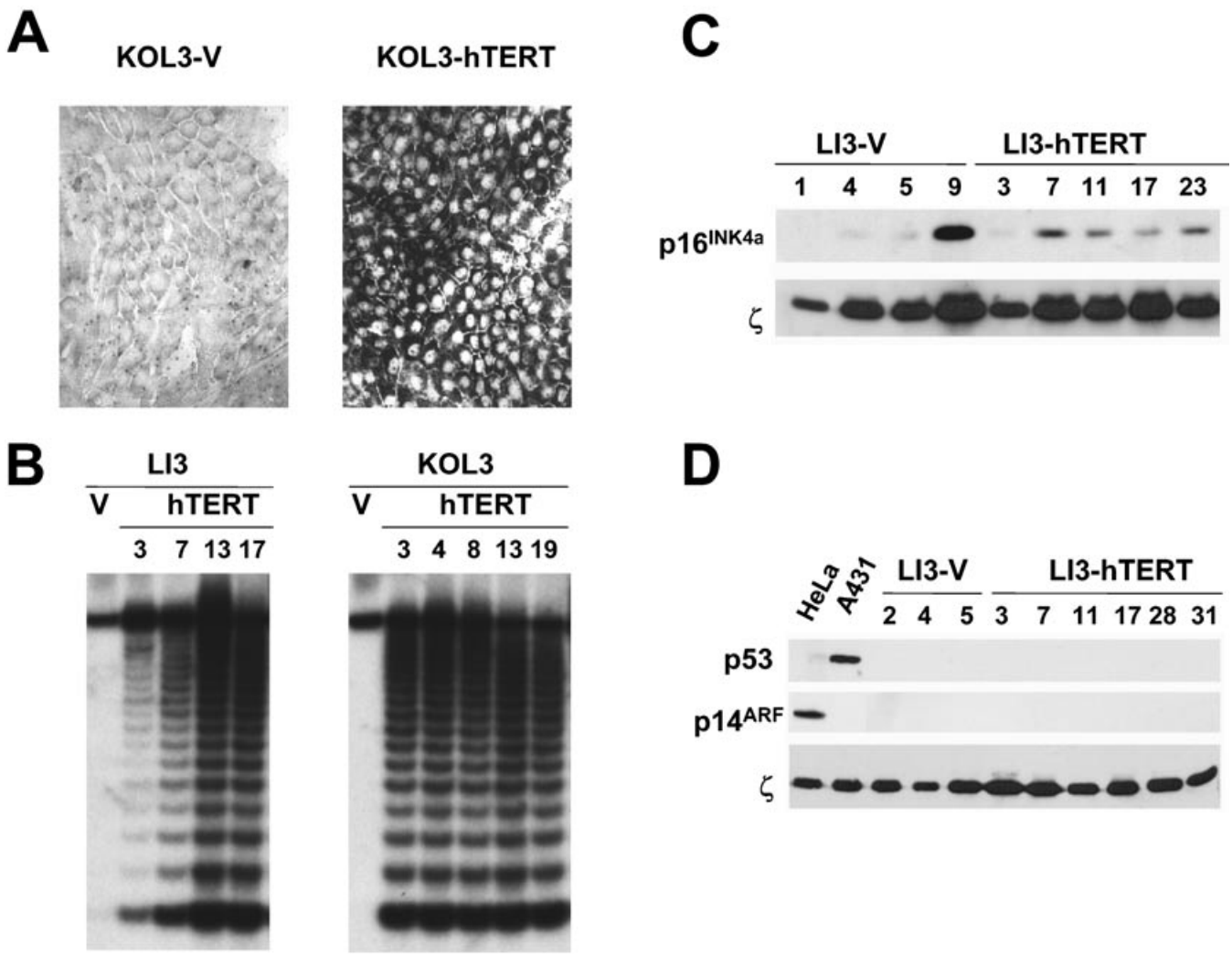

Fig. 3. hTERT transduction and $p16^{INK4A}$ expression. Infections with defective retrovirus were performed on three different strains of primary limbal (LI3, KOL3) and conjunctival (CON1) keratinocytes. A: In situ hybridization on KOL3 limbal colonies transduced with empty vectors (KOL3-V) or with retroviruses carrying a full-length hTERT cDNA (KOL3-hTERT). Cells were hybridized with a digoxigenin-labeled hTERT antisense-riboprobe. B: TRAP assay was performed on empty vector-transduced (V-lanes) and hTERT-transduced (hTERT lanes) limbal keratinocytes (strains LI3 and KOL3), at different cell passages (indicated by the numbers) after infection. Note: the difference in band intensity between the V-lanes of this panel and the lane 1 of the upper inset of Figure 1A is due to a much lower exposure time used for Figure 3B. C and D: Equal amounts of cell extracts prepared from empty vector-transduced (LI3-V) and hTERT-transduced (LI3-hTERT) LI3 limbal keratinocytes, at different cell passages (indicated by the numbers), were fractionated on 12.5% SDS-polyacrylamide gels and transferred to PVDF filters. Filters were immunostained with polyclonal antibodies to $p16^{INK4a}$ (C, p16) and 14–3-3ξ (C and D, ξ), and with monoclonal antibodies to p53 (D, p53) and $p14^{ARF}$ (D, p14). Note that $p16^{INK4A}$ was barely detectable in early-passage cells, but was consistently expressed during serial