Skin Aging: A Role for Telomerase and Telomere Dynamics?

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Abstract: Skin is a complex tissue composed of two very different compartments – the continuously renewing epidermis made up mostly by keratinocytes and the underlying matrix-rich dermis with the resting fibroblasts as its major cellular components. Both compartments are tightly interconnected and a paracrine mutual interaction is essential for epidermal growth, differentiation, and tissue homeostasis. Skin aging is commonly viewed as wrinkle formation, hair greying, and impaired wound healing. Nevertheless, the epidermis as the outermost shield needs to remain intact in order to guarantee an inside-out and outside-in barrier function throughout life time of a human being. Furthermore, the epidermis is one of the few regenerative tissues that express telomerase, the ribonucleoprotein complex that can counteract telomere erosion, one of the presently mostly favoured potential mechanisms causing cellular aging. This raises the question whether in the epidermis telomerase is able to counteract telomere erosion and thereby to prevents a telomere-dependent aging process and consequently which part of the skin is responsible for the most obvious changes associated with skin aging.

TELOMERE HYPOTHESIS OF CELLULAR AGING

Besides a number of hypothesis, the telomere hypothesis of cellular aging has gained considerable interest suggesting that progressive shortening of the ends of the chromosomes, the telomeres, is the mitotic clock that determines the onset of replicative senescence in normal somatic cells (reviewed in [1] and see Hazel ***et al.*** this issue). The chromosomes are capped by specialized structures that are comprised of tracts of hexanucleotid TTAGGG sequences and a number of specific proteins. Due to deficiencies of DNA polymerase α to replicate linear DNA up to the outermost end during lagging strand synthesis, also known as endreplication problem, as well as oxidative damage, the telomeres continuously loose part of their sequences and it has been proposed that this loss is the liming factor for the replicative capacity of a cell, i.e. telomeric loss is the counting mechanism - the internal clock of aging [2, 3].

In order to proliferate indefinitely, the cells must prevent telomere erosion and this is mostly achieved by up-regulation or ***de novo*** expression of the ribonucleoprotein complex telomerase. This enzyme, which has a reverse transcriptase activity (hTERT), is able to add telomeric sequences to the outer most ends of the telomeres and thereby stabilizes or even elongates the telomeres. As earlier studies demonstrated that telomerase is expressed in about 90% of all tumours while expression is absent in many somatic tissues [4, see articles of Opitz, of Hahn, of Harley, and of Greenberg in this issue], it was reasonable to propose that telomerase is essential for the indefinite growth capacity of tumor cells and that, because of lack of telomerase, continuous and eventually fatal telomere loss is the inevitable consequence with age in normal somatic cells [5].

TELOMERASE IS EXPRESSED IN THE EPIDERMIS OF THE SKIN

While telomerase deficiency was described for a number of normal somatic tissues it is now well documented that all regenerative tissues which either proliferate continuously such as hemato-poietic cells or the epidermis of the skin or periodically such as the endometrium are telomerase-positive (summarized in [6]). However, since e.g. peripheral blood lymphocytes were shown to be prone to a statistically significant telomere shortening with age, these data led to the interpretation that telomerase was too low in these tissues and therefore insufficient to counteract telomere erosion.

In the epidermis of the skin the situation may be less clear. The epidermis is a stratified and well differentiated epithelium that continuously renews every 3 to 4 weeks. It is mainly (95%) composed of keratinocytes that, starting from stem cells, gives rise to transit amplifying cells. While stem cells only rarely divide and are believed to be the reservoir for the epidermal's life-long proliferation, the transit amplifying cells more rapidly proliferate and after 4 to 6 divisions are destined to enter a well defined differentiation program [7]. They leave the basal layer and ascend through the different suprabasal layers up to the stratum corneum. The terminal step of differentiation includes flattening of the cells,

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loss of all cell organelles, and formation of cornified envelopes. These cells than form several layers of dead horn squames that are finally shed from the surface of the skin.

