Biochimica et Biophysica Acta 1792 (2009) 297–308

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Tissue context-activated telomerase in human epidermis correlates with little age-dependent telomere loss

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ARTICLE INFO

Article history:
Received 27 December 2008
Received in revised form 6 February 2009
Accepted 6 February 2009
Available online 12 February 2009

Keywords:
Age-dependent telomere attrition
Telomere length heterogeneity
In situ telomere length analysis
Organotypic culture
Histone acetylation

ABSTRACT

Telomerase- and telomere length regulation in normal human tissues is still poorly understood. We show here that telomerase is expressed in the epidermis in situ independent of age but was repressed upon the passaging of keratinocytes in monolayer culture. However, when keratinocytes were grown in organotypic cultures (OTCs), telomerase was re-established, indicating that telomerase activity is not merely proliferation-associated but is regulated in a tissue context-dependent manner in human keratinocytes. While not inducible by growth factors, treatment with the histone deacetylation inhibitor FK228 restored telomerase activity in keratinocytes grown in monolayer cultures. Accordingly, CHIP analyses demonstrated an acetylated, active hTERT promoter in the epidermis in situ and in the epidermis of OTCs but a deacetylated, silenced hTERT promoter with subsequent propagation in monolayer culture suggesting that histone acetylation is part of the regulatory program to guarantee hTERT expression/telomerase activity in the epidermis. In agreement with the loss of telomerase activity, telomeres shortened during continuous propagation in monolayer culture by an average of ~70 base pairs (bp) per population doubling (pd). However, telomere erosion varied strongly between different keratinocyte strains and even between individual cells within the same culture, thereby arguing against a defined rate of telomere loss per replication cycle. In the epidermis in situ, as determined from early-passage keratinocytes and tissue sections from different age donors, we calculated a telomere loss of only ~25 bp per year. Since we determined the same rate for the non-regenerating melanocytes and dermal fibroblasts, our data suggest that in human epidermis telomerase is a protective mechanism against excessive telomere loss during the life-long regeneration.

1. Introduction

The epidermis of the skin is a constantly renewing tissue that requires tightly controlled mechanisms for maintaining tissue integrity, to provide a life-long barrier against "inside out" water loss and "outside in" attacks such as bacterial infection or damage by UV radiation. The epidermis undergoes constant regeneration allowing the elimination of damaged cells. This is achieved by proliferation of the epidermal stem cells, giving rise to the so-called transit amplifying cells, the actual proliferating cells in the basal compartment, i.e. the stratum basale. After a few rounds of proliferation these cells terminally differentiate and form multiple differentiated cell layers until they turn into dead horn squames that build the stratum corneum. Additionally, melanocytes are also distributed throughout the basal layer and are likely to play a role in protecting the stem cells and proliferating basal cells.

The protective function of telomerase activity for telomere integrity could be another potential regulator of epidermal integrity. The human telomerase is a ribonucleoprotein reverse transcriptase composed of two major components, the RNA component (hTR) which functions as a template for adding new telomeric repeats, and the catalytic subunit (hTERT). A number of additional proteins are also required for the activation and inactivation of the complex [1–3]. While being active during embryogenic development, telomerase expression is subsequently inhibited in many adult human tissues. However, expression is maintained in regenerative tissues such as the hematopoietic system and the gastrointestinal tract (for review see

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0925-4439/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.bbadis.2009.02.005

[4]). Similarly in skin, telomerase is only expressed in the regenerative epidermis while the dermal fibroblasts which only proliferate rarely, e.g. upon wounding, are telomerase-deficient [5,6]. This could imply that telomerase is regulated in a proliferation-dependent manner and would function in counteracting replication-dependent telomere loss. However, it was reported that telomere length in skin declines with age in a statistically significant manner [7–10]. Since this was also in agreement with data from telomerase knock out studies [11], it was suggested that the level of telomerase observed in normal human epidermis is insufficient and thus functionally inactive. Intriguingly, however, and despite the fact of a monthly turn-over of the epidermis, the calculated rate of yearly telomere loss was only about 25 bp thus arguing for a contribution of telomerase in telomere length maintenance.

To better address the role of telomerase in human epidermis, we have explored the consequences of telomerase activity on telomere length regulation in the epidermis in situ and in keratinocyte cultures in vitro. In agreement with our previous studies [2,5,12] we show that telomerase is not constitutively expressed but that telomerase activity can be detected when the keratinocytes have formed an epidermal tissue (in the epidermis in situ an in organotypic cultures) but is largely absent in monolayer cultures. Furthermore, we provide evidence that histone acetylation is involved in this tissue context-dependent regulation. In agreement with this differential telomerase expression, also telomere length regulation differs in the epidermis in situ and in monolayer culture arguing for telomerase as being important for the life-long replication of the keratinocytes in human skin.

2. Materials and methods

2.1. Culture conditions

Keratinocyte cultures were established from human foreskin (infant and adult) or adult trunk skin (belly, breast, or upper leg, i.e. sites generally not excessively exposed to UV radiation) – approved by the Heidelberg Ethics Commission – and routinely passaged as previously described [13]. In addition, foreskin keratinocyte cultures from newborn donors were kindly provided by D. Spandau, Dept. Dermatology, Indiana University, Indianapolis, USA. The cells were propagated on lethally irradiated human feeder fibroblasts [14] in FAD medium containing 5% FCS and antibiotics [15] or grown in keratinocyte growth medium (Epilife, Cascade Biologics, Clonetics, Walkersville, USA) and split in a ratio of 1:3 to 1:5. The telomerase-positive human HaCaT skin keratinocytes line was used as the control [16]. Dermal human skin fibroblasts were isolated from explant cultures of human skin samples and routinely cultured as described [17]. Peripheral blood leukocytes were isolated from blood by standard procedures from individuals who all gave their consent for isolating DNA and measuring their telomere length.

Growth factors (EGF, TGFα, GM-CSF, G-CSF, IL-6, KGF, all purchased from R & D GmbH, Wiesbaden, Germany) were either dissolved in DMSO or PBS and added to the culture medium at a final concentration of 10 ng/ml medium 24 h after plating. The cells were incubated for 48 and 72 h with the addition of fresh growth factors after 48 h. The deacetylation inhibitor FK228 (NSC630176) was obtained from the Developmental Therapeutics Program (DTP) of the National Cancer Institute (http://dtp.nci.nih.gov), and dissolved as a stock solution in DMSO. Cells were treated at final concentrations of 0.1, 1, 10, 100 and 250 ng/ml for 3, 6, 12, 24 and 48 h or 1 week. Control samples were treated with an equivalent amount of DMSO.

2.2. Organotypic cultures (OTC)

Organotypic cultures of normal human keratinocytes were prepared as described [18]. After 1 and 2 weeks cultures were prepared for histology or TRAP assay. Histology was performed following standard protocols. For the TRAP assay, the epithelia were separated from the collagen substrate, frozen in liquid nitrogen and processed as described in Bachor et al. [19].

2.3. Telomerase (TRAP) assays

Cell lysis of cultured cells and tissues and telomerase assay were performed using the TRAPeze kit (Intergen Company, Oxford, UK, now sold by Q-Biogene, Heidelberg, Germany). 50 ng of total protein extract was used for each assay if not specified otherwise, and specificity was verified by RNase-inactivation (RNase, DNase-free, Roche, Mannheim, Germany) for selected samples. Products were separated in non-denaturing 10% polyacrylamide gels, visualized by auto radiography and PhosphorImager scanning (Fujifilm Bas-1500), and quantified with TINA 2.0 software.

2.4. Southern blotting

Genomic DNA was prepared from peripheral blood monocytes and keratinocytes using standard protocols. Five μg of DNA was digested with RsaI (Roche, Mannheim, Germany) at 37 °C overnight and processed as described [17]. Detection was performed using a CDP-Star kit (Roche, Mannheim, Germany), image analysis with AIDA software (version 2.0, Raytest Isotopenmeßgeräte GmbH, Straubenhardt, Germany), and statistical analysis with Graph Pad Prism Version 3.00 (GraphPad Software, Inc., San Diego, CA, USA).

2.5. Quantitative fluorescence in situ hybridization

Metaphases were prepared from normal keratinocytes and HaCaT cells (control with a stable mean TRFL of 4 kb, [17]) using standard protocols [20]. Quantitative Fluorescence in situ Hybridization (Q-FISH) was performed with the Cy3-labeled telomeric PNA probe Cat.-No. K5326 (DakoCytomation, Glostrup, Denmark) as described [17]. Generally, 15 metaphases were analyzed per cell type. Signals were quantified with the Telomere Quantifier Software (DakoCytomation, Glostrup, Denmark). Telomere lengths (in kb) of the keratinocytes were calculated by mathematical equation to the 4 kb mean telomere length of the HaCaT cells.