cultivation of hTERT-transduced cells that bypassed senescence. p53 and $p14^{ARF}$ were always undetectable. Cell extracts from HeLa and A431 cells (D) were used as positive controls for $p14^{ARF}$ and p53, respectively. Similar results were obtained in all strains.

Results

Ocular keratinocytes endowed with high proliferative potential exhibit high telomerase activity and low $p16^{INK4a}$ expression in culture

When serially cultivated, limbal keratinocytes underwent approximately 100 divisions before reaching senescence (Fig. 1A). Keratinocytes showed a progressive decrease of their colony-forming ability (Fig. 1B) that was accompanied by a continuous clonal evolution, as demonstrated by the progressive increase in the percentage of paraclones (Fig. 1C). 100% paraclones eventually developed, in coincidence with senescence. Similar results were obtained in 40 and 25 limbal and conjunctival strains, respectively. Telomerase activity was detected in limbal keratinocytes at early cell passages (Fig. 1A, upper inset, lanes 1 and 3), but was undetectable in cells approaching replicative senescence (Fig. 1A, upper inset, lane 9). In contrast (Fig. 1A, lower inset), $p16^{INK4a}$ was barely detectable in keratinocytes at early cell passages (lane 3), but was strongly expressed by keratinocytes approaching senescence (lanes 8 and 9). Colonies generated by early-passage limbal keratinocytes were formed mainly by small $p16^{INK4a}$-