The basal cells are tightly connected with the basement membrane that separates the epidermis from the underlying mesenchyme. While the epidermis is a cell-rich tissue, the dermis is a matrix-

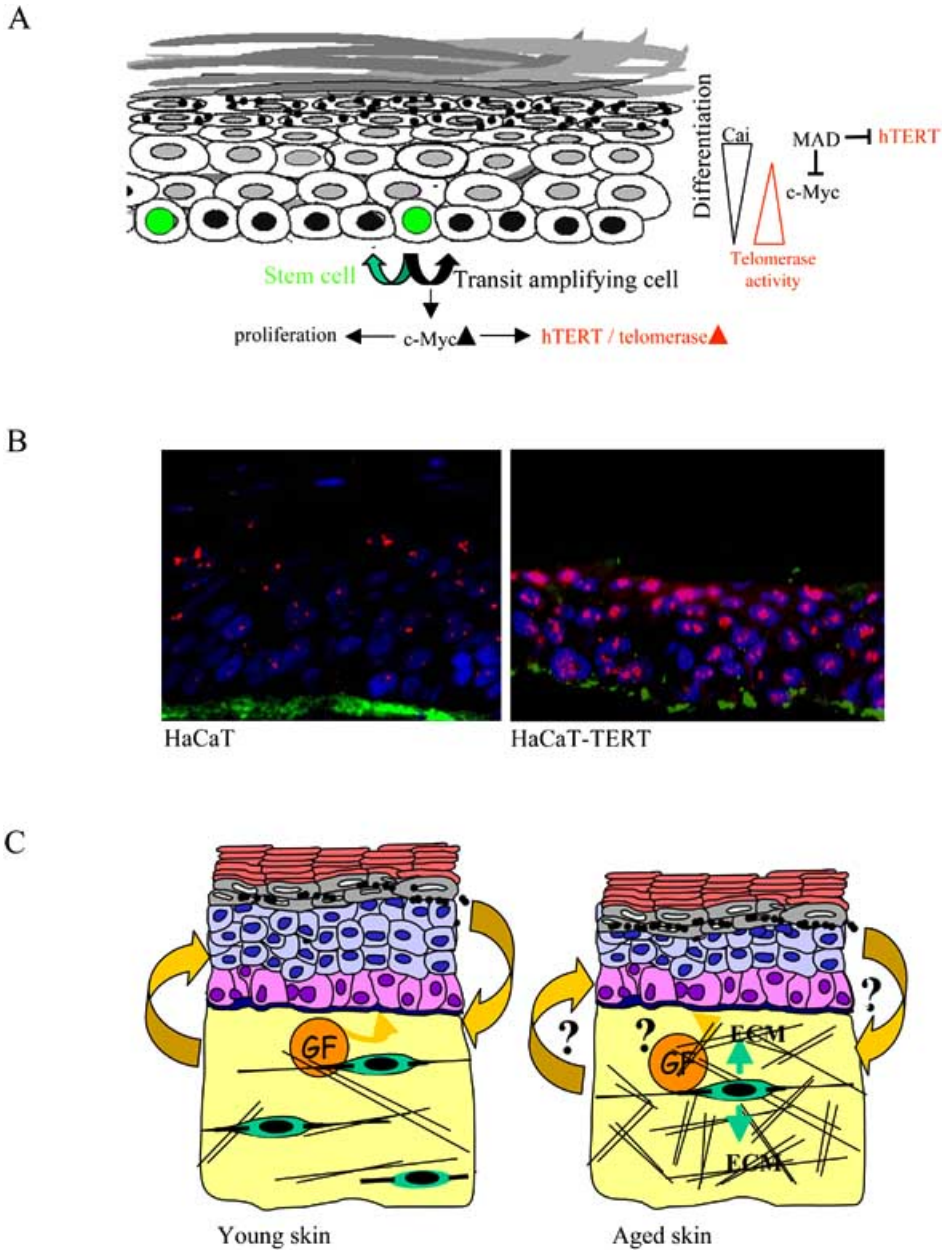

Fig. (1A). Schematical description of the stratified and terminally differentiated epidermis. In the basal layer the potential stem cells are marked in green and the transit amplifying cells in black. One potential stem cell division is indicated by arrows as is the potential up-regulation of c-Myc which results in increased proliferation as well as increase in hTERT/telomerase activity. Changes occurring during the differentiation process are marked on the right hand side. Intracellular calcium (Cai) is raising with differentiation while telomerase activity is decreasing. MAD is expressed in the suprabasal layers, replaces c-Myc, and inhibits hTERT transcription.

