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Contribution of Sp1 to Telomerase Expression and Activity in Skin Keratinocytes Cultured With a Feeder Layer

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The growth of primary keratinocytes is improved by culturing them with a feeder layer. The aim of this study was to assess whether the feeder layer increases the lifespan of cultured epithelial cells by maintaining or improving telomerase activity and expression. The addition of an irradiated fibroblast feeder layer of either human or mouse origin (i3T3) helped maintain telomerase activity as well as expression of the transcription factor Sp1 in cultured keratinocytes. In contrast, senescence occurred earlier, together with a reduction of Sp1 expression and telomerase activity, in keratinocytes cultured without a feeder layer. Telomerase activity was consistently higher in keratinocytes grown on the three different feeder layers tested relative to cells grown without them. Suppression of Sp1 expression by RNA inhibition (RNAi) reduced both telomerase expression and activity in keratinocytes and also abolished their long-term growth capacity suggesting that Sp1 is a key regulator of both telomerase gene expression and cell cycle progression of primary cultured human skin keratinocytes. The results of the present study therefore suggest that the beneficial influence of the feeder layer relies on its ability to preserve telomerase activity in cultured human keratinocytes through the maintenance of stable levels of Sp1 expression.

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The use of a feeder layer, such as irradiated fibroblasts, allows the in vitro expansion of colonies from single keratinocytes, and their subculture by passaging (Rheinwald and Green, 1975). This method has improved the culture of several human cell types originating from either normal or disease tissues (Sun and Green, 1977; Goulet et al., 1996; Pellegrini et al., 1999; Germain et al., 2000; Jean et al., 2008) providing models closer to native tissues for studying normal or pathologic cells by expansion in cultures without viral transformation. It fostered the development of clinical applications such as the culture of autologous epidermal and corneal limbal cells for the treatment of patients with skin burns and limbal epithelial stem cell deficiencies of the cornea, respectively, as well as the culture of oral mucosa epithelial cells (Green et al., 1979; Rama et al., 2010; Proulx et al., 2011). However, the molecular mechanisms by which the feeder layer increases cultured cells lifespan are poorly understood.

Telomeres are protective structures located at the ends of eukaryotic chromosomes. In human, they are constituted of 5–15 kbp of tandem DNA repeats (TTAGGG) (Blackburn, 1991). The telomere replication process by DNA polymerases causes the loss of 50–200 bp of telomeric DNA at each population doubling of cultured human somatic cells (Harley et al., 1990; Vaziri et al., 1994). When telomere erosion reaches a critically short length, cells undergo growth-arrest and enter replicative senescence (Harley et al., 1992; Stewart and Weinberg, 2000).

Sylvain L. Guérin and Lucie Germain contributed equally to this study.

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Telomeres are elongated by telomerase, a ribonucleoprotein constituted of two components: the telomerase reverse transcriptase catalytic subunit (TERT) and the telomerase RNA, the enzymatic component that add telomeric repeats de novo on chromosomal ends (Greider and Blackburn, 1987). While telomerase is active in 85–90% of all tumors, it is inactive in most somatic cells (Kim et al., 1994), except for those that are found in regenerating tissues such as the hematopoietic system and the epidermis (Harle-Bachor and Boukamp, 1996; Bachor et al., 1999; Cerezo et al., 2003).

The core promoter of human TERT (hTERT)-coding gene contains several target sites for the transcription factors c-Myc and Sp1 (Greenberg et al., 1999; Takakura et al., 1999; Kyo et al., 2000). c-Myc is closely associated with hTERT expression and increases telomerase activity in most cancer cells as well as in normal cells that overexpress this transcription factor (Wang et al., 1998; Greenberg et al., 1999; Wu et al., 1999). In cervical carcinoma and breast cancer cells, Sp1 has been shown to cooperate with c-Myc in order to activate hTERT transcription (Kyo et al., 2000; Marconett et al., 2011). Sp1 is ubiquitously expressed in mammalian cells and involved in a variety of biological processes such as cell cycle progression, cell survival and angiogenesis. The transcription factor AP-1 also binds the promoter of hTERT gene. However, unlike Sp1 and c-Myc that both activate hTERT gene transcription, AP-1 was found to repress its expression in HeLa cells (Takakura et al., 2005). Sp1 has also been reported to activate hTERT transcription independently of c-Myc (Verma et al., 2004; Wooten and Ogretmen, 2005).

The present study was undertaken in order to investigate the molecular mechanism by which the feeder layer increases the lifespan of cultured, normal human skin epithelial cells. Our results show that the extended lifetime of keratinocytes cultured with irradiated 3T3 (i3T3) as well as irradiated human fibroblast feeder layers (iHFLs) correlated with the maintenance of both Sp1 expression and telomerase activity over cell passages. Furthermore, we demonstrate that Sp1 significantly contribute to hTERT transcription in a variety of human cell types, including keratinocytes. Through a mechanism related to Sp1 stabilization, feeder layers contribute to sustain both telomerase expression and activity in human keratinocytes, therefore extending the in vitro lifespan of these cells.

Materials and Methods

This study was conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were approved by the hospital and Université Laval Committees for the Protection of Human Subjects and patients gave their written, informed consent for tissue isolation and cell culture.

Cell culture

Human skin keratinocytes were obtained from normal 9- and 7-day-old newborn foreskins (Nb1 and Nb2, respectively), or normal adult (Ad) skin specimens removed during face-lift surgery of 47-, 59-, 57-, 37-, and 45-year-old subjects (Ad1–Ad5, respectively). Upon isolation, keratinocytes were cultured in the presence of a lethally irradiated 3T3 mouse fibroblast feeder layer (i3T3) for the first three passages as described (Germain et al., 1993; Masson-Gadais et al., 2006). For the subsequent passages until growth arrest, keratinocytes were cultured with i3T3, or with irradiated human feeder layers (iHFL1, iHFL2) or without feeder layer in the same culture medium (Bisson et al., 2013). Growth rates were calculated as previously described (Bisson et al., 2013).

Human lung fibroblasts (WI-38 [ATCC, Manassas, VA]), skin (HSFCs, 34- and 38-year-old subjects), and corneal keratocytes (HCFCs, 26-day-old newborn or 34-year-old adult, [Proulx et al., 2010]) were cultured in DME containing 10% fetal calf serum (Hyclone, Logan, UT) and antibiotics. The Jurkat T lymphocyte leukemic cells (ATCC) were grown in RPMI medium 1640 (Invitrogen, Burlington, ON, Canada) containing 10% FBS and antibiotics.