2.6. Western blot analysis

Cell pellet preparation and further processing were performed as described [20]. Antibodies used were: anti-acetyl-H3, -acetyl-H4, or -acetyl-Lys (# 06-911, # 06-598, and # 21873, Upstate, Wolverton Mill South, UK), anti-histone H3 (ChIP Grade, ab1791, Abcam), and horseradish peroxidase-coupled secondary antibody (Dianova, Hamburg, Germany). Protein bands were detected using the enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech, Freiburg, Germany).

2.7. Chromatin precipitation (CHIP) analysis

CHIP was performed as described by Santoro and Grummt [21]. In brief, feeder cells were removed by short-term trypsin treatment, the keratinocytes were fixed with formaldehyde (1%) for 15 min at 37 °C, collected by scraping. and centrifuged. Cell pellets were resuspended in 460 μl Chip-dilution buffer (Upstate), and the DNAs were sheared and sonicated. The cell lysates were then precleared with protein A-agarose beads and incubated with anti-acetyl-histone H4 (Upstate # 06-866) antibody over night at 4 °C. The DNA–protein complexes were eluted with elution buffer (Upstate) for 2 h at 37 °C, followed by 65 °C over night. The complexes were then purified with phenol-chloroophorm, precipitated with ethanol and resuspended in ddH_2O. PCR was performed with primers: GGC CGG GCT CCC AGT GGA TTC G

(forward) and CAG CGG GGA GCG CGC GGC ATC G (reverse) for 5 min at 96 °C, 30 cycles of 30 s at 96 °C, 30 s at 58 °C, and 2 min at 68 °C and a final 6 min at 68 °C. The PCR products were fractionated on a 2% agarose gel. HaCaT cells were used as a positive controls and no antibody and histone 3 antibody (# 06-911, Upstate); not recommended for immunoprecipitation, as negative controls. To identify human keratin 16 (hK16) the following primers were used: AAA GCT GGG TGG GAA CTC TGA GCC (forward) and GGT GCC AAG GAG GGA GGT GAG C (reverse) for 5 min at 96 °C, 25 cycles of 30 s at 96 °C, 1 min at 58 °C, 1 min at 68 °C and a final 6 min at 68 °C.

2.8. RNA isolation and RT-PCR analysis

Total RNA was isolated from OCT-frozen tissue sections or from dermal equivalents using RNeasy according to manufacturer's instructions (Qiagen Hilden, Germany). One μg of total RNA from each sample was reverse transcribed to cDNA (Omniscript, Qiagen) as described in detail previously [22]. The cDNA template was used in a PCR reaction with the conditions and primers for hTERT and GAPDH as described [22].

2.9. 3D FISH analysis on skin sections

Simultaneous immunofluorescence/FISH analysis. Cells directly grown on glass slides or 5 μm skin cryostat sections were fixed in 3.7% formaldehyde for 15 min, washed, permeabilized with 0.2% Triton X-100, rinsed in ddH_2O, and air-dried. Cy3-labeled PNA probe (PNA Telomere FISH Kit K5326; DAKO A/S, Lustrum, Denmark) was added before denaturing the DNA for 3 min at 80 °C. After hybridization (2 h at 30 °C) the slides were washed at room temperature (rt) with 70% formamide/10 mM Tris–HCl, pH 7.2 (2×20 min), PBS (1 min), 0.1×SSC for 5 min at 55 °C, and PBS containing 0.05% Tween-20 (2×5 min at rt), and rinsed in ddH_2O. The slides were then shortly air dried, and incubated for 2 h at rt with an anti-keratin (DAKO Corporation, Carpinteria, CA, USA, dilution of 1:1000) and anti-vimentin antibody (Progen, Heidelberg, Germany, dilution 1:50). After washing with PBS, the slides were incubated with Cy2-conjugated anti-rabbit and AMCA-conjugated anti-guinea pig antibodies (Jackson ImmunoResearch Laboratories Inc., USA, at a dilution of 1: 50) 45 min at rt, washed with PBS, and covered with antifade solution (VectaShield, Vector Laboratories Inc., Burlingame, CA). Alternatively, telomere hybridization was counterstained with DAPI and combined with DTAF-conjugated anti-guinea pig antibody.

3D image acquisition, deconvolution microscopy, and image processing were principally performed as described [23] with a modification of the automation program (OpenLab software package) allowing deconvolution of >30 image stacks over night. Deconvolution of Cy3, Cy2, DAPI, AMCA and DTAF signals were performed individually with the same conditions for a given fluorochrome. The deconvolved images (stack of TIFF images) were further used for 3D reconstruction with Amira 3D visualization software (version 3.0, Indeed-Visual ConceptsGmbH, Berlin), and for telomeric signal intensity determination using ImageJ software (version 1.33u, Wayne Rasband, National Institutes of Health, USA).

3D reconstruction of telomeres (Cy3 emission, pseudo-colored in red) was performed by using the Amira module that computes an isosurface within a 3D scalar field. In addition, keratin (Cy2 and emission, in green), nuclei (DAPI emission, in blue), and vimentin staining (AMCA emission, in blue, or DTAF in green) were visualized with the Amira ProjectionView module; computing shadow projections of 3D images onto the three major planes (*xy*, *xz*, *yz*).

Quantification of the digitized fluorescent telomere signals was accomplished by using an ImageJ maximum Z Projection of deconvolved images. Projected telomeric signal intensities (TSI) with sizes greater than the threshold (background noise) were measured. Intensities for each telomeric spot were tabulated and exported to Microsoft Excel (Microsoft Corp.). GraphPad Prism (GraphPad Software, San Diego California, USA) was used for further data handling and statistical analysis. Statistical significance was determined using *t* test with a significance level of $p=0.05$ ($*0.01<p<0.05$, $**p<0.01$). For telomere shortening, linear regression and significance of deviation from zero were measured. A control skin sample with known TSI was included in each experiment. Analyses of the same regions on serial skin sections and co-hybridization with a centromere 7-specific PNA probe were used to confirm hybridization efficiency. All experiments were repeated at least twice.

To recalculate TSI into telomere length in kb, TSI of HaCaT, HaCaT-myc, human-, and mouse A9 fibroblasts were calculated and plotted against the TRFL values determined from the same cell populations by Southern Blot analysis. Chemilumi image exposures were digitized and transferred to Microsoft Excel, and were the basis for calculating the mean TRFL using the formula: $TRFL=\Sigma(ODi)/\Sigma(ODi/Li)$ where ODi is the integrated signal intensity at position i, and Li — the length of DNA fragment in position i. Calibration curves established this way were used for telomere lengths determination in skin sections.

2.10. Time lapse microscopy

To analyze telomere shortening on the single cell level passage 4 keratinocytes (~13 pd) were plated in keratinocyte growth medium (Dermalife, Lifeline Cell Technology, USA) in a slide flask (Nunc, Kat. Nr. 170920, Germany). After cell attachment, the flasks were tightly sealed (to keep CO_2 levels constant), and placed in the temperature-controlled incubator (37 °C) of the inverted microscope (Axiovert S100 TV, Carl Zeiss MicroImaging Inc., Germany). Live cell microscopy was performed at a low magnification (10X) and phase-contrast images (850×680 μm) were captured every 6 min for 92.8 h. At the end of monitoring, stacks of 929 images were exported to ImageJ program and used for further analysis. Each cell in this sector was labeled with a number (1,2,3….). After the each cell division, the two daughter cells were further labeled (1-1/1-2, 2-1/2-2 …). Each individual cell was followed for its next mitosis, allowing for the measurement of the number of mitoses and cell cycle times for each individual cell. After 92.8 h the cells were fixed and stained for telomeres (see above). 46 3D images of the monitored region were taken (63× objective) and the mean telomere signal intensity was measured and expressed in kb for each cell from the monitored region. Altogether 403 cells were monitored.

3. Results

3.1. Telomerase activity varies in keratinocytes from different donors

To study telomerase regulation in more detail we established keratinocyte cultures from 23 epidermal isolates (15 trunk skin and 8 foreskin samples) from different-age donors, including 5 samples from newborn foreskin, and determined their telomerase status by TRAP assay. As shown in Fig. 1A, TRAP activity in the different samples varied significantly from low to high levels with a few samples showing an activity comparable to or only slightly less than that of the immortal HaCaT skin keratinocytes, which were used as control for cells with high telomerase activity [22] (Fig. 1B). Most importantly, telomerase activity was not elevated in the newborn foreskin samples (Fig. 1C). Thus, our findings indicate that the expression level of telomerase is independent of donor age.