Fig. (1B). hTERT protein expression in organotypic cultures of HaCaT and HaCaT-TERT cells. Left: When HaCaT cells (spontaneously immortalized skin keratinocytes) [10] form a differentiated epithelium and frozen sections are labelled with an antibody against hTERT (Novocastra, 2C4), positive nuclei counterstained with Hoechst stain (in blue) can be detected in the basal and suprabasal layers. Note that the uppermost flattened cells show no staining. Right: HaCaT cells infected with hTERT are still able to stratify but lack terminal differentiation. Note the accumulation of hTERT protein in the uppermost cells (taken from [21]). The basement membrane is marked due to staining for collagen type IV (in green).

Fig. (1C). Schematic description of young (left) and aged skin (right). Note that the epidermis is thinner in aged skin, the dermis contains a reduced number of fibroblasts, increased amounts of extracellular matrix (ECM) and an increased number of fibres. While the paracrine interaction between epidermis and dermis in young skin is in part already well defined, little is known about this interaction in aged skin.

rich one with its major cellular component, the dermal fibroblast. Both, epidermal keratinocytes and dermal fibroblasts interact ***via*** expression of a number of growth factors and this regulatory loop is essential for the keratinocytes to continuously proliferate and to properly differentiate [8]. Thus, keratinocytes respond to a variety of environmental stimuli (matrix components, growth factors) in order to gain and maintain tissue homeostasis.

In the epidermis, telomerase is expressed in the basal layer and down-regulated upon differentiation in the suprabasal layers [9]. Trying to dissect the functionally different cells of the basal layer, there is increasing evidence that telomerase is low in the rarely dividing stem cells but increased in the more rapidly proliferating transit amplifying cells suggesting that telomerase is regulated with proliferation ([10]; Boukamp, unpublished results). This is also suggested from studies analysing different parts of the hair follicle [11]. Ramirez and co-workers showed that highest levels of telomerase were found almost exclusively in the bulb-containing fragment, the area of highest mitotic activity, while the bulge region, the area thought to comprise the stem cells, exhibited only weak telomerase levels. Similar as in other tissues, c-Myc is an important positive regulator of hTERT also in epidermal keratinocytes [reviewed in 12, 13]. Thus, up-regulation of telomerase from stem cell to transit amplifying cell is likely to occur in a Myc-dependent manner.

With differentiation telomerase is inhibited. Although the sequence of markers expressed during the process of epidermal differentiation is well defined since a long time, regulatory elements driving this process are only recently improve in our understanding [14]. One exception is calcium. It is well documented by a number of elegant early studies from Yuspa and colleagues that the shift from low to high calcium concentrations induces the expression of differentiation markers in cultured keratinocytes [15]. In agreement with this, it was also shown that in the epidermis ***in situ*** the calcium level rises significantly from the basal to suprabasal layers [16] implicating that calcium is a similar driving force of epidermal differentiation ***in vivo*** as ***in vitro***. A model of telomerase regulation in the epidermis is presented in Fig. (**1A**).