Human corneal epithelial cells (HCECs) were cultured with i3T3 from normal eyes of 3-, 44-, and 52-year-old subjects as described (Germain et al., 1999; Gaudreault et al., 2003). Human corneal endothelial cells (HCEndoCs) were isolated from normal eyes of 58-, 67-, and 83-year-old subjects and maintained in complete OptiMem-I medium as recently described (Proulx et al., 2010; Zaniolo et al., 2012). The uveal melanoma cell lines T97, T98, T108, T115, T128, T142, T143, and T151 were cultured as previously described (Landreville et al., 2011).

Nuclear and whole cell extract preparation

Nuclear extracts were obtained as described (Roy et al., 1991). To prepare whole cell protein extracts, cells were homogenized in lysis buffer containing 1% (v/v) Triton X-100, 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, and protease inhibitors (Complete, Roche Diagnostics, Laval, Canada). Protein concentration was determined by the Bradford procedure.

Electrophoretic mobility shift assay (EMSA)

DNA binding of Sp1 was monitored by EMSA as described (Gingras et al., 2009) using a 5′ ^{32}P-end-labeled, double-stranded oligonucleotide bearing the high affinity binding site for Sp1 as the labeled probe (Supplementary Table S1).

Western blot

Nuclear or whole cell proteins were analyzed by Western blot as described (Duval et al., 2012) using primary antibodies against: actin (C4, Cedarlane Laboratories Limited, Burlington, Ontario, Canada), c-Myc (9E10) and Sp1 (PEP 2, Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with peroxidase-conjugated goat anti-mouse (Chemicon International, Temecula, CA) or goat anti-rabbit (Sigma–Aldrich, Oakville, Ontario, Canada) secondary antibodies.

Telomerase repeat amplification protocol (TRAP) assay

Telomerase catalytic activity was determined by the TRAP assay, using the TRAPeze® Detection Kit (Chemicon International, Temecula, CA) and TRAPeze® XL Telomerase Detection Kit (Millipore, Temecula, CA) according to the manufacturer's instructions. The TRAP assay was performed using 0.3 μg of the protein extract. PCR cycles were 94°C/30 sec, 59°C/30 sec, and 72°C/1 min for 30 cycles on a thermocycler. The PCR products were resolved on a 12.5% polyacrylamide non-denaturing gel. For the TRAPeze® XL kit, 0.6 μg of the protein extract was used and 36 cycles were performed during the PCR amplification. The PCR products were then transferred in an opaque 96-well plate and fluorescence was measured at 495/516 nm excitation/emission for fluorescein (telomerase activity) and at 600/620 nm for sulforhodamine (internal control) with a Varioskan Flash Multimode Reader (Thermo Scientific, Hudson, NH). The relative fluorescence unit was calculated from a ratio of telomerase activity and the internal control as recommended by the manufacturer.

Telomere length assay

Telomere length was measured with the terminal restriction fragment (TRF) assay using the *TeloTAGGG Telomere Length Assay* (Roche Molecular Biochemicals, Indianapolis, IN) as recently described (Rochette and Brash, 2010) and the results analyzed by chemiluminescence with the Fusion FX7 (Vilber-Lourmat, Marne-la-Vallée, France).

Gene expression profiling

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Toronto, ON, Canada). Details regarding the biological replicates are provided in the supplementary materials. Cyanine 3-CTP-labeled cRNA targets were prepared from 50 ng of total RNA using the Agilent One-Color Microarray-Based Gene Expression Analysis kit. Then 600 ng cRNA was incubated on a G4851A SurePrint G3 Human GE 8 × 60K array slide (60 000 probes, Agilent Technologies Canada, Mississauga, ON, Canada). Slides were then hybridized and scanned on an Agilent SureScan Scanner. Data were analyzed using the ArrayStar V4.1 (DNASTAR, Madison, WI) software for scatter plots and generation of the heat maps of selected genes of interest. All microarray data presented in this study comply with the Minimum Information About a Microarray Experiment (MIAME) requirements.

Lentivirus production and cell transduction

The details regarding the production of lentiviruses that overexpress Sp1 shRNAs are provided in the Supplementary Methods. HaCaT cells were plated in 6-well plates at a density of 350,000 cells/well and incubated overnight at 37°C. The medium was removed and 700 μl of the virus suspension was added to the cells (overnight at 37°C) along with 1.3 ml of fresh medium and Hexadimethrine bromide (Polybren; Sigma Aldrich Co., Mileaukee, WI) (Sigma) at a final concentration of 4 μg/ml. Blasticidine (Sigma) was added to the culture medium at a concentration of 10 μg/ml 48 h following virus transduction and cells were allowed to grow for an additional 48 h. Expression and DNA binding of Sp1 was finally determined in the blasticidin-resistant HaCaT cell population.

Quantitative PCR (qRT-PCR)

Total RNA was isolated from HaCAT cells, HaCAT cells transduced with the empty plenti6/V5-U6 lentiviral vector (HaCAT/Ctl$^-$), or HaCAT cells transduced with a derivative from plenti6/V5-U6 that expresses a shRNA directed against the Sp1 transcript (HaCAT/shSp1) and reverse transcribed using random hexamer primers following the manufacturer's protocol for the synthesis of the first strand cDNA (Superscript II; Invitrogen, Burlington, ON, Canada). Equal amounts of cDNA were run in quadruplicate and amplified in a 20 μl reaction containing 10 μl of 2× Brillant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies), 500 nM of upstream and downstream primers, and 5 ng of cDNA target. No-template controls were also used as recommended. The mixture was incubated at 95°C for 3 min, and then cycled at 95°C for 10 sec and at 60°C for 20 sec 35 times using the QIAGEN Rotor-Gene Q real-time cycler. Amplification efficiencies were validated and normalized to the GAPDH mRNA transcript and quantity of target genes were calculated according to a standard curve. Primers were designed using Primer Quest Design Tool (IDT Integrated DNA Technology) and are listed in Supplementary Table S1.

Statistical analyses

Student's *t*-test was performed for comparison of the groups in qRT-PCR analyses. Differences were considered to be statistically significant at $P < 0.001$. All data are also expressed as mean ± SD.