3.2. Telomerase activity is rapidly down-regulated in monolayer culture in vitro

To determine how proliferation influences telomerase activity, six keratinocyte strains were propagated in culture until they reached

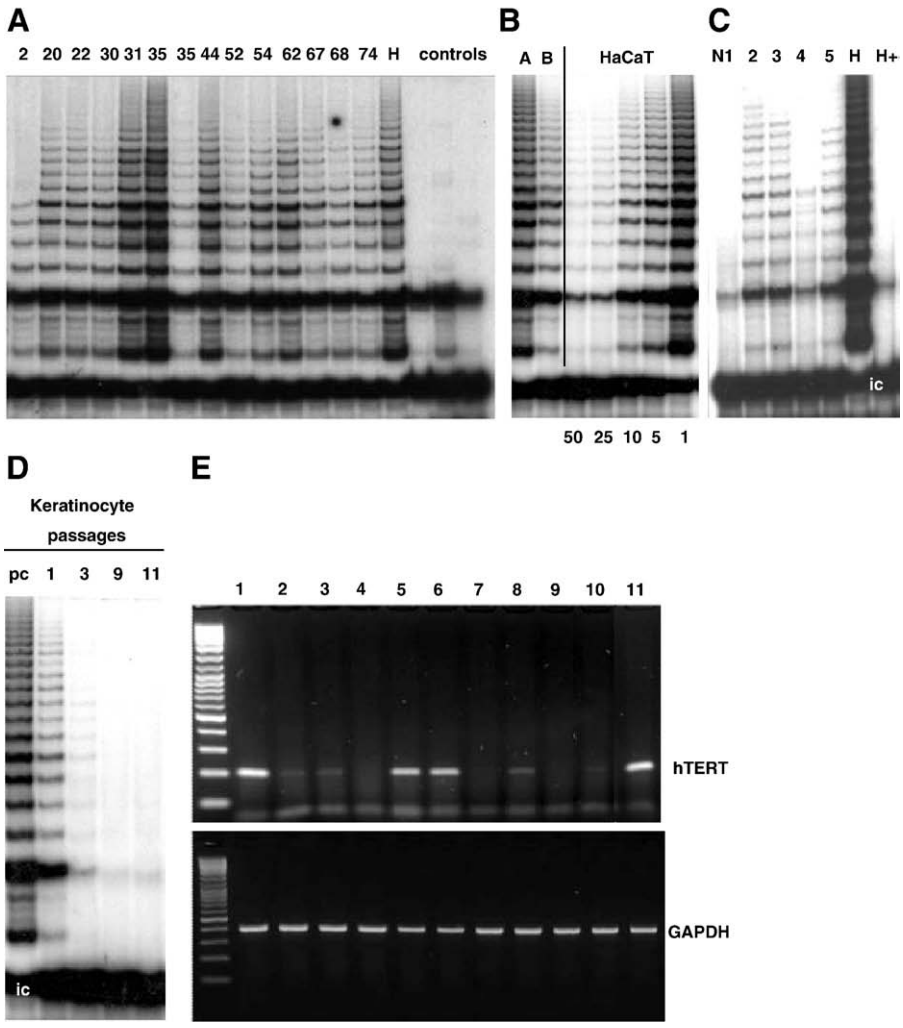

Fig. 1. Telomerase is expressed in early-passage keratinocytes but downregulated with further propagation in monolayer culture. (A) Telomerase activity was determined by TRAPeze assay in first passage keratinocytes from different-age donors all being telomerase-positive though exhibiting different levels of telomerase activity. HaCaT cells (H) serve as positive control and HaCaT cells treated with RNase, fibroblasts, and dilution buffer as negative control. (B) In a titration experiment dilutions of 1:50 (50), 1:25 (25), 1:10 (10), 1:5 (5) and undiluted (1) HaCaT extracts were compared with undiluted extracts from two normal keratinocyte strains thereby showing that telomerase levels in the normal cells can be as high as in the immortal keratinocytes. HaCaT extracts were diluted with extracts from telomerase-negative fibroblasts to guarantee similar protein concentrations. (C) Also second-passage keratinocytes from five (1 to 5) newborn (N) foreskin exhibit different levels of telomerase activity. HaCaT cells (H) serve as positive and HaCaT cell treated with RNase (H+) as negative control. (D) During further propagation in monolayer culture telomerase activity declines with increasing passage number (pc = primary culture, 1, 3, 9, and 11 = passage number). (E) Only freshly isolated or primary culture keratinocytes exhibit good levels of hTERT expression (RT-PCR). Expression of GAPDH is used as a loading control. Data from two individual keratinocyte strains are shown. The first represents: freshly isolated cells (1) and passage 3 (2), 5 (3), and 7 cells (4), the second one: intact epidermis (5) freshly isolated keratinocytes (6) and cells from passage 3 (7) primary culture (8) and passages 2 (9) and 4 (10). RNA from HaCaT cells (11) served as a positive control.

terminal growth arrest i.e. cellular senescence. In four of these strains which primarily exhibited high levels, telomerase activity declined within ~10 pd (Fig. 1D). In the other two strains that only exhibited low levels in early passage cultures, telomerase activity became undetectable even more rapidly (data not shown).

To determine how the culture-dependent telomerase inhibition was regulated, we next investigated hTERT expression by RT-PCR and found that hTERT mRNA was strongly reduced (Fig. 1E). This suggested that telomerase activity was inhibited in monolayer cultures by transcriptional silencing of the hTERT gene.

3.3. Telomerase activity cannot be restored by conventional culture conditions

Despite telomerase inhibition, all keratinocytes continued to proliferate in monolayer cultures for another 20 to 30 population doublings (pd), demonstrating that proliferation *per se* was not causal for telomerase expression. However, it is well established that keratinocyte growth and differentiation in the epidermis in situ is promoted through specific growth factors induced upon mutual interaction of the keratinocytes with the underlying dermal fibroblasts [24–26]. One of these factors is EGF which has been shown earlier to activate hTERT expression upon binding to the *ets* binding site in the hTERT promoter [27]. Interestingly, neither co-cultivation of the keratinocytes with fibroblast feeder cells [14], nor propagating them in keratinocyte growth medium containing a set of growth factors (including EGF) was able to maintain telomerase activity. To exclude that specific factors were missing or were present in insufficient amounts, we further propagated three keratinocyte strains in the presence of 10 mM/ml EGF/TGFα, IL-6, G-CSF, GM-CSF, and KGF, applied either individually or in combination. Cells were harvested after 48 h and 72 h and assayed for telomerase expression analyses but none of these factors was able to re-stimulate telomerase expression in monolayer cultures (Fig. 2A).

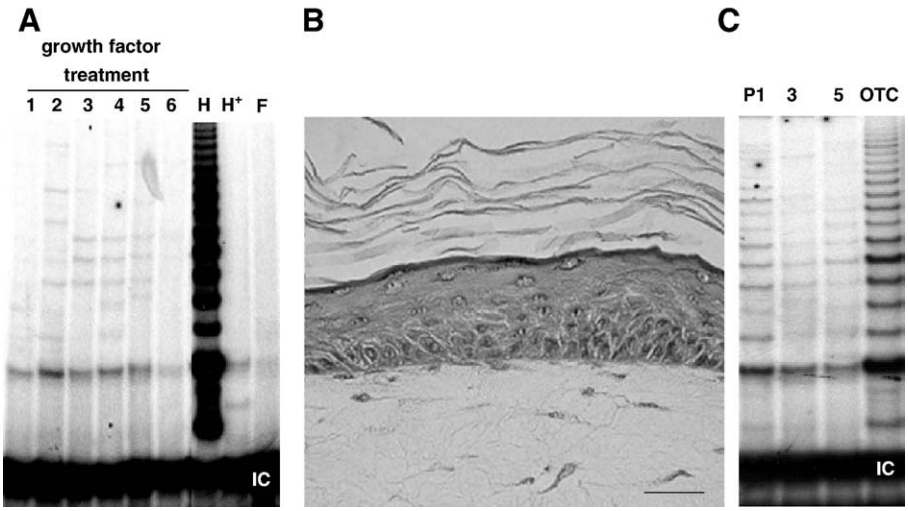

Fig. 2. Telomerase is re-expressed in culture when the keratinocytes are allowed to form an epidermis-like epithelium. (A) Growth factors cannot overcome the culture-dependent telomerase inhibition. TRAPeze detection of telomerase activity 48 h after growth factor treatment. 1 = control, 2 = EGF/TGFα, 3 = IL-6, 4 = GM-CSF, 5 = G-CSF, 6 = KGF. HaCaT cells (H) are used as a positive control and HaCaT cell lysates treated with RNase (H+), telomerase-negative human skin fibroblasts (F) as negative controls. IC = internal 36 bp standard. (B) Keratinocytes were grown in organotypic cultures (on a collagen gel with integrated fibroblasts and air exposed). H & E staining of a two-week-old culture showing a stratified and well-differentiated epidermis. (C) TRAPeze detection of telomerase activity in a keratinocyte strain that was propagated in conventional cultures and analyzed at passages (p) 1, 3, and 5, shows a clear decline in telomerase activity. 3rd passage keratinocytes from this strain were grown in OTCs and re-express increased levels of telomerase activity. IC = internal 36 bp standard; bar = 30 μm.