TELOMERASE IS PRESENT IN PROLIFERATING CELLS AND DOWN-REGULATED WITH DIFFERENTIATION

This prompted us to determine the role of calcium on telomerase regulation and we found that in cell free systems calcium but not e.g. magnesium was able to inhibit telomerase activity [10]. This inhibition could be abrogated by the calcium chelator EGTA and was reversible. A similar effect was obtained in intact cells when increasing the intracellular calcium concentration by thapsigargin and abrogating the calcium effect by pre-incubation with BAPTA thus demonstrating that calcium affects telomerase activity in keratinocytes. We also have evidence that this inhibition is mediated indirectly ***via*** calcium-binding proteins (Rosenberger ***et al.*** in preparation) and it is now tempting to speculate that calcium is a primary step in the differentiation-dependent inhibition of telomerase. This scenario is supported by early ***in situ*** hybridisation experiments studying hTERT expression in newborn foreskin [17]. Here it was shown that hTERT expression was not only found in the proliferatively active basal cells but also in suprabasal layers and the authors suggested that hTERT is present both in actively cycling and in resting cells. Accordingly, hTERT protein can be detected in nearly all suprabasal spinous layers but not the flatted terminally differentiated ones (Fig. **1B**).

By interfering with the telomerase complex, this calcium-dependent inhibition of telomerase activity is still reversible. Only in a second and irreversible step, hTERT transcription is inhibited due to transcriptional repression by MAD [18], the expression of which is increased with differentiation and is expressed in the suprabasal layers of the epidermis [19, 20] (Cerezo and Boukamp, unpublished results). The early reversible inhibition of telomerase is likely to be of functional importance for the keratinocytes which upon e.g. wounding can regain proliferative activity. Furthermore, when hTERT regulation was abrogated due to constitutive over-expression in the immortal HaCaT skin keratinocytes, the terminal step of differentiation, i.e. formation of a stratum granulosum and a stratum corneum were prevented [21].

AGING OF CULTURED EPIDERMAL KERATINOCYTES CORRELATES WITH TELOMERASE DOWN-REGULATION AND PROGRESSIVE TELOMERE EROSION

How does aging affect the epidermis? ***In vitro*** propagated keratinocyte were shown to suffer from continuous telomere shortening [22] (Moshir ***et al.*** submitted) correlated with a rapid down-regulation of telomerase activity [23] (Moshir ***et al.*** submitted). So far, it is still a matter of debate whether this finally leads to telomere-dependent cellular aging or whether a tissue culture/stress-dependent checkpoint pathway that represents a telomere-independent mechanism of replicative aging is responsible for cellular senescence of the keratinocytes as recently discussed by Wright and Shay [24]. In accordance with that, Counter ***et al***. [25] showed that keratinocytes expanded in culture from skin biopsies and allografted to severely burned patients exhibited significantly shorter telomeres compared to non-cultured skin from the same individual as well as compared to healthy (>80 year-old) donors. Unfortunately, telomerase activity was not measured in the cultured

keratinocytes and after transplantation but the authors determined telomere lengths of skin biopsies from healthy donors of similar age [25]. Due to the high variability in telomere length between individuals, even between newborns [26], however, it still remains open whether the telomere shortening is a mere consequence of ***in vitro*** expansion or continues to occur during ***in vivo*** expansion.

Own data suggest that telomerase is reactivated in keratinocytes when allowing them to grow in organotypic cultures and to form a stratified and well differentiated epidermis [21] (Moshir ***et al.*** submitted). So far we have not follow telomere length regulation under these conditions and it, therefore, must remain elusive whether the level of telomerase is sufficient to hinder further telomere loss. Irrespective of that, however, the finding that telomerase is reactivated demonstrates that organotypic cultures provide a growth situation and environment - epithelial-mesenchymal interaction, polarity of the epithelial cells, and interaction with the extracellular matrix - similar to the situation ***in situ*** and therefore would be superior to conventional cultures which are inadequate to unravel the role of telomerase for the aging process of epidermal keratinocytes in the intact skin.

AGING OF THE SKIN: HAIR GREYINGAND IMPAIRED WOUND HEALING

What are the typical signs of skin aging? In skin the most obvious changes are profound textural (steady increase in wrinkles and skin thinning) as well as pigmentary changes. Accordingly, hair greying and hair loss (alopecia) are commonly used as typical criteria for skin aging also in mouse models. In addition, delayed or impaired skin wound healing is used to characterize aging. Correspondingly, late generation mice (6^{th} generation mice) deficient for telomerase due to disruption of the mouse telomerase RNA gene (mTERC-/-) exhibit an early onset of hair greying, alopecia, and reduced capacity to respond to wound healing [27].