Results

The feeder layer influences telomerase activity and Sp1 expression in cultured human keratinocytes

Although many studies have described the beneficial influence of a feeder layer on the morphologic and physiologic properties of various human cell types, little is known on whether this positive influence is determined by alterations in telomerase expression and/or activity. We therefore evaluated the effect of the widely used i3T3 feeder layer on telomerase expression and activity in human skin keratinocytes. As the promoter of hTERT gene is a Sp1 target, we monitored the expression of this transcription factor. Newborn (Nb) and adult (Ad) keratinocytes cultured with or without i3T3 from passage p4 until senescence were compared. Keratinocytes from three different donors (referred as Nb [Figs. 1A,D and F], Ad1 [Figs. 1B,E and G], and Ad2 [Supplementary Figs. S1 and S2]) were tested.

Co-culturing along with an i3T3 feeder layer clearly increased the number of cell passages that Nb keratinocytes can sustain in culture by four more passages (Fig. 1A and Supplementary Fig. S1A). Ad1 and Ad2 strains reached terminal differentiation at p7 and p8, respectively, when grown without i3T3, whereas cells cultured with i3T3 had not yet terminally differentiated when experiments were stopped at p9 (data for Ad1 cells are shown in Fig. 1B and Supplementary Fig. S1B; data for Ad2 are presented in Supplementary Fig. S2). The elevated Sp1 expression level observed at p2 progressively decreased to low but yet detectable levels at p10 in Nb keratinocytes cultured with i3T3 (Fig. 1A), whereas Sp1 expression fluctuated over cell passages in Ad1 (Fig. 1B) and Ad2 cells (Supplementary Fig. S2) grown with i3T3, as we previously reported (Masson-Gadais et al., 2006). As shown in Figure 1C, no expression of Sp1 was observed in i3T3, confirming that the Sp1 signal we observed originated from keratinocytes (Nb, Ad1, and Ad2 cells) and not from the feeder layer. Disappearance of telomerase activity seems to correlate quite well with a corresponding reduction of Sp1 expression in Nb1 keratinocytes (compare Figs. 1A and D), and Ad2 (Supplementary Fig. S2) but not in Ad1 keratinocytes (compare Figs. 1B and E) cultured with i3T3. When keratinocytes were grown without i3T3, Sp1 expression was lost in the early passages, concomitantly with the early occurrence of senescence in Nb, Ad1, and Ad2 cells (Figs. 1A,B; and Supplementary Fig. S2). Moreover, an obvious reduction of telomerase activity occurred in these cells (Figs. 1D and E) when cultured without feeder layer.

The human feeder layer preserves telomerase activity of cultured human keratinocytes

We examined to which extent the co-culture with a human (iHFL1, iHFL2) or mouse (i3T3) feeder layer was affecting telomerase activity in keratinocytes. Keratinocytes grown on either i3T3 or iHFL2 were still proliferating when cultures were stopped at passage 18 and an appreciable level of telomerase activity was detected at each passage (the average telomerase expression level was 0.084 and 0.099 relative fluorescence units [RFU] for keratinocytes grown with i3T3 and iHFL2, respectively; Figs. 2B and D). Keratinocytes grown on the iHFL1 also had telomerase activity (average expression level of 0.054 RFU) but a drastic reduction was observed at passage 17, which correlated with keratinocytes reaching growth arrest (Fig. 2C). The lowest telomerase activity was observed in the condition where no feeder layer was used (average expression level of 0.041 RFU Fig. 2A). Incidentally, keratinocytes grown without a feeder layer reached growth arrest at passage 8, which is much earlier than their counterparts grown on the three tested feeder layers.

Telomerase enzymatic activity was reported to fluctuate in relation to culture condition in i3T3 fibroblasts (Borras et al., 2004). To eliminate the possibility that i3T3 contributed to the telomerase activity measured in keratinocytes cultured with a feeder layer, we have evaluated the telomerase activity level in i3T3 fibroblasts alone. Given that human dermal fibroblasts can be utilized as an alternative to i3T3 feeder layers to support keratinocytes growth (Braye et al., 2000; Auxenfans et al., 2013), telomerase activity was also assessed in iHFL.