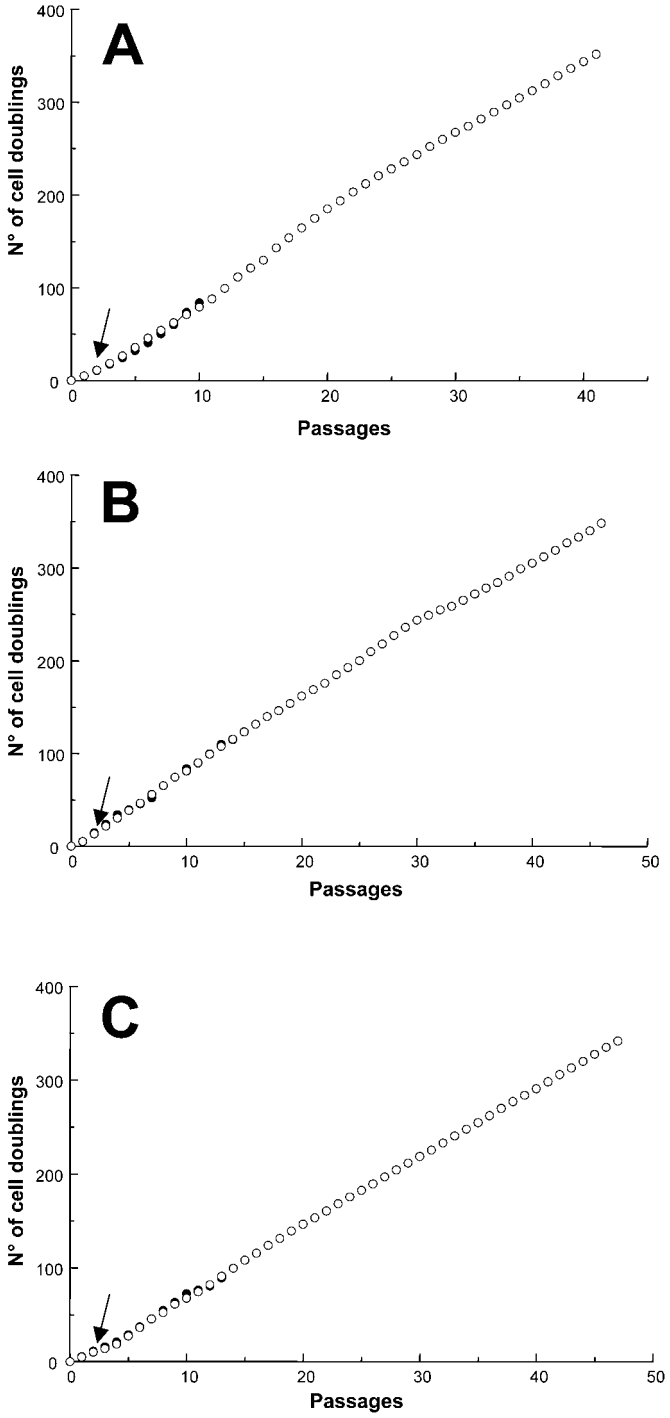

Fig. 4. Proliferative potential of hTERT-transduced cells. Primary LI3 (A), KOL3 (B) and CON1 (C) keratinocytes transduced with hTERT (open circles) or with an empty LXSN vector (filled circles) were serially cultivated and the number of cell doublings was calculated. Control cells underwent replicative senescence, while hTERT-transduced cells proliferated indefinitely. Arrows indicate keratinocyte infection.

negative cells (Fig. 2A), while colonies generated by late-passage senescent cells were formed by large $p16^{INK4a}$-positive cells (Fig. 2B) cells. We did not observe expression of p53 and $p14^{ARF}$ in aging keratinocytes (not shown). Similar results were obtained in 5 and 3 limbal and conjunctival strains, respectively.

These data confirm what we previously observed with epidermal cells (Dellambra et al., 2000): (*i*) keratinocytes endowed with high proliferative capacity possess telomerase activity, but not $p16^{INK4a}$; (*ii*) replicative senescence correlates with clonal evolution and inactivation of telomerase; (*iii*) senescence-dependent growth arrest correlates with $p16^{INK4a}$ expression.

Enforced expression of hTERT extends the lifespan of keratinocyte cultures in the presence of a normal $p16^{INK4a}$ expression

Infections with defective retrovirus carrying a full-length hTERT cDNA were performed on three different strains of primary limbal (LI3, KOL3) and conjunctival (CON1) keratinocytes. Clonogenic cells were transduced with an efficiency close to 100%. Southern analysis showed multiple bands, resulting from numerous proviral integrations in a heterogeneous transduced cell population (not shown, see also (Mathor et al., 1996)). In situ hybridization showed abundant levels of exogenous hTERT transcripts in hTERT-transduced keratinocytes (Fig. 3A, KOL3-hTERT), but not in empty vector-transduced cells (Fig. 3A, KOL3-V). Similar results were obtained in all transduced strains.

As shown in Figure 4 (A – C, filled circles), LI3 (A), KOL3 (B) and CON1 (C) cells transduced with an empty vector underwent 95, 137 and 97 cell generations before senescence, respectively. In contrast, hTERT-transduced keratinocytes bypassed replicative senescence (Fig. 4A – C, open circles) and continued to divide at the same rate of young keratinocytes. To date, LI3, KOL3 and CON1 hTERT-transduced cells underwent 351, 348 and 350 cell doublings, respectively, have been passaged 41, 46 and 47 times, respectively, and have been serially cultivated for more than one year. Bypass of senescence was not preceded by a "crisis" or by a slow growth phase (Fig. 4), suggesting that most of the transduced cells contributed to the lifespan extension.

As shown in Figure 3B (V lanes), telomerase activity was barely detectable in empty vector-transduced cells. hTERT-transduced keratinocytes maintained high levels of telomerase activity during serial cultivation and after bypass of replicative senescence (hTERT lanes). As shown in Figure 3C, $p16^{INK4a}$ was barely detectable in empty vector-transduced (LI3-V) and hTERT-tranduced (LI3-hTERT) keratinocytes at early cell passages, while it was abundantly expressed at later cell passages in empty vector-transduced cells in coincidence with the onset of cell senescence.