This phenotype was even more pronounced in mice lacking both telomerase and the ataxia-telangiectasia gene (***Atm***), causing a generalized proliferation defect [28]. Not only were hair greying and alopecia increased but there was also a significant delay in hair re-growth. A similar severe phenotype was described for mTERC-/- mice crossed with mice deficient for the gene, which is mutant in the premature aging Werner syndrome - the ***WRN*** helicase, which is involved in recombination, replication and repair [29, 30]. These mice suffered from premature hair loss and impaired healing of acute wounds as measured by a delay in wound closure and a reduced number of cells that could be labelled with 5-bromo-deoxyuridine in the sites of re-epithelialization [31]. On the other hand, no skin effect was described for mice constitutively expressing mTERT [32] as well as mice expressing mTERT specifically in epithelia (under the keratin 5 promoter, see [33]). Instead, these latter showed an increased wound healing rate, suggesting that telomerase actively promotes proliferation in the skin keratinocytes.

SKIN AGING A MATTER OFMELANOCYTIC AND DERMAL CHANGES

Colour of the epidermis and hair is provided by melanocytes, which, derived from neural crest, are localised in the basal layer of the epidermis and produce melanin that is distributed to the keratinocytes (reviewed in [34]). Here it serves as a supranuclear cap to protect the nucleus from environmental damage, e.g. UV radiation [35]. Also in hair, melanocytes are responsible for transferring pigment to the differentiating keratinocytes and thus for determining hair colour. Consequently, hair greying is due to a reduced number of melanocytes allowing only fewer melanosomes to be incorporated into cortical keratinocytes of senile hair shafts. Eventually, no melanogenic melanocytes remain in the hair bulb (reviewed in [34]). Thus, hair greying is an exhaustion phenomenon and if at all an aging rather than a differentiation phenomenon of melanocytes. Since no telomere length studies exist from melanocytes of young and aged individuals it still remains to be resolved whether a telomere length-dependent mechanism is responsible or whether the decrease in melanocytes is due to other as yet unknown processes.

Of interest in this context is the extensive melanogenic activity of an only limited number of pigmented bulbar melanocytes. The melanocytes can reside in the hair bulb for up to 10 years. Thereafter they are replaced by melanocytes derived from an outer root sheath reservoir. Thus, melanocytes are resting cells and in agreement with that no telomerase activity could be detected in cultured normal melanocyte [36, 37], while an increase was seen with progression from nevi to melanomas [38, 39].

The second characteristics of aging skin is impaired wound healing. An early and important step during wound healing is that macrophages and fibroblasts express embryonic fibronectin that serves as a primary extracellular matrix in order to facilitate wound repair [reviewed in [40]. This matrix provides the basis for a different set of fibroblasts that then are able to secrete migration-stimulating factors. Thus, the onset of wound healing is performed by macrophages and fibroblasts, again cells that generally only proliferate rarely and are commonly viewed as telomerase activity-negative. Interestingly, the balance between matrix synthesis and degradation is impaired during skin aging and it is well demonstrated that senescent cells display an altered pattern of matrix metalloproteinases, a group of enzymes responsible for remodelling the collagen and elastin matrix [reviewed in 41]. It was

further suggested that impaired wound healing resulted from the accumulation of senescent cells with an altered cellular phenotype and a decreased ability to divide in response to damage and/or cell loss [42-44]. Stephens ***et al***. [45], however, found that chronic wound fibroblasts demonstrated no signs of senescence. Instead, they often diametrically opposed from ***in vitro*** senesced fibroblasts. Mendez ***et al***. [46], on the other hand, showed that fibroblasts cultured from venous ulcers displayed characteristics of senescence including a reduced growth rate. They could also show that neonatal foreskin fibroblasts treated with venous ulcer wound fluid significantly increased the number of aged cells and reduced the growth rate. All this suggests that for impaired wound healing of venous ulcers disease-specific alterations rather than aging phenomena of the wound fibroblasts as well as the environment with pro-inflammatory cytokines must be considered.