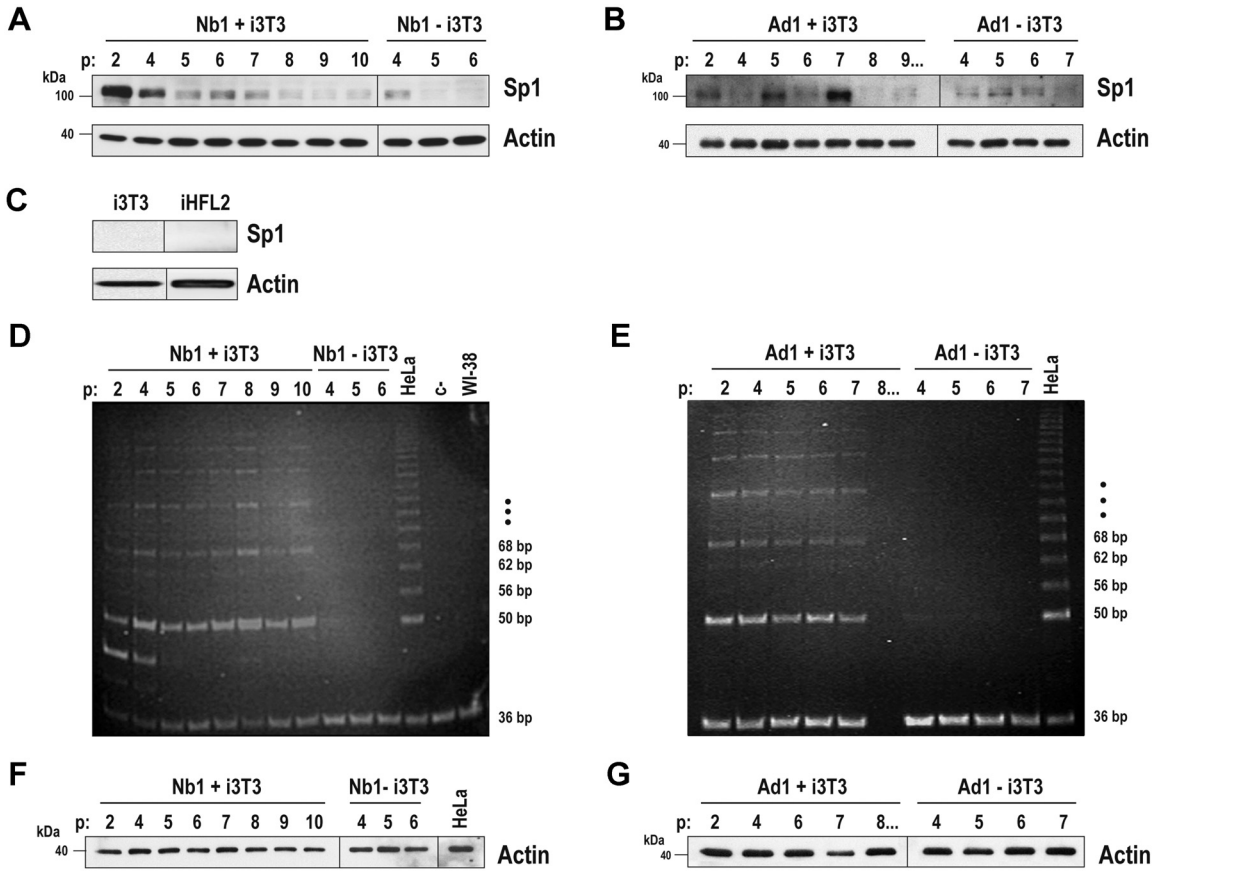

Fig. 1. Telomerase activity and Sp1 expression in human keratinocytes cultured with a feeder layer. Nuclear proteins (A,B) and whole cell extracts (D,E) were prepared at each cell passage (p) from newborn (Nb1) (A,D,F) and adult (Ad1) (B,E,G) skin keratinocytes grown with (+3T3) or without (−3T3) i3T3 to monitor the expression of Sp1 by Western blot in Nb1 (A) and Ad1 (B) keratinocytes. C: Western blot analysis of Sp1 in i3T3 and iHFL1 feeder cells. Actin expression was used as a normalization control. D,E: Telomerase activity was measured by TRAP assays. A protein sample from HeLa cells was used as a positive control for the TRAP reaction. c−: negative control performed without the addition of proteins. WI-38: TRAP assay performed with whole cell extract from telomerase-negative WI-38 cells. 36 bp: internal PCR amplification control band. F,G: The protein samples used in (C,D) were Western blotted with the anti-actin antibody for normalization purpose.

Approximately 3% and 8% of iHFLs and i3T3, respectively, remain in the cell culture after keratinocytes were harvested for the measurement of telomerase activities at near-confluence (Bisson et al., 2013). Therefore, telomerase activity was analyzed in 30, 15, and 3 ng total proteins, which represents 10%, 5%, and 1%, respectively, of the typical amount of protein extract (300 ng) normally used to analyze telomerase activity in keratinocytes cultured with a feeder layer. The results presented in Supplementary Figure S3 indicate that a significant, but low telomerase activity could be observed only when 30 ng of i3T3 protein extracts was used. Since the ratio of i3T3 remaining in keratinocyte cultures at near-confluence was estimated to be near 8%, it cannot be excluded that a low, but significant, proportion of the signal observed might have resulted from the feeder layer. On the other hand, the telomerase activity from the iHFL protein extract was consistently found to be negligible (iHFL2; Supplementary Fig. S3). In addition, and as noted for i3T3, iHFLs do not express Sp1 (Fig. 1C). Therefore, analyses conducted using a human feeder layer (iHFL) rather than mouse i3T3 are likely to give a more realistic assessment of the level of telomerase expressed by human keratinocytes.

In order to analyze the effect of different kinds of feeder layers on telomere loss, the telomeres lengths were measured in keratinocytes when co-cultured along with three different types of feeder layers. The same cells and culture conditions as those presented in Supplementary Figure S3 were used in this experiment the Sp1 expression levels in keratinocytes used in this experiment (Ad3 or Kfl57) were published in Bisson et al. (2013). As shown in Figures 2E and F, an important telomere loss occurs between passages 6 and 14 (for iHFL1) or passages 6 and 17 (for i3T3 and iHFL2; over the course of 11 passages, which corresponded to ~53 population doublings, keratinocytes in the iHFL2 condition lost ~2.2 kpbs of telomeres). Interestingly, the telomere loss occurred twice as fast for keratinocytes cultured with iHFL1 (410 ± 55 bp/passage) in comparison to when they are cultured with the other two feeder layers (Fig. 2F; 190 ± 12 and 169 ± 29 bp/passage for keratinocytes grown with iHLF2 and i3T3, respectively). Incidentally, keratinocytes cultured on iHFL1 had a lower average telomerase activity (0.050 RFU) than iHLF2 (0.097 RFU; compare Fig. 2C with D). There were no significant differences in telomere loss from cells cultured with either i3T3 or iHFL2. The telomere loss occurred faster in conditions