3.4. Telomerase is regulated in a tissue context-dependent manner in the human keratinocytes

One major difference between the co-culture of keratinocytes with feeder fibroblasts and growth of the keratinocytes in the epidermis in situ is their three-dimensional organization within the tissue. In addition to the multilayered nature of the epidermis, the basal cells, tightly connected to the basement membrane, exhibit a cuboidal morphology as compared to their largely flattened cell shape observed in monolayer culture. This determines cell polarity and most likely an altered sensitivity towards activating or inhibiting factors provided by the dermal fibroblasts.

To address the role of tissue organization, we performed organotypic cultures (OTCs) with 2nd and 4th-passage keratinocytes being plated on a dermal equivalent that consisted of a collagen type I gel containing human dermal fibroblasts. Within two weeks the keratinocytes formed a three-dimensional tissue with all the characteristics of a stratified epidermis (Fig. 2B). Most importantly, telomerase activity was absent or only faintly expressed in monolayer cultures but was clearly detectable in keratinocytes grown in the OTCs (Fig. 2C). This result was confirmed with a second independent keratinocyte strain (data not shown).

3.5. Histone acetylation/deacetylation is part of the regulatory program of telomerase activity in human skin keratinocytes

One possible explanation for the absence of hTERT expression after addition of e.g. EGF in monolayer cultures could be that the hTERT promoter might not be accessible. Epigenetic modulation, i.e. DNA CpG-methylation and histone acetylation, known to modulate the chromatin state, have previously been show to modify hTERT/telomerase expression [28]. Furthermore, histone deacetylation inhibitors reactivated telomerase in a number of cell types [29–32].

Therefore, we treated the keratinocytes in monolayer cultures with the histone deacetylase inhibitor FK228 (now known as romidepsin) (for reviews see [33,34]) and measured TRAP activity after 3, 6, 12, and 24 h. As shown in Fig. 3A, telomerase activity recurred in a time-dependent manner with maximal activity at 24 h (Fig. 3A). Neither increasing concentrations of FK228 (here shown for 250 ng/ml) nor combining FK228 with EGF did enhance the level of telomerase activity further (data not shown), suggesting that at 100 ng/ml of FK228 was sufficient to completely inhibit deacetylation and thus maximally stimulate telomerase activity in the keratinocytes. Interestingly, repeated treatment with 100 ng/ml FK228 – in order to maintain telomerase activity for long-term growth in monolayer cultures – caused differentiation of the cells within one to two weeks (data not shown). Even with reduced concentrations (≤0.1 ng/ml) the cultures terminally differentiated within the following weeks. Thus, long-term treatment with histone deacetylation inhibitors and with that global inhibition of deacetylation is incompatible with continuous proliferation and not a useful experimental approach to maintain telomerase activity in cultured (monolayer) keratinocytes. Dermal fibroblasts, telomerase-negative in monolayer culture as well as in the skin in vivo [5], did not become telomerase-positive under these treatment conditions (see Fig. 3A), indicating that hTERT activation in fibroblasts requires alternative/additional regulatory steps.

Since the deacetylation inhibitor FK228 was able to reactivate telomerase, we reasoned that histones were predominantly deacetylated in monolayer cultures but became acetylated upon FK228 treatment. To test this, we performed Western Blot analyses with keratinocytes before and 24 h after treatment with FK228 by using antibodies that specifically recognize acetylated histones (anti-acetylated histones H3 and H4 as well as an antibody recognizing the acetylated lysines in all histones). All three antibodies showed little or no acetylation in the untreated keratinocyte cultures but increased acetylation after a 24 h treatment with FK228 (Fig. 3B). This was not due to changes in histone expression *per se*, as the overall level did not change (Fig. 3D).

3.6. Histone acetylation correlates with tissue-dependent telomerase activity in the epidermis

To determine whether histone acetylation may also be responsible for the shift in telomerase expression in the epidermis in situ versus keratinocytes in monolayer cultures, we compared the acetylation profile of keratinocytes freshly isolated from the epidermis versus 3rd passage keratinocytes and found a similar difference in histone