Similarly, remodelling the dermis in aged skin is altered due to illegitimate crosslinking of the collagen and elastin fibrils thereby loosing elasticity and gaining an inflextible texture including wrinkles (Fig. **1C**). The intrinsic age-dependent effect is difficult to dissect from UV-induced chronical damage where it could be shown that long-term exposure to artificial UV radiation caused a disorientation of the collagen fibres [47]. Irrespective of that, however, alterations in the matrix also cause an altered interaction with the fibroblasts and consequently this also alters their expression profile. Since the epidermal keratinocytes are dependent on the "proper" interaction with the dermal fibroblasts, the massive changes in the dermis are likely to be of profound consequence for epidermal growth and differentiation and may in part account for the changes seen in the epidermis (Fig. **1C**).

CHARACTERISTICS OF EPIDERMAL AGING

So, what are the most prominent age-dependent changes in the epidermis? One obvious change is flattening of the dermo-epidermal junction (see Fig. **1C**). While in young skin epidermis and dermis are interwoven by intradermal villous cytoplasmatic projections, also called rete ridges, these are lost in aged skin thereby explaining the increased fragility to shear stress and blister formation [reviewed in 48]. The reason for this structural change is, however, still elusive. Accordingly, one cannot exclude at present that remodelling of the dermal matrix as described above is at least in part responsible for flattening of the dermo-epidermal junction.

A second criterion is an overall thinning of the epidermis (reviewed in [48], see Fig. **1C**). Interestingly, this epidermal atrophy seems to affect predominantly the stratum spinosum, the early differentiation stage, while the stratum granulosum and the stratum corneum, important for skin function, that is protection against water loss and influx of bacteria and chemicals, remains largely unaltered [49;50]. Trans-epidermal water loss as measured by water vapour is thought to increase with age and thus to account for drying of aged skin. However, it was recently shown that water loss is low at young age, increased during young adulthood and maturity, and decreases again in old age [51]**.** Thus, despite epidermal thinning with age, the reason for which is still elusive, these data do not allow to conclude that only young skin is well hydrated and has a good barrier function. Instead, also aged epidermis still seems to function properly.

Despite the reduced thickness of the epidermis with aging a nearly constant turnover rate of the keratinocytes is maintained throughout the life span of a human being [40] and the thickness of the stratum corneum does not change with age [52]. Accordingly, keratinocytes even from elderly individuals are still able to form a nice epidermis in organotypic cultures and when pre-selected for potential stem cells they still exhibit a life span largely exceeding that of mass population of younger donors (Boukamp, unpublished observation). Nevertheless, it was shown that the number of senescent cells increases with age. Dimri ***et al***. [42] described a special form of ß-galactasidose (SA-ß-Gal) which can be depicted at pH6 and which now commonly serves as a biomarker of replicative senescence in some tissues. In the epidermis of young donors, they found only few SA-ß-Gal-positive cells in the basal layer in half of the samples, while SA-ß-Gal-positive cells were always seen in the epidermis of old donors and the number was generally increased. Sparsely and randomly distributed SA-ß-Gal-positive cells were also seen in the dermis of old donors, tentatively identified as fibroblasts [42]. Interestingly, the epithelium of the hair follicles, associated sebaceous glands, and eccrine glands and ducts were generally positive and this was independent of donor age. Since telomere length is difficult to measure in these individual cells, it still remains to be determined what SA-ß-Gal-positivity stands for and why it recognizes all hair follicle keratinocytes but only "aging ones" in the interfollicular epidermis.

IS THE EPIDERMIS TRULY AGING?

As an outer shield, the epidermis has to cover and protect the body. Consequently, the turnover rate remains nearly constant throughout the life time of a human being. So far, there is no evidence for loss of telomerase activity with aging ***in vivo*** and in agreement with that recent findings do not provide evidence for a massive and statistically significant age-dependent decline in telomere length ([53]; Moshir ***et al.*** submitted). This seem to contradict earlier reports where an age-dependent telomere loss was described for total skin [54; 55] as well as isolated epidermis [56]. In this latter study a

yearly telomere erosion rate of approximately 36 base pairs (bp) was calculated.