Notably, hTERT-transduced keratinocytes that bypassed senescence maintained the expression of $p16^{INK4a}$ (Fig. 3C, LI3-hTERT) and generated regular and smooth-bordered colonies formed by small $p16^{INK4a}$-positive cells (Fig. 2C), suggesting that hTERT-mediated lifespan extension did not require $p16^{INK4a}$ inactivation. p53 and $p14^{ARF}$ (Fig. 3D) were undetectable in control (LI3-V) and hTERT-transduced (LI3-hTERT) cells throughout the lifespan of the cultures. Similar results were obtained in all transduced strains.

hTERT-transduced keratinocytes respond to growth-inhibitory stimuli and exhibit normal differentiation properties

Protein kinase C (PKC) inhibits keratinocyte proliferation and induces the keratinocyte terminal differentiation program

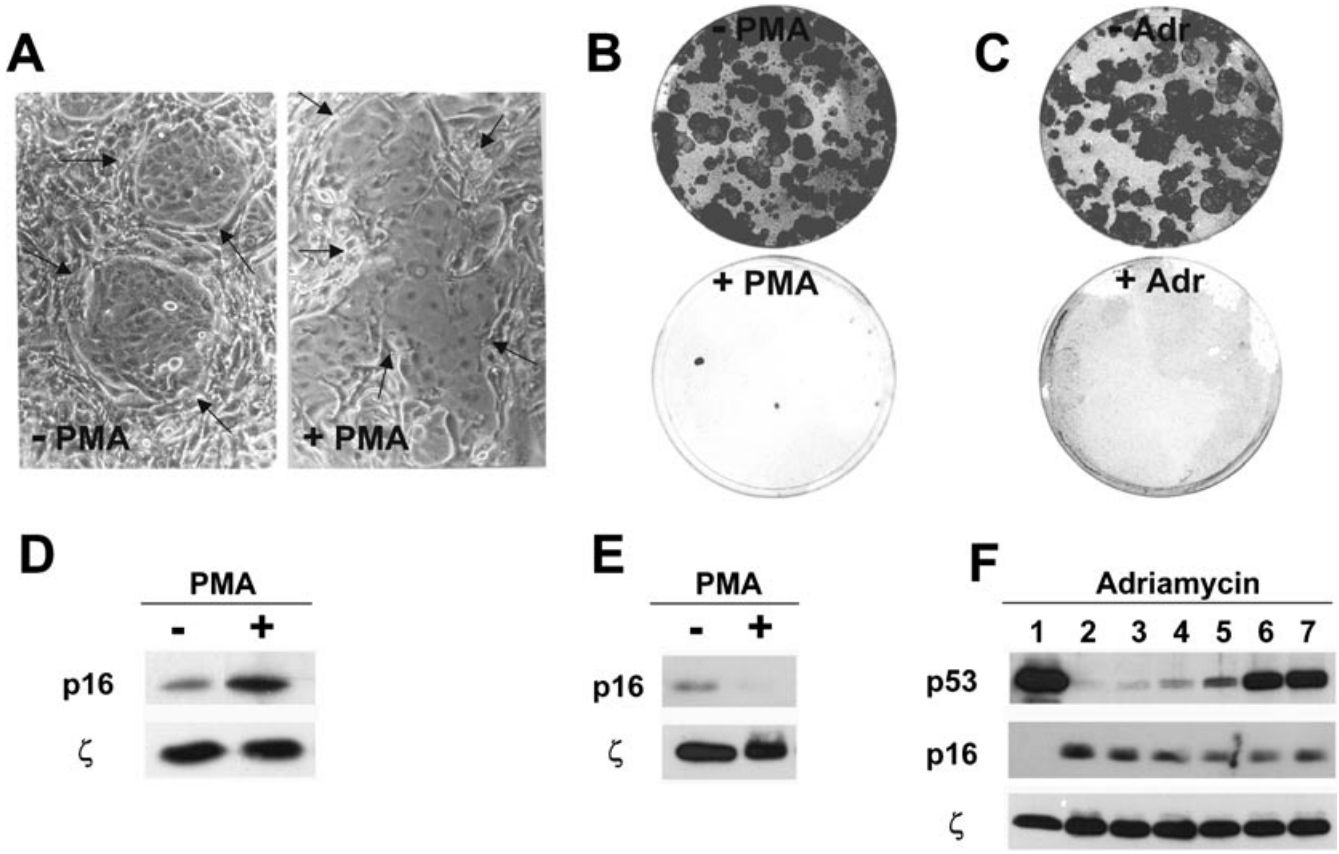

Fig. 5. PMA and adriamycin induce growth arrest in hTERT-transduced cells. hTERT-transduced limbal keratinocytes (strains KOL3 and LI3, 15 and 10 passages after bypass of senescence, respectively) were exposed to PMA (10 ng/ml) (A, B, D and E) and adriamycin (0.05–2 μg/ml) (C and F). KOL3 data are shown. A: After 4 days of PMA exposure, limbal colonies (+PMA, arrows) became irregular and were formed by flattened and enlarged cells, whilst control colonies (−PMA, arrows) were smooth and formed by small keratinocytes. B and C: Cells exposed to PMA (7 days) and to adriamycin (0.51 μg/ml, 1 day) were trypsinized and subjected to colony-forming efficiency assay (B and C, respectively). Both PMA and adriamycin treatment caused a dramatic decrease in the clonogenic ability and a complete growth arrest. D: Equal amounts of cell extracts prepared from untreated (−) and PMA-treated (+) hTERT-transduced cells were fractionated on 12.5% SDS-polyacrylamide gels, transferred to PVDF filters and immunostained with polyclonal antibodies to $p16^{INK4a}$ (p16) and 14–3-3ξ (ξ). Note that PMA-mediated growth arrest was accompanied by an increase of $p16^{INK4A}$. E: hTERT-transduced limbal cells were maintained in culture in the presence of PMA (10 ng/ml). Cells were growth arrested and could not be sub-cultivated. After 4 weeks, rare clones of proliferating cells emerged that resumed the ability to proliferate. Western blot analysis (performed as in (D), after one cell passage) showed that these cells did not express $p16^{INK4A}$ (E, +). F: hTERT-transduced keratinocytes were exposed (1 day) to 0, 0.05, 0.1, 0.2, 0.5, and 1.0 μg/ml of adriamycin (lanes 2–7). Equal amounts of cell extracts prepared from adriamycin-treated cells were then fractionated on 12.5% SDS-polyacrylamide gels, transferred to PVDF filters and immunostained with monoclonal antibodies to p53 (p53) and with antisera to $p16^{INK4a}$ (p16) and 14–3-3ξ (ξ). Note that adriamycin-mediated growth arrest was accompanied by a strong increase of p53, but not of $p16^{INK4a}$. Cell extract from HeLa cells (lane 1) was used as positive control for p53.

(Dlugosz and Yuspa, 1993). Phorbol ester-mediated stimulation of PKC accelerates clonal evolution and generation of paraclones, hence it induces keratinocyte growth arrest (our unpublished data). hTERT-transduced keratinocytes maintain the capacity to respond to PKC. As shown in Figure 5A (+PMA, arrows), hTERT-transduced limbal keratinocyte colonies exposed to phorbol-myristate-13-acetate (PMA) were irregular and formed by flattened and enlarged cells, whilst control colonies (−PMA, arrows) were smooth-bordered, regular and formed by small keratinocytes. PMA induced a dramatic decrease of the colony-forming ability (Fig. 5B) and growth arrest of hTERT-transduced limbal cells. This effect was associated to increased expression of $p16^{INK4a}$ (Fig. 5D), but not of p53 or $p14^{ARF}$ (not shown).

Notably, after approximately 4 weeks of PMA exposure, occasional clones of dividing cells emerged that resumed the ability to proliferate and could be serially cultivated in the presence of PMA. These cells did not express $p16^{INK4a}$ (Fig. 5E), confirming that inactivation of $p16^{INK4a}$ is required for continuous keratinocyte growth under certain conditions (Ramirez et al., 2001).

TGFβ1 inhibits keratinocyte proliferation and its effect is abolished in $p16^{INK4a}$-defective hTERT-transduced epithelial cells (Stampfer et al., 2001). Proliferation of hTERT-transduced keratinocytes was strongly inhibited by exposure to 30 ng/ml of recombinant TGFβ1 and this effect was accompanied by elevation of $p16^{INK4A}$, further suggesting that $p16^{INK4a}$ was functional in hTERT-transduced cells (not shown).

Escape from replicative senescence can also be achieved by a different mechanism, which involves the inactivation of p53 (Sherr and DePinho, 2000; Hanahan and Weinberg, 2000). To asses whether a p53-dependent cell cycle checkpoint was