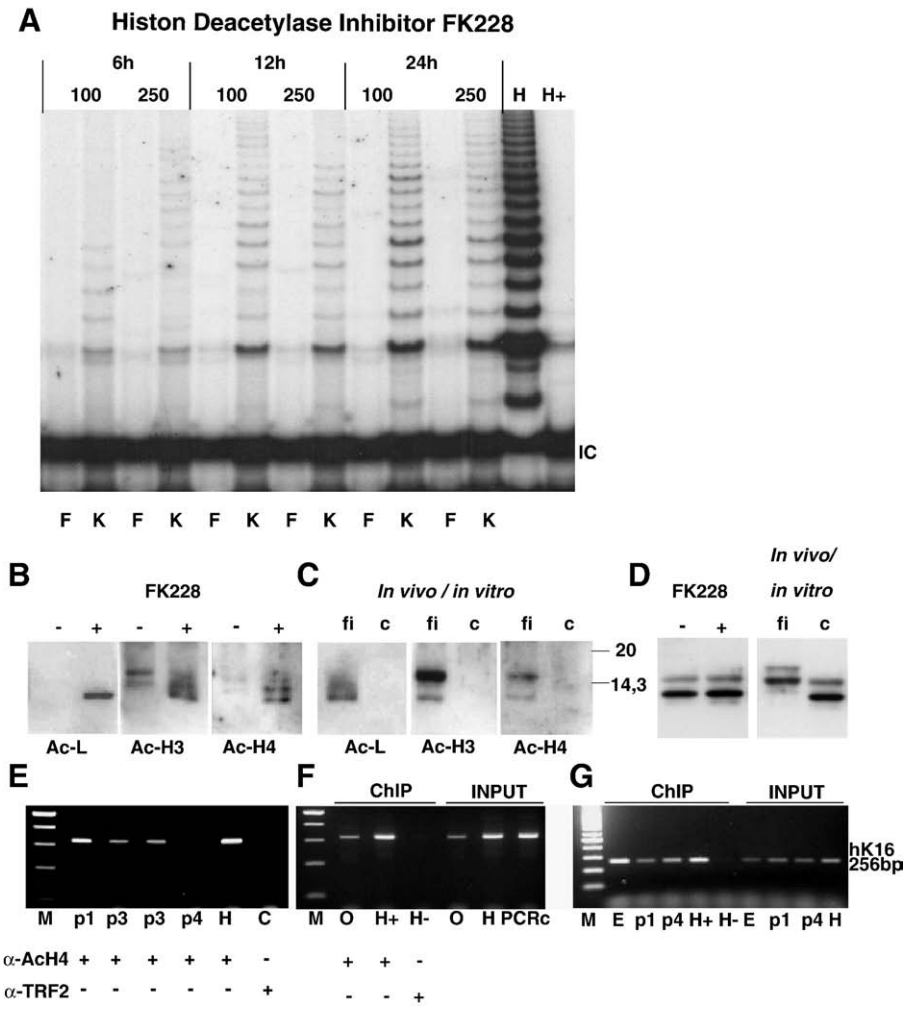

Fig. 3. Histone deacetylation is involved in telomerase regulation. (A) The histone acetylase inhibitor FK228 can reactivate telomerase in the cultured keratinocytes (K) but not in dermal fibroblasts (F). Cells were treated with FK228 (100 and 250 ng/ml) and telomerase activity (TRAPeze assay) was determined after 6, 12, and 24 h. An untreated lysate from HaCaT cells (H) served as positive and RNase treated HaCaT cells (H+) as negative control. IC = internal 36 bp standard. (B–D) Western blot analyses demonstrate differences in histone acetylation using different histone antibodies (antibody recognizing all acetylated lysines (ac-L) and antibodies against the acetylated histones 3 (ac-H3) and histone 4 (ac-H4)). (B) Comparison of cultured keratinocytes without (−) and with (+) FK228 treatment and (C) comparison of keratinocytes freshly isolated from the epidermis (in vivo = fi) versus cultured 3rd passage keratinocytes (in vitro = c). For all three antibodies the same cell combination is shown. (D) As a control, non-acetylated histones are present in similar amounts in the absence (−) and presence (+) of FK228 as well as in vivo (fi) and in vitro (c). (E–G) Chromatin immunoprecipitation study (CHIP) for hTERT and keratin 16 promoter DNA precipitated with an anti acetyl histone 4 antibody to investigate (E) different keratinocyte passages (passages 1, two independent samples from passage 3, and passage 4 cells). HaCaT cells served as a positive control, and an irrelevant antibody (anti TRF2 antibody) as a negative control. (F) CHIP analysis performed with DNA isolated from keratinocytes grown in organotypic culture (O) and HaCaT cells (H) (positive control). PCRc is a PCR positive control. (G) CHIP analysis of epidermal isolates and cultured keratinocytes (passage 1 and 4) and HaCaT cells (H) analyzed for human keratin 16 (hK16) promoter DNA. 10% of the primary solution was used as input DNA in F and G.

acetylation as seen with the FK228-treated keratinocytes (Fig. 3C). The freshly isolated keratinocytes showed distinct bands with the three different antibodies while essentially no bands were seen with 3rd-passage keratinocytes.

To confirm the role of histone deacetylation (and by analogy chromatin compaction) on hTERT promoter inhibition, we performed chromatin precipitation studies (CHIP) with different passage keratinocytes. While hTERT promoter fragments from first-passage keratinocytes precipitated with an antibody specific for the acetylated histone 4 (acetyl H4), the amount of hTERT fragments decreased with further propagation in monolayer culture and could no longer be detected at later passages (Fig. 3E). This result was confirmed with a different keratinocyte strain (data not shown) suggesting that deacetylation-dependent silencing of the hTERT promoter was not unique for a specific keratinocyte population.

If acetylation/deacetylation was involved in telomerase regulation in the epidermis in situ and in monolayer culture, reactivation of telomerase in organotypic cultures should require hTERT promoter acetylation. Indeed, CHIP analysis demonstrated the presence of acetylated hTERT promoter fragments in the epidermis formed in organotypic cultures (Fig. 3F). As a gene known to be actively transcribed both in the regenerating epidermis in situ and in monolayer culture, we also investigated for keratin 16 promoter acetylation and found no changes (Fig. 3G), further strengthening the specificity of hTERT promoter deacetylation for the inhibition of telomerase activity.

3.7. Telomere length declines in a replication-dependent manner during propagation in monolayer cultures

Although it was suggested that telomerase activity of human keratinocytes would be insufficient to counteract telomere erosion, our finding that telomerase was only detectable when keratinocytes they had formed an epidermis but not when they were grown in monolayer cultures prompted us to determine whether telomere shortening in monolayer culture would correlate with a more promi-

nent telomere loss than in the epidermis in situ despite the fact that the epidermis regenerates every three to four weeks and accordingly, also the stem cells should also go through a number of replications per year.

To address this, we first propagated different keratinocyte strains up to cellular senescence and investigated their telomere lengths at different passage levels by determining their telomere restriction fragment lengths (TRFL). Two keratinocyte strains derived from infant foreskins, exhibited an initial mean TRFL of about 10 kilobases (kb) and senesced after >30 pd with a mean TRFL of 6 to 7 kb, giving rise to a calculated loss of 100 to 130 bp per pd (one example is shown in Fig. 4A, left panel). Keratinocytes derived from an adult foreskin or from different sites of trunk skin, on the other hand, varied strongly in their rate of telomere loss, ranging from 18 to 125 bp per pd (one example is shown in Fig. 4A, right panel). A similar variation was observed when performing Q-FISH analysis (Fig. 4B and C). Altogether these data argue for a telomeric loss of ~100 bp per pd for the keratinocytes when being propagated in monolayer cultures.

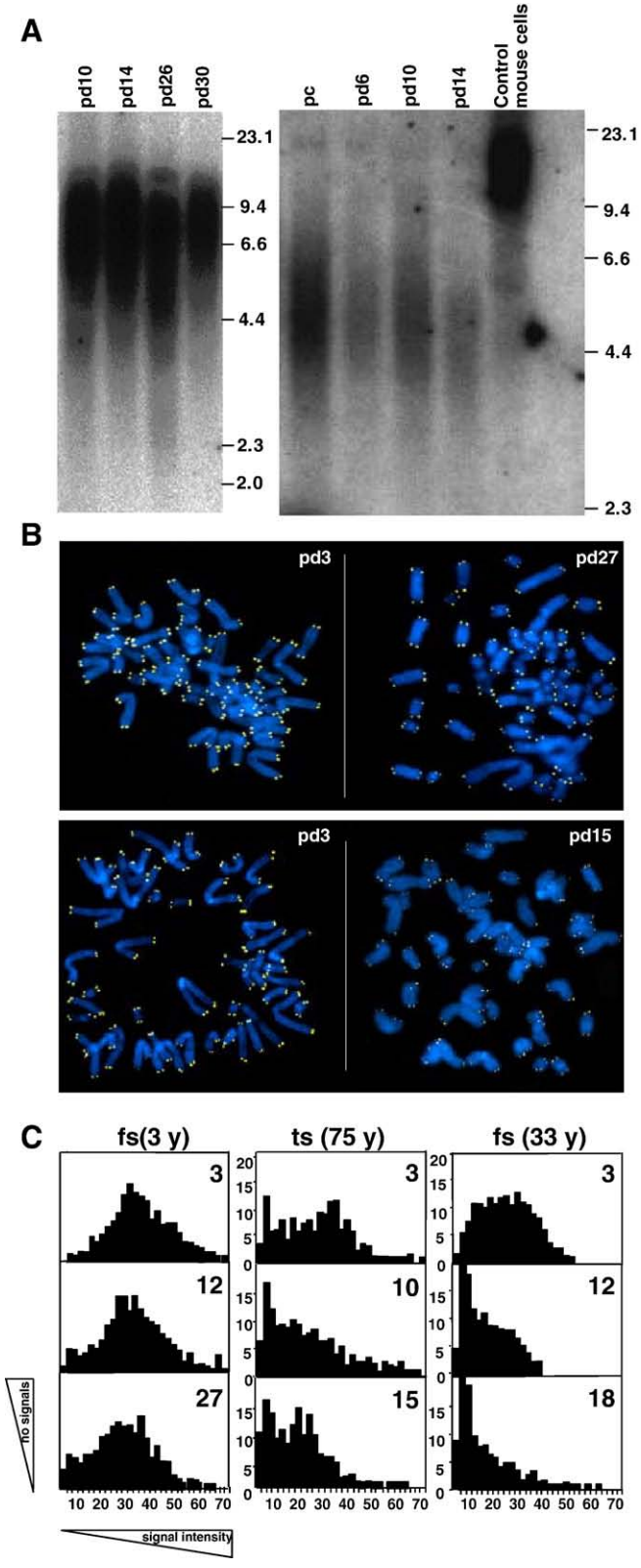

Fig. 4. Telomere lengths decrease with consecutive propagation of human skin keratinocytes in monolayer culture. (A) Telomere-specific Southern blot from keratinocytes isolated from the epidermis of an infant (age 1, left panel) and an adult donor (age 24, right panel) and cultured up to cellular senescence. Different passages were analyzed for TRFL. Mouse A9 cells with a mean TRFL of ~11 kb were used as a control. (B) Fluorescence in situ hybridization (FISH) images of metaphase spreads from an early and late passage of infant keratinocytes (pds 3 and 27, lower panel) and adult (pds 3 and 15, upper panel), respectively. Yellow: telomeric FISH signals and blue: DAPI counterstained chromosomes. (C) Quantitative FISH (Q-FISH) analysis from keratinocytes from, foreskin from a 3-year-old donor (pds 3, 12 and 27), trunk skin from a 75-year-old donor (pds 3, 10, and 15) and foreskin from a 33-year-old donor (pds 3, 12 and 18). Each curve represents the mean of at least 10 metaphases.