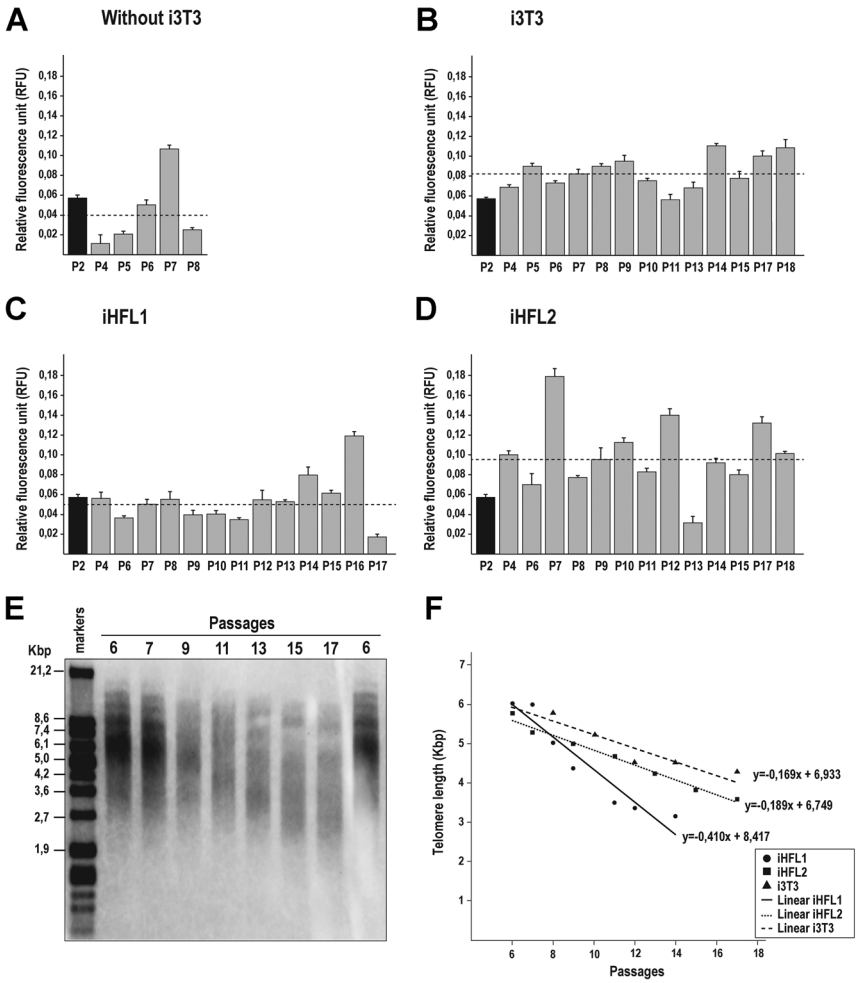

Fig. 2. Influence of a human or mouse feeder layer on the telomerase activity and telomere shortening in human keratinocytes. Telomerase activity was measured by fluorescence analyses in Ad3 keratinocytes cultured (A) without a feeder, or in the presence of either (B) irradiated mouse i3T3 or (C,D) a human feeder layer (iHFL1 and iHFL2). Relative fluorescence units are shown along the *y*-axis whereas cell passages are indicated along the *x*-axis. The dashed lines represent the average telomerase expression level for each feeder layer. E: Shortening of telomeres was evaluated in keratinocytes cultured with a human feeder layer (iHFL2) using terminal restriction fragment (TRF) Southern blot. F: The keratinocytes used in part (E) were cultured along with either a mouse (i3T3), or human (iHFL1 and iHFL2) feeder layer. Keratinocyte's telomeres length was then evaluated for each condition at multiple passages using TRF.

where Sp1 was reduced earlier (Bisson et al., 2013). We could not observe whether the telomere loss occurs faster in keratinocytes grown without a feeder layer as these cells reached terminal differentiation very early near passage 6.

Maintenance of hTERT expression correlates with Sp1 but not c-Myc expression in cultured human keratinocytes

Since Sp1 and c-Myc are both important transcription factors for hTERT gene transcription, we exploited gene expression profiling on microarrays in order to examine their presence in total RNAs isolated from a variety of primary cultured human cells including skin epithelial cells (Nb2/Ad4) and fibroblasts (HSFCs), corneal epithelial cells (HCECs), corneal stroma keratocytes (HCFCs), and corneal endothelial cells (HCEndoCs) (Proulx et al., 2010). As shown on the heatmap depicted in Figure 3A (and also presented as linear signals on Fig. 3B), hTERT gene expression was very low and fairly uniform in all cell types examined, unlike Sp1 whose basal level of expression was much more elevated (28-fold higher on average). As with Sp1, c-Myc expression was very high in both types of epithelial cells (HCECs and Nb2/Ad4) whereas its expression was lower in the fibroblast cell strains (HCFCs and HSFCs) and endothelial HCEndoCs. To investigate which of Sp1 or c-Myc expression correlates the best with that of hTERT, we conducted microarray analyses on a set of uveal melanoma cell lines, some of which we knew were expressing variable levels of Sp1 (Landreville et al., 2011). Except for T151 cells, basal expression of hTERT was much higher in these cancer cell lines (Figs. 3C and D) than in untransformed, primary cultured cells (Figs. 3A and B). Interestingly, hTERT expression perfectly correlates with that of Sp1; that is, cell lines expressing very low levels of hTERT (such as T97, T98, T108, and T151) have the lowest Sp1 expression whereas those with the higher levels of hTERT (such as T111, T128, T142, and T143) express high Sp1 levels (Figs. 3C and D). On the other hand, many cell lines expressing low hTERT levels (such as T97, T98, T108, and T151) also express high c-Myc levels. In contrast, cells having high hTERT levels (such as T142 and T143) often have low c-Myc