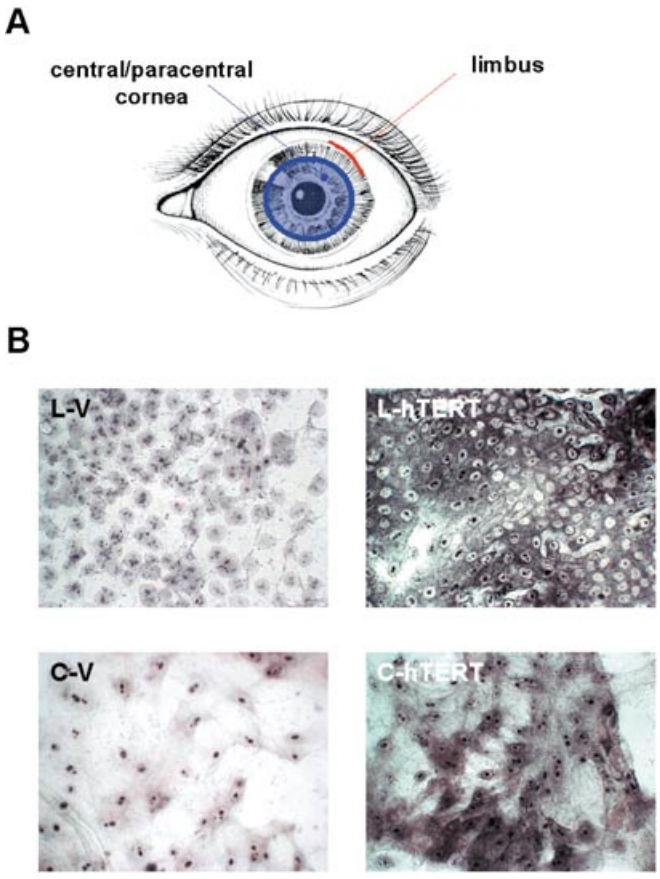

Fig. 6. hTERT expression in limbal and corneal keratinocytes. Keratinocytes were isolated from the entire central/paracentral corneal surface (C) (as indicated by the blue dashed area in (A)) and from a 1-cm limbal biopsy (L) (A, red bar) taken from the eye of 4 different organ donors (strains 76, 77, 78, 12861). Cells were cultivated for 7 days, then infected with defective retrovirus carrying an empty vector or a full-length hTERT cDNA. In situ hybridization was performed on empty vector-transduced (L78-V and C77-V) and hTERT-transduced (L78-hTERT and C77-hTERT) keratinocyte colonies 2 passages after infection. Note that exogeneous hTERT was detected both in limbal and corneal hTERT-transduced cells.

maintained in our cells, keratinocytes were treated with the DNA intercalator adriamycin. As shown in Figure 5C, adriamycin induced a dramatic decrease of the colony-forming ability and a complete growth arrest of hTERT-transduced keratinocytes. Differently from PMA, however, adriamycin-induced growth arrest was associated with a strong increase of p53, but not of $p16^{INK4a}$ (Fig. 5F).

Collectively, these data show that hTERT-transduced keratinocytes extend their lifespan in the presence of $p16^{INK4a}$, can up-regulate expression of $p16^{INK4a}$, can respond to growth inhibitory stimuli and to DNA damage by a p53-mediated growth arrest.

We did not observe significant differences between growth rates of control cells (at early cell passages) and of hTERT-transduced keratinocytes (after bypass of senescence). hTERT-transduced keratinocytes had the same growth requirements like control cells, namely they depended on feeder layer, FCS and EGF for optimal growth, and exhibited anchorage-dependent growth. Detailed G-banding performed on hTERT-transduced keratinocytes after bypass of senescence revealed that cells had the normal complement of 46 chromosomes and no obvious translocation or abnormalities (data not shown).

Keratin type 3 (K3) and keratin type 19 (K19) are expressed by limbal/corneal and conjunctival keratinocytes, respectively. Furthermore, conjunctival keratinocytes can generate goblet cells (Wei et al., 1997; Pellegrini et al., 1999a). hTERT-transduced limbal and conjunctival cells maintained the proper expression of K3 and K19, whilst hTERT-transduced conjunctival cells maintained the capability of generating normal amounts of goblet cells (not shown).

Telomerase extends the lifespan of limbal but not corneal keratinocytes

To investigate whether hTERT was able to extend the lifespan of keratinocytes endowed with different proliferative potential, hTERT transduction was performed on primary limbal and corneal keratinocytes isolated from the limbus and the central/paracentral cornea of 4 different organ donors. Given the low clonogenic ability and proliferative capacity of corneal cells (Pellegrini et al., 1999a), the entire corneal surface was used to isolate central/paracentral keratinocytes, whilst a 1 cm limbal biopsy was used to obtain limbal cells (Fig. 6A). In situ hybridization showed abundant levels of hTERT transcripts in limbal (Fig. 6B, L-hTERT) and corneal (Fig. 6B, C-hTERT) hTERT-transduced keratinocytes, but not in empty vector-transduced cells (Fig. 6B, L – V, C – V).

Cells were then serially cultivated. As shown in Figure 7, corneal cells (C-76 and C-78) transduced with empty vectors (V) or with hTERT (hTERT) showed a progressive decline of their colony-forming ability during cell passages. Similarly, limbal cells (L-76 and L-78) transduced with empty vectors (V) progressively lost their clonogenic potential. The complete loss of clonogenic ability coincided with the onset of senescence in all cases. In sharp contrast, however, hTERT-transduced limbal cells (Fig. 7, L-76 and L-78, hTERT) bypassed senescence and showed a progressive increase of their colony-forming ability, which remained constant after bypass of senescence. Similar results were obtained in all strains (not shown).