While this average estimate already implicated major variations between different keratinocyte populations, we aimed for establishing the rate of telomere loss in individual keratinocytes. For this, keratinocytes were plated at low density and followed by live cell imaging over a period of 92.8 h. Individual cells were marked and after mitoses the two daughter cells were followed for further replication. Those daughter cells that differed in their subsequent replication number where chosen for telomere length measurement. Telomere length measurements were performed by in situ hybridization of the telomeres with a telomere-specific PNA probe, deconvolution microscopy, 3D-telomere reconstruction [23,35]. Measurement of the individual signal intensities was performed and the mean of all telomeres per nucleus was then compared for each “daughter pair”.

Being able to identify 14 daughter pairs that differed in at least one replication round (2/14 differed in 2 and 1/14 differed in 3) we found an unexpected heterogeneity (Fig. 5). First, most but not all daughter cells remained synchronized in their replication time. Second, the cell cycle time varied from 12 to 35 h between different daughter pairs and third, telomere erosion varied from 45 to 1044 bp with no obvious correlation between the rate of telomere loss and cell cycle time. These results implicate that even within the same keratinocyte population there is no defined rate of replication-dependent telomere loss.

3.8. Telomere length heterogeneity rather than an age-dependent decline is characteristic for early-passage human skin keratinocytes

To determine telomere length regulation in human epidermis in situ we measured the mean TRFL from 31 early passage keratinocyte samples from donors ranging from newborn (0 year) to the age of 89. The six newborn foreskin samples showed a homogeneous distribution with a mean TRFL of 9 to 10 kb (Fig. 6A). The samples from the infant and adult donors (3 foreskins and 22 trunk skins), on the other hand, differed (Fig. 6B). Instead of a steady age-dependent decline, these samples showed strong inter-individual telomere length heterogeneity with differences ranging from 11 to 5 kb. Interestingly, the largest differences were seen in the group of 30 to 50 years while variations were less pronounced in samples from older donors (≥60 years) and the oldest donor (89) actually showed a similar mean TRFL as the youngest donors with 10 kb. From these data we calculated decline of ~25 kb loss per year and this decline was not statistically significant (*p* value of 0.1027; Fig. 6C).

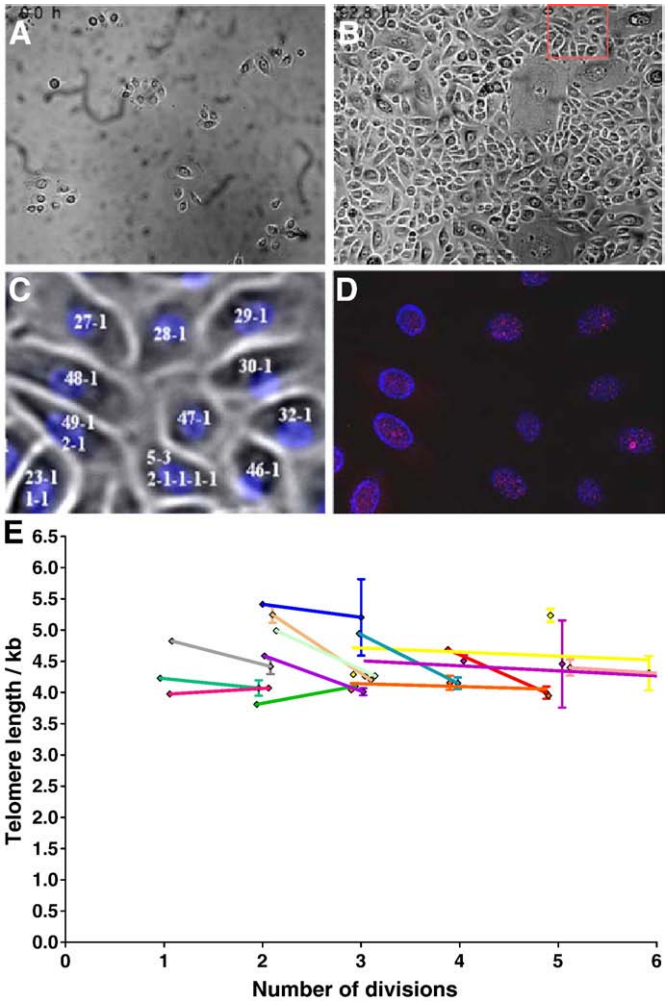

Fig. 5. Telomere loss differs between individual cells of the same population. Live cell microscopy combined with 3D-telomere length measurement was used to identify telomere length between different daughter cells (A) phase contrast micrographs of the keratinocyte cultures at the start (24 h after plating — time point 0) and (B) at the end (time point 92.8 h) of the live-cell-imaging experiment. (C) In a given area each mitosis was marked with a specific number and the daughter cells were followed and marked again when they went through the next mitosis. (D) Same area after in situ hybridization with the telomere-specific PNA probe (DakoCytomation, Glostrup Denmark) in red. Nuclei are counterstained with DAPI (in blue). (E) In 14 daughter pairs that differed in at least one replication round we found an unexpected heterogeneity, telomere erosion varied from 45 to 1044 bp. The mean telomere length and the error of mean are shown for cells from each replication round as well as the linear regressions between the replication rounds. The differences between the different regression slopes were not statistically significant ($p = 0.2665$), therefore, one slope could be calculated for all 14 cells — corresponding to a telomere loss of 322 bp/division.

3.9. Telomere length measurement on intact skin sections confirms the minimal age-dependent decline

Since establishing keratinocytes in culture may represent selection for specific subpopulations, we next adapted our 3D telomeric FISH analysis for telomere length analysis on tissue sections and combining it with immuno-labelling, we evaluated telomere length of the epidermal keratinocytes in skin samples from 12 different donors (ranging from 2 to 81 years of age). In addition, this technique allowed the distinction between the different cell types, i.e. keratinocytes, melanocytes, and fibroblasts present within the same skin section (Fig. 7A).

We first investigated the telomere lengths for the epidermal keratinocytes. Their respective mean lengths integrated well into the age-dependent pattern seen by Southern blot analysis (Fig. 7B and see Fig. 6C diamonds). Accordingly, combining all data and calculating their linear regression, we now obtained a statistically significant (p-value of 0.0181) yearly loss of 25 bp.

3.10. Epidermal differentiation does not contribute to telomere loss

To determine whether and how differentiation would contribute to telomere length regulation, we next studied samples from 14 different donors by dividing the epidermis into basal, suprabasal, and upper part (as indicated in Fig. 7C) and measured telomere length in at least 5 individual areas of each specimen (Fig. 7D). We found one sample with both suprabasal und upper part containing shorter telomeres than the basal layer, one sample with the upper layers containing shorter and two where the upper layers contained slightly longer telomeres. In most (10/14) samples, however, telomere lengths were very similar in all planes, indicating that epidermal differentiation does not contribute to telomere loss.

3.11. Telomere length maintenance is a characteristic of human skin

Since the in situ technique allowed us to distinguish between different cell types within the same skin sample, we also investigated for telomere length of the non-epidermal cells, the melanocytes, which make up ~10% of the basal layer of the epidermis, and the fibroblasts which reside in the underlying dermis. It is important to note that different from the epidermal keratinocytes both cell types only rarely proliferate in intact skin. As shown in Fig. 7B, for each individual tissue sample, telomeres were longer in the fibroblasts and melanocytes as compared to the respective keratinocytes. Actually, both cell types (within the same sample) exhibited highly comparable telomere lengths and unexpectedly, all three cell types (keratinocytes, fibroblast, and melanocytes) revealed very similar and statistically non-significant regression slopes (epidermis: p-values of 0.0907, dermal fibroblasts: p-value of 0.1972, and melanocytes p-value of 0.0886) (see Fig. 7B).

Finally and as a control for a different proliferation-active tissue, we also investigated hematopoietic cells, i.e. peripheral blood lymphocytes (PBL). It is well accepted that PBLs exhibit age-dependent telomere attrition [36] and in agreement with these previous data, we found a statistically significant reduction with a p value of 0.0010 when establishing the mean TRFL for PBLs from different-age donors (Fig. 7F).