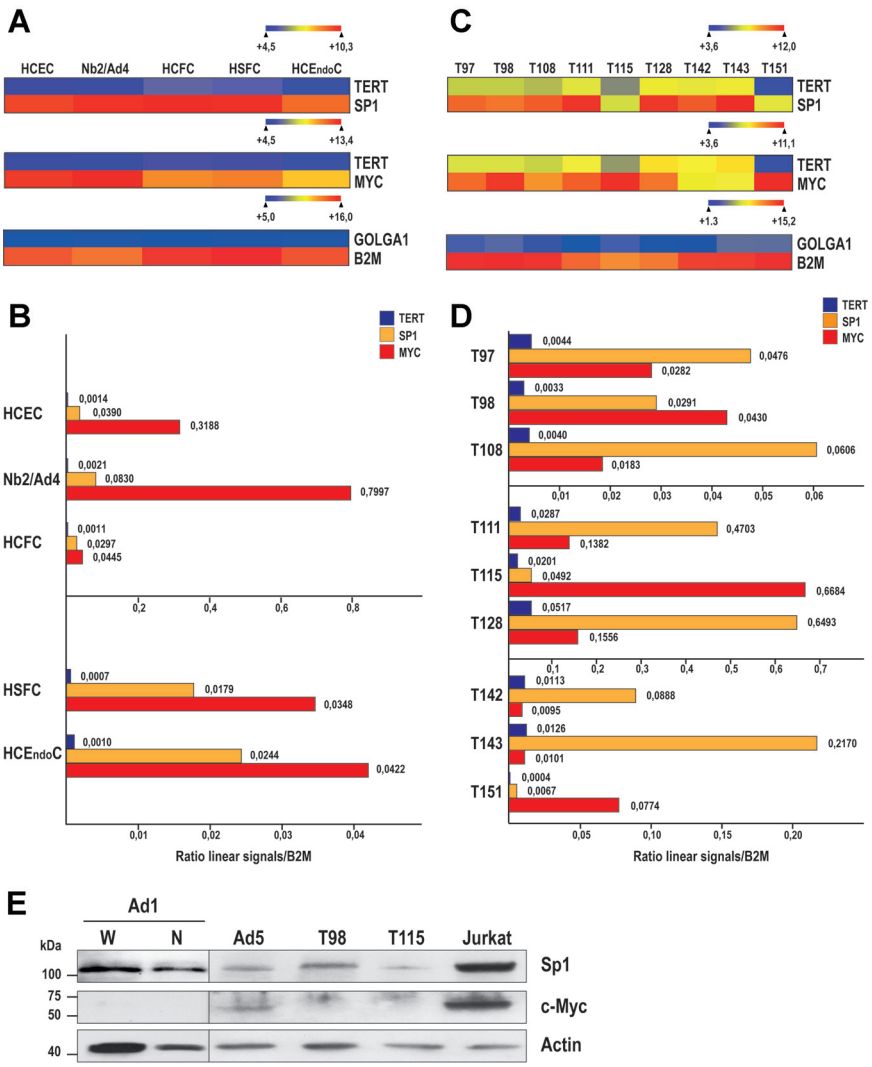

Fig. 3. Analysis of telomerase, Sp1 and c-Myc expression in keratinocytes and other tissue cultured cells. Heatmap representation of telomerase (TERT), Sp1 and c-Myc genes level's of expression in (A) primary cultures of Nb2/Ad4 keratinocytes, HSFC, HCEC, HCFC, and HCEndoC, or (C) in uveal melanoma cell lines (T97–T151). Microarray data for the housekeeping genes β2-microglobulin (B2M) and golgin subfamily A member 1 (GOLGA1) that are expressed, respectively, to very high and low levels in all types of cells are also shown. Genes in dark blue are those whose expression is very low whereas highly expressed genes are shown in orange/red. Data for each group of cultured cells are also presented as linear signals normalized to those of B2M (B,D). (E) Whole cell (W) and nuclear (N) extracts were prepared from Ad1 and Ad5 keratinocytes co-cultured with i3T3 and blotted with c-Myc and Sp1 antibodies. Whole cell proteins were also obtained from the uveal melanoma cell lines T98 and T115, and Jurkat cells (positive control for c-Myc expression). Actin expression was also monitored for normalization purpose.

expression (Figs. 3C and D). As revealed by Western blot analysis, c-Myc protein level was either totally absent or only barely detectable in the nuclear (N) or whole cell (W) protein fractions of Ad1 keratinocytes grown with i3T3 (Fig. 3E). On the other hand, a nuclear extract from the positive control cell line Jurkat yielded a strong c-Myc signal whereas no signal was obtained with a similar extract prepared from human fibroblasts that have previously been reported to express either no or only very weak levels of c-Myc (Kyo et al., 2000). This result prompted us to examine expression of c-Myc in other types of cells including Ad5 keratinocytes and the uveal melanoma cell lines T98 and T115 that also express, besides hTERT, different Sp1/c-Myc ratios. Low but detectable levels of c-Myc were observed in Ad5 but not in T98 and T115 cells (Fig. 3E). Sp1 could easily be detected in Ad1 and Ad5 keratinocytes, as well as in the other cell types examined.

Suppression of Sp1 reduces the activity and expression of hTERT and alters the long term growth of keratinocytes cultured with a feeder layer

To demonstrate the contribution of Sp1 to expression of hTERT in keratinocytes, we suppressed Sp1 expression in the immortalized human keratinocyte cell line HaCaT (Schurer et al., 1993) and examined its influence on the expression and enzymatic activity of telomerase. HaCaT cells were used to conduct this experiment because human skin keratinocytes transduced with the shSp1-expressing lentiviruses did not survive the blasticidin selection. We therefore transduced HaCaT cells with lentiviruses encoding two different shRNAs directed against human Sp1. Endogenous Sp1 gene expression was very efficiently suppressed (by near 70%) in HaCaT cells when transduced with lentiviruses encoding shSp1 but not by

an empty lentivirus expressing no shRNA (HaCaT/Ctl$^-$), as revealed by the significant reduction of Sp1 binding in EMSA (Fig. 4A) and the important reduction in the amount of Sp1 protein in Western blot (Fig. 4B; results are shown for the Sp1 shRNAs shSp1–2 but similar results were also obtained with the shSp1–1 expressing lentivirus). The suppression of Sp1 observed at the protein level was also confirmed at the transcriptional level by qPCR (Fig. 4C, top part). Most importantly, suppressing Sp1 expression drastically reduced transcription of the hTERT gene (reduction of 12- and 65-fold in HaCaT/shSp1–1 and HaCaT/shSp1–2 cells, respectively, relative to the level observed in HaCaT/Ctl$^-$; Fig. 4C, bottom part) as well as hTERT enzymatic activity in HaCaT/shSp1 cells (Fig. 4D).

To circumvent the need for blasticidin selection required when cells are transduced with plenti6/V5-U6 derivatives, we cloned a new Sp1 shRNA into the pNL-SIN-GFP lentiviral vector (kindly provided by Dr. Bryan R. Cullen, Duke University Medical Center, Durham, NC) to create the derivative pNL/shSp1–3. This shSp1-encoding lentivirus was then transduced into primary cultured human skin keratinocytes in order to evaluate the influence of suppressing Sp1 expression on the ability of these cells to be passaged in culture. Human skin keratinocytes (Ad1) transduced at passage p3 with the negative control pNL-LUC lentivirus that expresses a shRNA against luciferase were still able to form nice colonies very similar to uninfected cells upon passaging at p4 and p5 (Fig. 5, phase contrast; Ad1/shLUC-2). However, the ability of keratinocytes to form colonies was entirely lost right at the first passage (p4) following transduction at p3 using the shSp1-encoding pNL/shSp1–3 lentivirus (Fig. 5, phase contrast; Ad1/shSp1–3). qPCR analyses demonstrated the dramatic extinction of the Sp1 transcript at both p4 and p5 in cells