Thus, enforced expression of hTERT induced bypass of senescence in limbal but not corneal keratinocytes, suggesting that telomerase is sufficient to immortalize only human keratinocytes endowed with high proliferative potential. To confirm this observation, limbal cultures were serially cultivated and cells were transduced at early cell passages and at 1 – 2 passages before the onset of replicative senescence. This last condition mimics the residual proliferative potential possessed by corneal cells. Enforced hTERT extended indefinitely the lifespan of limbal cells at early cell passages but was unable to do so in cells approaching replicative senescence (not shown).

Discussion

Enforced telomerase activity induces immortalization of several human somatic cell types (Bodnar et al. 1998; Vaziri and Benchimol 1998; Yang et al., 1999). However, it is controversial whether telomerase is sufficient to immortalize human keratinocytes (Kiyono et al. 1998; Jarrard et al. 1999; Dickson et al. 2000; Farwell et al. 2000; Jones et al. 2000; Stampfer et al., 2001;

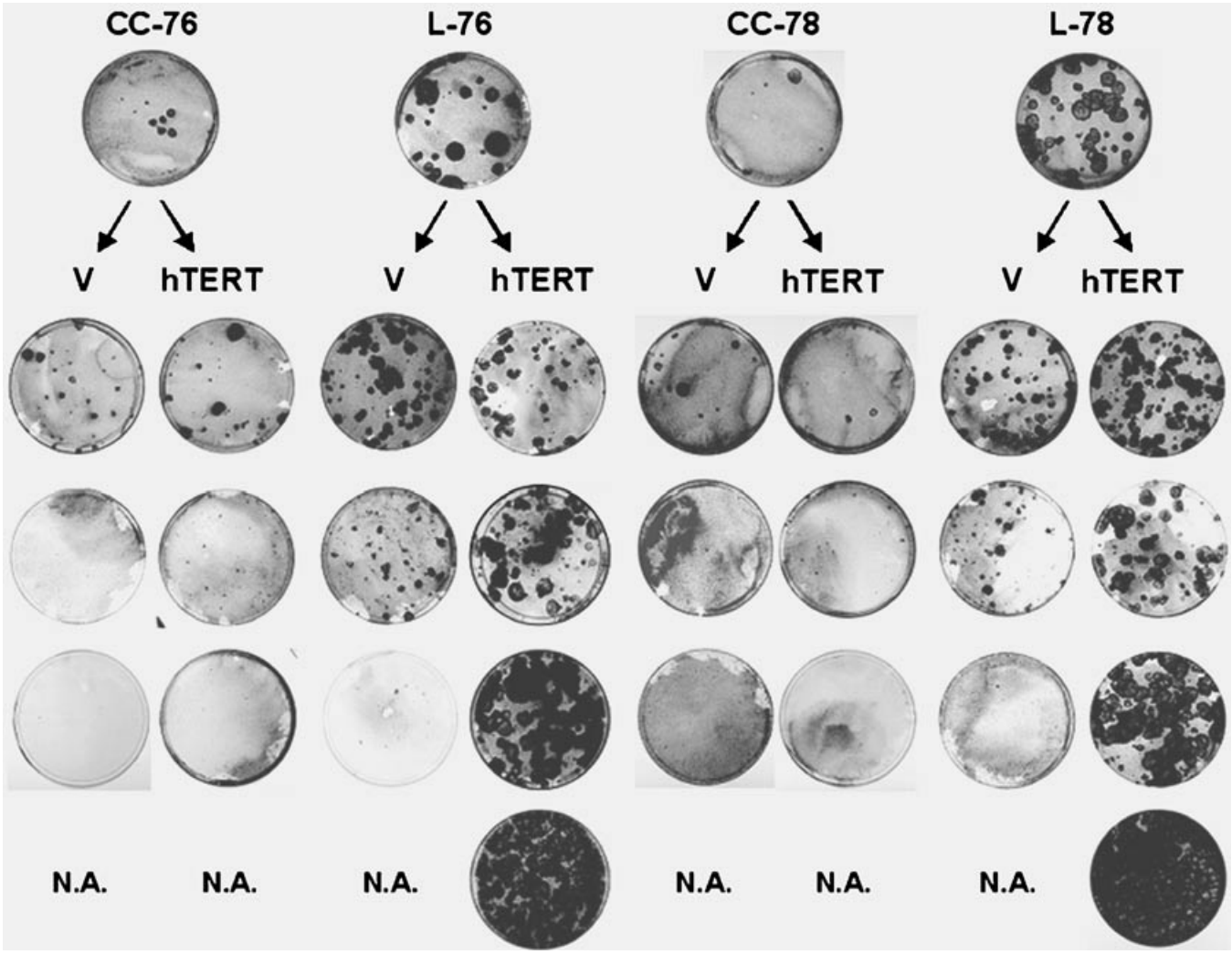

Fig. 7. Clonogenic ability of transduced limbal and corneal cells. Limbal (L76 and L78) and corneal (C76 and C78) keratinocytes transduced with an empty LXSN vector (V) or with hTERT cDNA (hTERT) were serially cultivated. Clonogenic ability was evaluated at each cell passage. Dishes were stained with rhodamine B after 12 days of cultivation. N. A. indicates 'not available' since cells reached replicative senescence. Single dishes at the top of the Figure indicate the clonogenic ability of cells isolated from the primary culture, before infection.

Ramirez et al., 2001; Rheinwald et al., 2002; Harada et al., 2003).

We show here that telomerase extended the lifespan of human limbal and conjunctival, but not corneal, keratinocytes without the inactivation of the $p16^{INK4a}$/Rb pathway or the abrogation of the p53 pathway.

That the limbus is the site of stem cell precursors of the corneal epithelium is clear for several reasons: basal limbal keratinocytes lack keratin 3 (Schermer et al., 1986), are slow-cycling (Cotsarelis et al., 1989), possess telomerase activity and an extensive proliferative capacity (Lindberg et al., 1993, Pellegrini et al., 1999a). These cells generate holoclones (Pellegrini et al., 1999a), express the p63 transcription factor (Pellegrini et al., 2001), and are capable to regenerate the limbal/corneal epithelium when grafted in patients suffering from limbal stem cell deficiency (Pellegrini et al., 1997; Tsai et al., 2000; Rama et al., 2001). In contrast, the corneal epithelium is devoid of slow-cycling cells (Cotsarelis et al., 1989) and of