4. Discussion

4.1. No significant age-dependent telomere loss of keratinocytes in human epidermis in situ

The epidermis, as the outermost barrier of the human body depends on mechanisms that guarantee life-long tissue integrity. One of these mechanisms is its constant regeneration which allows for a steady elimination of “damaged” cells from the basal proliferative compartment by passing through a defined program of differentiation and desquamation as dead horn squames. This continuous regeneration, however, also bears the risk of constant telomere shortening and, therefore, exhaustion of the proliferative potential of the epidermis in aged skin due to critically short telomeres. Indeed, evidence existed for a significant age-dependent decline in telomere length for peripheral blood lymphocytes ([37]; for review see [38]) as well as human skin [7–10]. However, in contrast to blood lymphocytes, the rate of telomere loss in human epidermis or in complete skin was small with a range of 9 to 36 bp/year. Only one study reported on a rate of 75 bp/year [8]. Our data confirm the significant rate of age-dependent telomere loss in blood lymphocytes. Here it is noteworthy that our PBL samples were derived from members of two related families, thus restricting genetic differences. Our data also support the low rate of telomere loss in human epidermis, thereby

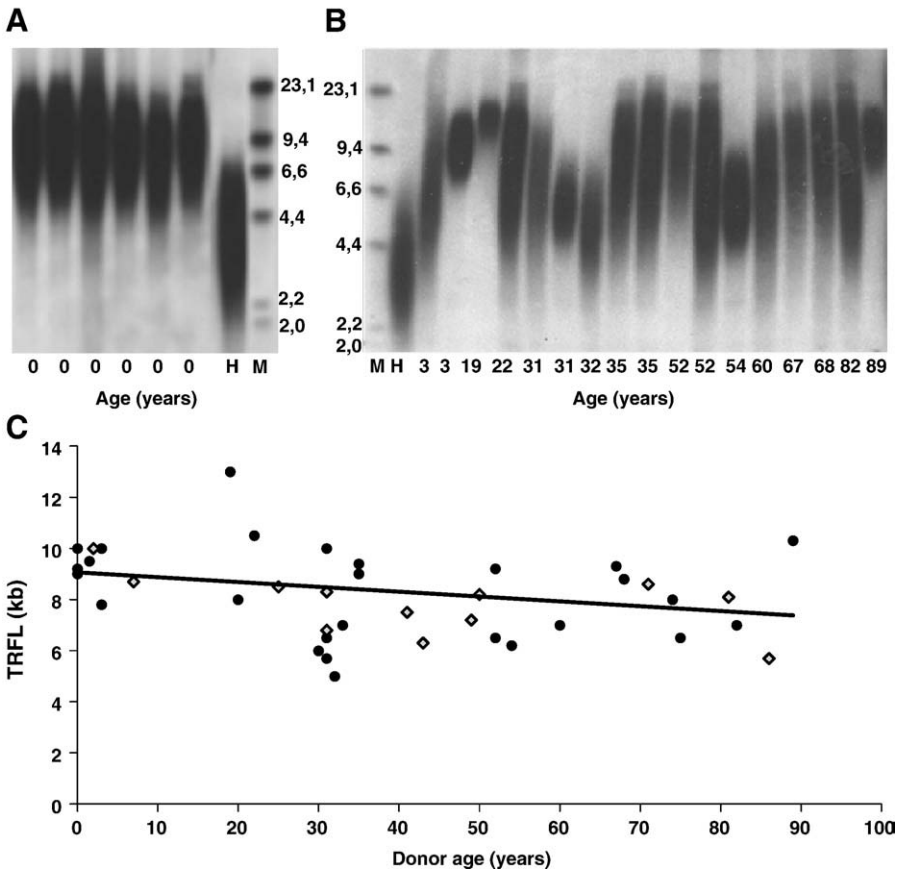

Fig. 6. Telomere length heterogeneity not telomere loss characterizes human skin keratinocytes from different-age-donors. (A–B) Telomere-specific Southern blot analysis of six second-passage foreskin keratinocyte strains from newborn donors (last lane = HaCaT cells) (A), and freshly isolated or first passage culture keratinocytes from different-age-donors (first lane = HaCaT cells) (B). (C) Quantification of the mean TRFL with indicated donor age. Linear regression of the 31 samples (black circles) demonstrates lack of statistical significance (p value of 0.1027) for age-dependent telomere attrition in the human skin keratinocytes. Grey diamonds represent the data obtained from the 3-dimensional telomere length analysis on tissue sections from different-age donors (see Fig. 7).

arguing for potential differences in the regulation of telomere length maintenance between these two tissues.

We further demonstrate that in the same skin sections where we measured telomere length of keratinocytes, the fibroblasts in the dermis and the melanocytes within the basal layer of the epidermis both exhibit longer telomeres and a similar minor age-dependent regression rate (see Fig. 7B). This finding is in good agreement with TRFL data from Sugimoto et al. [10] reporting on longer telomeres in the dermis than the epidermis and a regression rate of 11 bp/year for the dermis and 9 bp/year for the epidermis. Although only a few samples were available from very young donors so far, the difference in telomere length between epidermal keratinocytes versus dermal fibroblasts and melanocytes already seemed to be established early in life. If this is correct and provided that the different cell types start with a similar telomere length, these results implicate a rapid telomere loss during early epidermal development, i.e. a phase when even epidermal stem cells are supposed to proliferate heavily. During this time, apparently, telomerase is not sufficient for telomere length maintenance. Thereafter, when proliferation is restricted to maintaining tissue homeostasis, however, the level of telomerase seems adequate to minimizes telomere erosion to a similar rate as seen in the non-regenerating cells, i.e. to only a few bp per year.

A rapid telomere loss was described for PBLs of young children (>1 kb per year) followed by an apparent plateau between the age of four and young adulthood and gradual attrition later in life [37]. The authors proposed that loss of telomeric repeats in hematopoietic cells is a dynamic process that is differentially regulated in young children and adults. Besides a potential rapid telomere loss discussed for the embryogenic development, we did not detect obvious phases of telomere attrition in the human epidermis thereafter. It is intriguing to note that all three skin cell types exhibit a similar rate of telomere loss. Considering the fact that fibroblasts and melanocytes hardly ever proliferate, it is tempting to suggest that the rate of 11 bp/year reported by Sugimoto [10] and ~25 bp/year determined here, may not even be proliferation-related. If so, the more this argues for active telomere lengths maintenance throughout life time of the constantly proliferating keratinocytes. Along this line, Counter et al. [39] found that in patients with severe burns the epidermal transplants derived from keratinocytes expanded in monolayer cultures had shorter telomeres than in non-cultured skin transplants from the same individuals or those in skin of healthy donors older than 80 years. From this they concluded that due to the great loss of telomeric DNA the engrafted cells might have a shortened lifespan, which could have negative repercussions on the long-term viability of these grafts. Having now shown that telomerase is inhibited in culture but can be reactivated in cells that are allowed to form an epidermal tissue, such concerns may be invalid since in the tissue context telomerase may be reactivated and protect against consecutive severe telomere attrition. This said, it is tempting to suggest that intrinsic skin aging occurs rather independently of telomere shortening. The mechanism how telomere length is maintained, however, seems to differ and to depend on the cell type. We postulate a telomerase-based mechanism for the epidermal skin keratinocytes, while dermal fibroblasts and melanocytes maintain their long telomeres due to their low proliferation rate.