Interestingly, the authors observed large differences in telomere length between eight neonates ranging from 11.7 to 14.8 kb. Thus, telomere lengths of 15.9 kb in a 51, 12.3 kb in a 68, 10.7 kb in a 92, and 11.1 in a 101 year-old donor, as shown in their study, are still in that common range [56]. Our study, on the other hand, showed a rather constant mean telomere length of about 10 kb for keratinocytes from six new borns (Moshir ***et al.*** submitted). However, we observed large variations in mean telomere lengths between young adult individuals of similar age. Whether this variance is genetically determined [57-59] or may be due to stress-dependent factors including extensive UV-damage (for review see [60]) still needs to be determined. The relatively low erosion rate with 36 bp per year calculated by Nakamura ***et al***. [56], however, seems rather minimal in the light of these high variations and when taking into consideration that the epidermal turn-over rate is 3 to 4 weeks throughout the entire life. This is even more questionable when considering that the stem cells are supposed to be reduced in number with aging and thus the remaining cells have to replicate more often in order to guarantee epidermal integrity.

Telomere maintenance mechanisms are particularly crucial for the epidermis. The epidermis is prone to a number of damages as well as acute and chronic stress. These include in particular chronic UV exposure which damages DNA preferentially at guanine residues. In agreement with the high content of G residues in telomeres, UV exposure was shown to accelerate telomere shortening [61]**.** This also could explain our finding that in some skin samples, particularly from donors around the age of 30, the mean telomere length varied significantly and in part was only 5 kb while skin samples from elderly donors still exhibited a mean TRFL > 8kb (Moshir ***et al.*** submitted). In order to prevent premature telomere exhaustion, telomerase could thus particularly be required for "repair of such damaged telomeres" and in agreement with that earlier findings demonstrated increased telomerase activity in sun-exposed ***versus*** sun protected skin areas [62, 63]. Counteracting and thereby restoring a certain telomere length may, therefore, be essential for epidermal keratinocytes to allow life-long proliferation and with that life-long overall function. This interpretation is also substantiated by a recent publication demonstrating that stress has a strong impact on telomere length of peripheral blood lymphocytes [64]. On the other hand, when epidermal keratinocytes are propagated in culture, ***in vitro*** growth is correlated with loss of telomerase activity and this in turn correlates with a telomere erosion rate largely exceeding the 36 bp per population doubling ([67, 68]; Moshir ***et al.*** submitted). Miyata ***et al***. [69] calculated a telomere erosion rate of about 80bp and this was independent of culture conditions used. If this is roughly correct, an erosion rate of only 36 bp per year ***in vivo*** largely argues in favour of our hypothesis that telomerase is active in epidermal keratinocytes ***in situ*** - if not to stably maintain telomere length to at least minimize telomere erosion and to counteract telomere loss in the case of massive damage.

CONCLUSION

Taken together, the presently most favoured telomere hypothesis of cellular aging suggests that as a consequence of continuous telomere erosion critically short telomeres cause telomere dysfunction and thereby induce cellular senescence. The skin provides an interesting example to critically examine whether this hypothesis may also account for ***in vivo*** aging. On the one hand, the continuously proliferating epidermal keratinocytes express telomerase and thus may at least in part be able to counteract telomere loss. On the other hand, the dermal fibroblasts or neural crest-derived melanocytes only rarely proliferate and thus telomere erosion seems to be less prominent than other stress-related mechanisms. Furthermore, as outlined above, many age-related processes primarily affect the dermis and only as a consequence of their essential mutual interaction with the epidermis may also cause the epidermis to suffer from these changes. Thus, skin aging ***per se*** may be a dermal rather than an epidermal phenomenon. This may further suggest that in addition to the replication-dependent telomere erosion as established for e.g. cultured fibroblasts and keratinocytes, other telomere-independent mechanisms may account for the age-related processes in the skin.

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