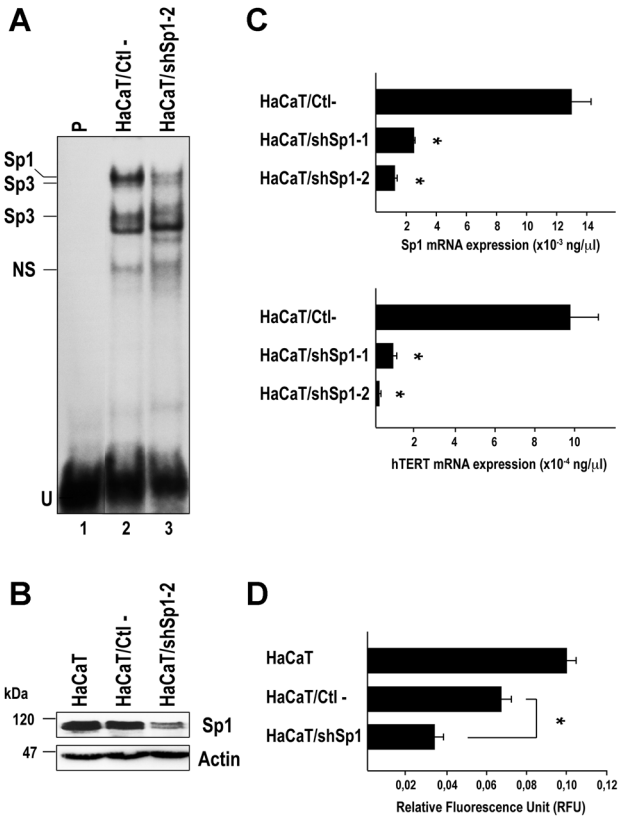

Fig. 4. Suppression of Sp1 expression in HaCaT cells and influence on telomerase expression and activity. (A) Suppression of Sp1 expression in HaCaT cells transduced with an empty lentivirus (HaCaT/Ctl$^-$) or with a lentivirus expressing an shRNA directed against Sp1 (HaCaT/shSp1) was monitored by EMSA, using the high affinity binding site for Sp1 as the DNA labeled probe. NS, non-specific complex; U, unbound probe. (B) Expression of Sp1 was monitored by Western blot using the extracts from part (A). Proteins from non-transduced HaCaT cells were also used as negative controls. Actin expression was also monitored for normalization purposes. (C) Suppression of Sp1 and its influence on hTERT gene expression was monitored by qPCR in HaCaT cells transduced (HaCaT/shSp1–1 and HaCaT/shSp1–2) or not (HaCaT/Ctl$^-$) with two recombinant lentiviruses that each express a shRNA against the Sp1 mRNA transcript. *Values considered to be statistically significant from those obtained in control HaCaT/Ctl$^-$ cells (*P*-value $<$0.001). (D) TRAP assays were conducted using total protein extracts from the cells described above (HaCaT, HaCaT/Ctl$^-$ and HaCaT/shSp1 to monitor telomerase activity in Sp1-suppressed HaCaT cells. Telomerase activity is expressed as relative fluorescence units (RFUs).

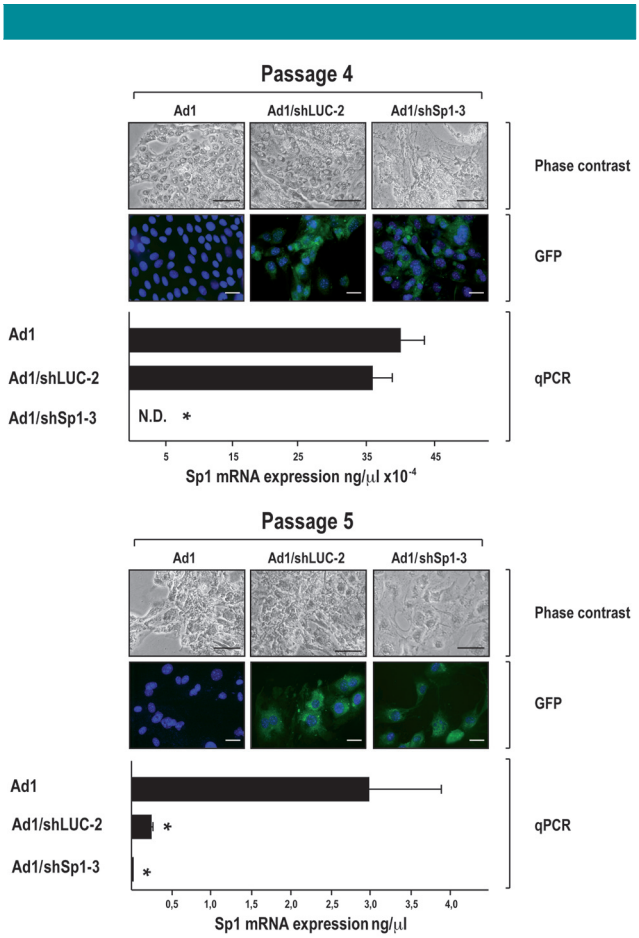

Fig. 5. Influence of suppressing Sp1 expression on the long-term growth of keratinocytes. Human skin keratinocytes (Ad1) transduced at passage 3 with the lentiviral vectors pNL/shLUC-2 and pNL/shSp1–3 were cultured further to passages p4 and p5. Formation of cell colonies was examined at both p4 and p5 by phase contrast microscopy whereas GFP expression was monitored by immunofluorescence analysis (in green). Nuclei were counterstained with Hoechst 33258 reagent and appear in blue. Expression of Sp1 was also monitored at the mRNA level by qPCR. Standard deviation is provided. N.D., not detectable. *Values considered to be statistically significant from those obtained in control Ad1 (*P*-value $<$0.001).

transduced with the Sp1-encoding shRNA (Fig. 5, qPCR; Ad1/shSp1–3), a reduction that did not result from less efficient transduction of these cells as nearly all cells expressed GFP in immunofluorescence analysis (Fig. 5; GFP). On the other hand, Sp1 expression remained unaffected at p4 in cells transduced with the pNL-LUC-2 lentivirus (Fig. 5, qPCR; Ad1/shLUC-2) but decreased by approximately 10-fold at p5, indicating that keratinocytes may suffer from viral transduction over cell passaging. In spite of this reduced Sp1 expression in pNL-LUC-2-transduced keratinocytes at p5, the level of Sp1 mRNA remaining in these cells was by far superior to that from keratinocytes transduced with pNL/shSp1–3.