holoclone-forming cells (Pellegrini et al., 1999a). Cells from this region exhibit low clonogenic ability, reduced proliferative capacity (Pellegrini et al., 1999a), express keratin 3 in the basal layer (Schermer et al., 1986), while do not express p63 (Pellegrini et al., 2001). The limbus, however, contains also TA cells originated by stem cell divisions, and destined to replace the renewing corneal epithelium (Lehrer et al., 1998).

The observation that enforced hTERT expression induced the bypass of replicative senescence of limbal but not corneal keratinocytes suggests that telomerase is sufficient to extend the lifespan of keratinocytes endowed with high proliferative potential such as stem cells (and probably TA cells with high residual proliferative capability), but not of keratinocyte TA cells. Accordingly, sustained telomerase activity did not extend the lifespan of limbal cells approaching replicative senescence after serial propagation in culture. Thus, keratinocytes might reach a point of their replicative history beyond which their lifespan cannot be extended by telomerase.

Stimulation of PKC induced both growth arrest and expression of $p16^{INK4A}$ in the hTERT-transduced limbal keratinocytes. Clones of immortal $p16^{INK4A}$-negative cells emerged after prolonged exposure of hTERT-transduced limbal cells to phorbol esters. Since these cells were already immortal before PKC stimulation, it seems unlikely that the inactivation of the $p16^{INK4a}$/Rb pathway is responsible for the immortalization process. Stimulation of PKC is known to induce rapid clonal evolution and premature senescence in human keratinocytes, mimicking the stress due to inadequate keratinocyte culture conditions (Ramirez et al., 2001). Our data strengthen the notion that replicative senescence is conceptually different from premature cell senescence (Ramirez et al., 2001). Inactivation of the $p16^{INK4a}$/Rb pathway seems to be required for the bypass of stress-induced premature senescence. In our opinion, the same argument might also apply to the premature senescence induced by oncogenes, such as *Ras* (Serrano et al., 1997).

In summary, our data suggest that telomere-dependent replicative senescence may be a general property of all human somatic cells and argue against a telomere-independent mechanism for counting cell divisions in epithelial cells, at least in keratinocytes endowed with a high proliferative capacity.

Acknowledgements. This work was supported by FIRB, I.S.S., Telethon (grants B53 and GAT0201) and by the EEC (grant n° NMP2-CT-2003 – 504017). We thank Dr. Enzo Calautti for critical reading of the manuscript.

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