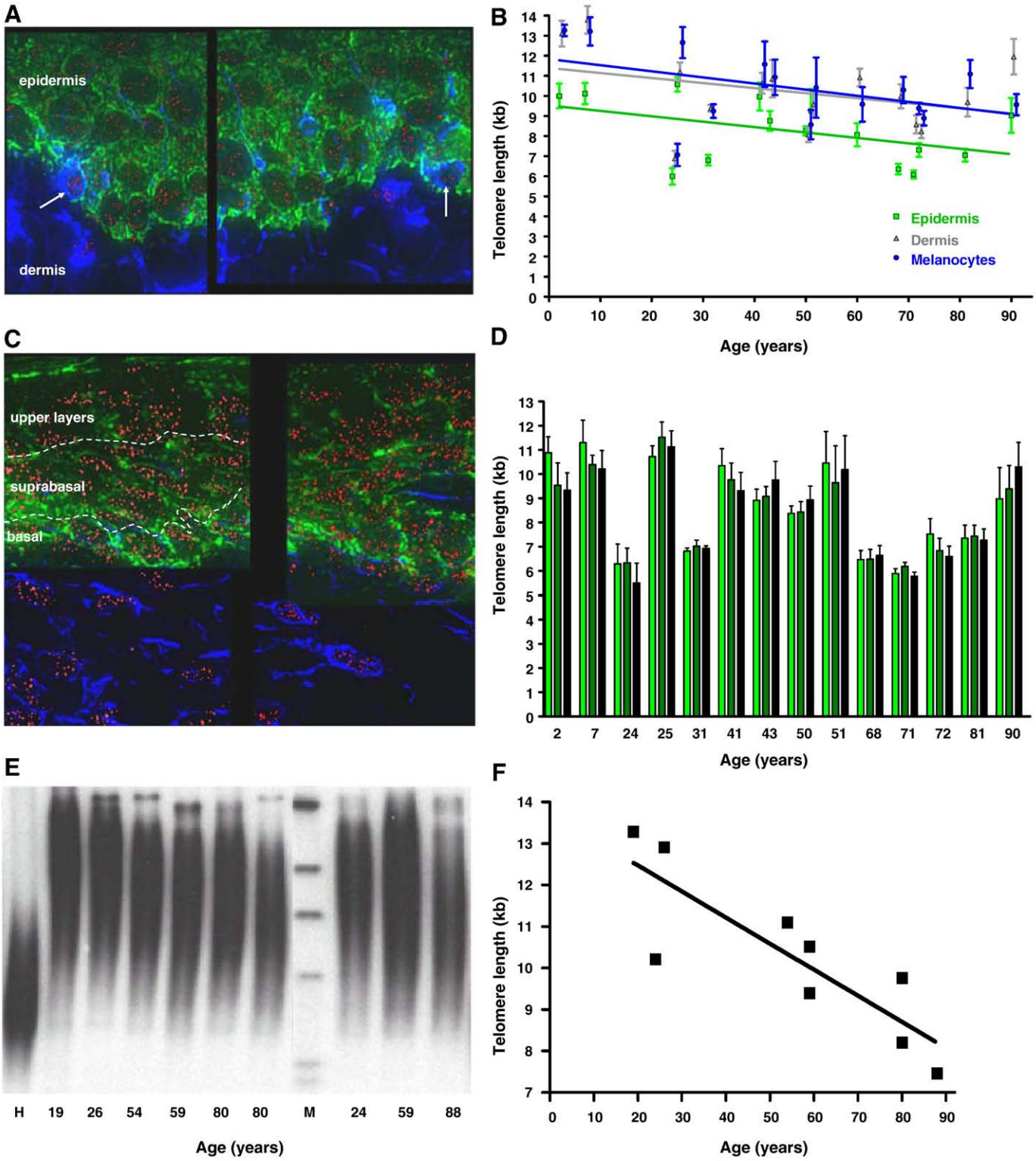

Fig. 7. Three-dimensional analysis of telomere length on skin sections verifies Southern Blot data. (A) Human skin section stained for keratin (green) marking the epidermal keratinocytes (epidermis) and vimentin (blue) marking the fibroblasts in the dermis as well as melanocytes (marked with arrows) in the basal layer of the epidermis. The telomeres, hybridized with a telomere-specific PNA probe, are presented in red. Epidermis and dermis are indicated. (B) Graphic presentation of the mean telomere signal intensities of keratinocytes (green), melanocytes (blue), and dermal fibroblasts (grey) in skins from different-age donors, demonstrates that telomeres in the proliferating keratinocytes are generally shorter than in the non-proliferating melanocytes or dermal fibroblasts but that all exhibit similar regression slopes. The respective donor age is indicated (*X*-axis). (C) Human skin stained for keratin (epidermis in green), vimentin (dermis in blue) and telomeres (telomere-specific PNA probe in red). The epidermis is divided by dotted lines into basal, suprabasal, and upper layers (as indicated). (D) Graphic presentation of the mean telomere signal intensities and standard error of the mean in basal (light green), suprabasal (dark green), and upper layers (black) of the epidermis of different age donors. (E) Telomere restriction fragment length analysis demonstrates a significant age-dependent telomere decline in peripheral blood leukocytes (PBL) from different-age members of two related families (right and left panel). (F) Their telomere lengths show a statistically significant age-dependent decline (*p* value of 0.0010). In all Southern blots, HaCaT cells (H) with a stable mean TRFL of 4 kb were included as control as was the size markers λHind III).

4.2. Histone acetylation contributes to hTERT/telomerase expression in the epidermal tissue

In line with the differences in telomere length regulation in the epidermis in situ and in keratinocytes in conventional cultures we also find a clear difference in telomerase regulation. Telomerase is expressed when the keratinocytes grow as an epidermis either in situ or in organotypic cultures but is inhibited in monolayer culture. Since proliferation is ongoing under both conditions, actually, proliferation is increased in monolayer culture as compared to the epidermis in situ, telomerase is not constitutively active in the keratinocytes and accordingly dispensable for keratinocyte growth. But it is obviously crucial in the tissue context. This said, it is noteworthy that the epidermis is a tissue that is highly prone to stress and damage. It is, therefore, tempting to hypothesize that besides counteracting proliferation-dependent telomere loss telomerase may similarly be essential for counteracting damage-dependent telomere loss in order to maintain epidermal tissue integrity and maintenance throughout life time.

Our data suggest that epigenetic remodelling of the hTERT promoter is an important regulatory step in the concert of tissue-dependent telomerase activation in human epidermis. While histone acetylation correlates with an active hTERT promoter in freshly isolated or early passage keratinocytes, hTERT is silenced upon passaging in monolayer culture and this in turn correlates with its promoter deacetylation. In organotypic cultures the hTERT promoter became reacetylated and accordingly telomerase re-expressed. Furthermore, keratinocytes in monolayer cultures re-expressed telomerase activity when treated with the deacetylation inhibitor FK228. It was recently shown that hTERT was hypoacetylated in human mesenchymal stem cells (hMSC) in culture and that telomerase could be reactivated by the deacetylation inhibitor Trichostatin A [40]. This may implicate that also in these cells hTERT is acetylated and active in vivo and that the observed deacetylation could be a “culture”-dependent trait as we suggest for the human epidermal keratinocytes.

Histone deacetylation coupled with a gradual accumulation of hTERT promoter methylation was reported previously for differentiation-dependent hTERT/telomerase repression in the human HT teratocarcinoma, human HL60 acute myeloid leukaemia cells, and mouse embryonic stem cells [41]. Our finding that histone acetylation plays a role in the tissue context-dependent telomerase regulation supports the idea that histone acetylation/deacetylation regulate telomerase activity in physiological processes. Importantly, this type of regulation also allows for downstream regulatory processes. EGF was reported to be a potent telomerase activator, stimulating hTERT transcription through an ets motive in the hTERT promoter [27] and that withdrawal of EGF or amphiregulin inhibited telomerase activity in culture [42]. In our experiments EGF was not able to induce telomerase activity but this can now be explained by hTERT promoter deacetylation which made the hTERT promoter inaccessible for EGF in the monolayer cultures. On the other hand, growth factors such as EGF and TGFα play an important role in stimulating keratinocyte growth and differentiation in the epidermis in situ [25,26]. Being active and accessible in the tissue context, our data, therefore, do not exclude that EGF may be part of the regulatory network controlling activation and inhibition of hTERT/telomerase activity in the process of epidermal regeneration and tissue homeostasis.

Altogether, we have demonstrated that telomerase is expressed in human epidermal keratinocytes in a tissue organization-dependent manner and that in the epidermis from skin from different age donors this expression is correlated with an only minor age-dependent telomere loss. This suggests that telomerase is functionally active and acts as a telomere lengths maintenance mechanism in human epidermis. Since the corresponding melanocytes and dermal fibroblasts show a similar low rate of telomere loss, we have to conclude that the overall telomere erosion is obviously not a driving force of skin aging. As we also show that telomerase is inhibited in monolayer cultures, telomere maintenance is not a constitutive trait but is extrinsically regulated. We provide evidence that part of this regulatory program is hTERT promoter acetylation, thereby guaranteeing an active hTERT transcription in the epidermis. In monolayer cultures, on the other hand, hTERT is silenced by histone deacetylation causing telomerase inhibition and accordingly a more extensive replication-dependent telomere loss. Therefore, our results support our hypothesis that telomerase is not required for proliferation *per se* but is part of the regulatory network required for life-long epidermal tissue regeneration.

Acknowledgements

We would like to thank Elke Tomakidi and Iris Martin for their excellent technical assistance as well as Dan Spandau, University of Indiana, Indianapolis, for kindly providing the newborn foreskin keratinocytes. We also would like to thank DAKO Cytomation for generously providing us with telomeric PNA probe. We wish to extend our thanks to Magareta Müller for her valuable discussion and advice and Yetunde Odunsi and Angelika Lampe for their help in editing the manuscript.

This work was supported by grants from Sander Stiftung, EC (LSHC-CT-2004-502943), Landesstiftung Baden-Württemberg, Deutsche Krebshilfe e.V., and BMBF (03NUK003) and the NAR Verbundprojekt “Stem Cells and Aging”(all to PB).

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