Discussion

Skin keratinocytes were the first human diploid normal cell types to be expanded in vitro to a large extent without viral transduction. Indeed, with the appropriate culture conditions, large colonies can grow from single cells and a large number of cells can be obtained by serial cultivation (Rheinwald and Green, 1975). Because they are closer to native tissues than cells modified by viral transduction are, in vitro expansion of unmodified cells represents a good model for physiological or pharmacological studies on normal skin epithelial cells (Lavoie et al., 2013). Moreover, the possibility to produce cell cultures from a very small biopsy represents an interesting alternative for the study of human skin diseases in which large biopsies are difficult to obtain (Jean et al., 2009). In this study, we provided evidence for the preservation of telomerase activity in cultures of normal human cells in vitro. Besides preserving telomerase activity, the presence of a feeder layer was shown to delay the extinction of Sp1 in human skin keratinocytes (Masson-Gadais et al., 2006). Our finding supports the hypothesis that the gene encoding the hTERT telomerase catalytic subunit is a transcriptional target of Sp1 in keratinocytes. Consequently, the sustained expression of Sp1 in keratinocytes cultured with a feeder layer may help preserve telomerase expression. It then, in turn, delays telomere shortening thereby avoiding the progression of keratinocytes into replicative arrest and senescence, resulting in an increased number of potential passages in culture.

The regulation of hTERT gene promoter activity by Sp1 has been documented in cancer cells (Wooten and Ogretmen, 2005). Physical interaction of Sp1 with the hTERT promoter has been demonstrated through in vivo ChIP analyses (Won et al., 2002; Kim et al., 2008b; Marconett et al., 2011; Xu et al., 2013) as well as in vitro EMSA (Kyo et al., 2000), some of these studies being also conducted in keratinocytes (Kyo et al., 2000; Kim et al., 2008b). However, it is rather c-Myc, alone, or in cooperation with Sp1, that is, more often associated with hTERT gene transcription in immortalized cells (Wu et al., 1999; Kyo et al., 2000), as well as in normal human mammary epithelial cells and human fibroblasts modified to over-express c-Myc (Wang et al., 1998; Greenberg et al., 1999). The absence of telomerase activity in normal mammary epithelial cells before the introduction of c-Myc (Wang et al., 1998) is consistent with our results as such mammary cells were cultured in the absence of a feeder layer. The feeder layer has been suggested to promote Sp1 stabilization and accumulation in the nucleus by increasing its glycosylation in cultured keratinocytes (Masson-Gadais et al., 2006), thereby preventing its degradation by the proteasome (Han and Kudlow, 1997; Duval et al., 2012). Elevated telomerase activity in keratinocytes was only observed when they were cultured with fibroblast feeder layers. The concomitant expression of Sp1 and telomerase activity reported in the present study suggests that Sp1 significantly contribute to hTERT gene expression in normal human keratinocytes. Indeed, suppression of Sp1 expression by RNAi in human skin keratinocytes not only caused a significant reduction in telomerase activity but also drastically reduced (and almost completely suppressed, in HaCAT/shSp1–2 transduced cells) hTERT gene transcription thereby supporting an important role played by Sp1. This result is further supported by the fact that suppression of Sp1 expression also eliminated the long-term growth of these cells, which is rather coherent with the fundamental role that Sp1 plays in the cell cycle progression (Abdelrahim et al., 2002; Grinstein et al., 2002). These results are also consistent with the site-directed mutagenesis experiment reported by Racek et al. (2005) who noted a severe reduction in hTERT promoter activity when all five Sp1 sites are mutated in vitro. It is noteworthy that similar patterns of Sp1 and hTERT, but not c-Myc expression was observed in uveal melanoma cell lines that also express the hTERT gene to various levels, which is also consistent with a major regulatory function for Sp1 in the transcription of that gene. Interestingly, it has been recently proposed (Zhang et al., 2012) that c-Myc activation may be insufficient to trigger the expression of hTERT primarily because c-Myc also co-stimulates the expression of E2F1, a multifunctional transcription factor that has been demonstrated to repress hTERT transcription by interacting directly with hTERT promoter (Crowe et al., 2001; Elliott et al., 2008; Lacerte et al., 2008). This is also consistent with the promotion of epidermal stem cell differentiation and depletion of stem cells reported when c-Myc is constitutively activated in the epidermis (Waikel et al., 2001; Frye et al., 2003).

The telomerase activity fluctuation we observed with keratinocytes passaging in culture could not be explained solely by the Sp1 expression variations we recently reported (Bisson et al., 2013) and suggests that other mechanisms must somehow contribute to post-translational regulation of hTERT besides a direct action of Sp1 to the transcription of the hTERT gene. Indeed, although a fairly good correlation can be observed between Sp1 expression and telomerase activity in Nb and Ad2 cells, no such conclusion can be reached for Ad1 cells (refer to Fig. 1 and Supplementary Fig. S2). Telomerase is a large ribonucleoprotein complex whose function can be regulated by telomerase-associated proteins after transcription. Among those regulators, the p23 and Hsp90 chaperones bind to hTERT and appear essential to the assembly of active telomerase (Holt et al., 1999; Forsythe et al., 2001). Hsp90 has been shown to bind the hTERT promoter thereby enhancing hTERT expression and telomerase activity in the oral cancer cell line SCC4 (Kim et al., 2008a). Beside, kinases that phosphorylate telomerase, such as PKCα and Akt, act as positive regulators of its activity (Li et al., 1998; Kang et al., 1999), while phosphorylation by c-Abl rather inhibits telomerase activity (Kharbanda et al., 2000). Post-translational modifications of hTERT therefore constitute an additional mean by which telomerase activity may be regulated and represent an attractive hypothesis to account for the loss of telomerase activity in keratinocytes cultured without a feeder layer. Interestingly, while hTERT glycosylation has not been documented to date, the MKRN1 ubiquitin ligase has been shown to interact with hTERT and might therefore promote hTERT ubiquitination and subsequent degradation, accompanied by a decrease of telomerase activity and a shortening of telomere lengths (Kim et al., 2005). Further investigation is needed to determine if the presence of feeder cells influence the post-translational state of hTERT, as well as its interaction with regulatory proteins.

In conclusion, our study demonstrates that culturing human keratinocytes with a feeder layer allows the preservation of telomerase activity and increases the lifespan of cultured keratinocytes derived from normal human skin of both adult and newborn subjects. Unraveling the mechanism by which the feeder layer extends the lifespan of human keratinocytes

through preservation of telomerase activity is an important step towards the improvement of keratinocyte cultures. The preservation of telomerase activity may explain the long-term survival of cultured epidermal sheets after grafting suggesting that stem cells are preserved during keratinocyte expansion on a feeder layer in vitro.

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