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Review

Telomere length maintenance in stem cell populations

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ABSTRACT

The maintenance of telomere length is essential for upholding the integrity of the genome. There is good evidence to suggest that telomere length maintenance in stem cell populations is important to facilitate the cell division required for tissue homeostasis. This is balanced against the requirement in long lived species for proliferative life span barriers for tumour suppression; the gradual erosion of telomeres provides one such barrier. The dynamics of telomeres in stem cell populations may thus be crucial in the balance between tumour suppression and tissue homeostasis. Here we briefly discuss our current understanding of telomere dynamics in stem cell populations, and provide some data to indicate that telomeres in human embryonic stem cells may be more stable and less prone to large-scale stochastic telomeric deletion.

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1. The importance of telomere length in stem cell populations

The regenerative capacity of human tissues declines with age and the incidence of cancers increases; both these processes may be driven by a decline in stem cell function [1–5]. There has to be a balance, in long-lived organisms such as humans, between maintaining regenerative potential on one hand, and tumour suppression on the other. Several studies focusing on $p16^{INK4A}$, a key mediator of replicative senescence, suggest that this may be the case. In the bone marrow of mice, $p16^{INK4A}$ levels increase with age in haematopoietic stem cells, but not in other cell types [6]; this correlated with a decline as a function of age, in the ability of these cells to reconstitute the immune system of irradiated mice, whereas $p16^{INK4A-/-}$ mice had more stem cells and were better able to reconstitute an immune system. Similar observations were made in neurons from the forebrain, where again $p16^{INK4A}$ levels increased with age, which correlated with a decline in proliferation, this was to some extent ameliorated in $p16^{INK4A-/-}$ mice [7]. As $p16^{INK4A}$ is a tumour suppressor, the deficiency of protein leads to an increased frequency of cancer [8], and yet these data show that the absence of $p16^{INK4A}$ can slow most aspects of stem cell ageing, thus highlighting the balance between tumour suppression and regenerative capacity. This is likely to result from multiple factors such as Bmi1 that promote proliferation, balanced against tumour suppression for example by $p16^{INK4A}$ [9–11]. One mechanism that may contribute to fine tune this balance may be telomere length, whereby stem cells may need to maintain telomeres at a length that provides sufficient replicative capacity for tissue homeostasis, versus the requirement to minimise telomere length and replicative capacity as a tumour suppressive mechanism.

Telomere length is an important determinant of telomeric function, in the presence of a functional DNA damage response short telomeres elicit a DNA damage checkpoint, that leads to either replicative senescence or apoptosis [12]. Telomere-dependent replicative senescence represents the tumour suppressive function of telomeres [13,14]; the corollary of which may be an age-related accumulation of senescent cells [15,16]. The presence of senescent cells not only reduces the proportion of mitotically active cells but may also, but by virtue of exhibiting a more catabolic phenotype they can actively degrade the tissue microenvironment [17]. Interestingly even the presence of a small proportion of senescent cells, may render the tissue microenvironment more permissive for tumour progression [18]. The importance of telomere length from the standpoint of ageing and cancer is exemplified by the telomerase knock out mice. Where after several generations telomeres erode sufficiently to confer a phenotype, whereby the presence of short telomeres leads to defects in proliferative tissues that mirror some age-related phenotypes in humans. In addition to a shortened lifespan these include, immunosenescence, alopecia, hair greying and intestinal atrophy and increased rate of tumour formation [19–22]. These phenotypes could be partially rescued in the context of mutations in the DNA damage checkpoint responses, for example the absence of p53 rescued some of the proliferative defects, but this was accompanied by an increase in the rate of tumour formation [23]. Interestingly in the context of Terc and p21 mutations, the rescue of age related phenotypes was not accompanied by an increase in tumourigenesis, despite rescuing proliferative and self renewal detects in stem cell populations; these observations indicate that, p53/p21 dependent, telomere driven, replicative senescence was driving the age-related

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phenotypes, and furthermore, that apoptosis may be tumour protective [24]. Some of the phenotypes exhibited by the telomerase knockout mice mirror those observed in individuals with dyskeratosis congenita (DC): a disease characterised by mutations in genes that result in impaired telomere length maintenance, such as components of the telomerase complex that results in haploinsufficiency for telomerase activity [25–27], or components of the shelterin complex [28]. DC patients exhibit reduced haematopoiesis, reduced number of haematopoietic progenitors and colony forming ability [29,30], and chromosomal instability phenotypes [31,32]. In addition, like telomerase knockout mice, DC patients display disease anticipation, whereby the severity and age of onset of the disease, get progressively worse from one generation to the next [33]. Failure of telomere length maintenance has been implicated in other bone marrow failure syndromes including Fanconi anaemia, Shwachman–Diamond syndrome, Diamond–Blackfan anaemia and aplastic anaemia [34–37]. Thus clinical data together with mouse models indicate telomere length maintenance is important in the context of human disease, the ageing process and cancer; it appears likely that telomere length maintenance in the stem cells compartments may play a crucial role in these processes.

2. Telomere length dynamics in human stem cell populations

Data concerning telomere length and telomerase activity in stem cells is limited. Adult stem cell populations undergo cell division comparatively infrequently [38] and therefore, based on the end replication problem, stem cells would be predicted to suffer limited telomere erosion, irrespective of telomerase expression. The most extensively studied stem cells are those of the haematopoietic system. Haematopoietic stem cells (HSCs) are known to express telomerase [39,40] yet they appear to be subjected to telomere loss as a function of age [41]; telomerase activity is insufficient to maintain telomere length in HSCs and thus the proliferative potential of these cells may decline with age. However telomerase is up regulated following immune stimulation of B and T cells [42–46], this is sufficient to reduce the rate of telomere erosion, and some cases result in telomere lengthening [47–49]. Importantly the up regulation of telomerase activity following stimulation, confers a proliferative lifespan extension compared to cells that do not express telomerase, this allows for repeated clonal expansions in response to the antigen [48]. However this is not unlimited and ultimately telomere erosion and the loss of proliferative potential may underlie some aspects of immunosenescence [49,50].

In the epidermis, telomerase is expressed by cells specifically on the basal layer [51], yet telomere length decreases with age [52]. Spermatogonial stem cells express high levels of telomerase [53–55], this is considered to maintain telomere length in the germline for subsequent generations. Indeed to date the male germline is the only tissue that has shown to exhibit telomere lengthening as a function of age [56,57]. There is evidence to suggest that there is a gradient in telomere length from the stem cell compartments which decreases in more differentiated cell populations. This was exemplified by the lingual mucosa, where *in situ* hybridisation revealed the longest telomeres in the basal cells [58]. Further evidence using *in situ* methods in mice also showed distinct gradients of telomere length, with the longest telomeres observed in the stem cell compartments of the skin, small intestine, cornea, testis and brain [59]. Of particular importance, this work included the observation that the telomere length in the stem cell compartments appears to shorten with age [59]. Thus the evidence from humans and mice, indicates that stem cells maintain telomeres at a longer length, relative to the other cells within the tissue in which they reside. This is probably a function of a combination of telomerase activity together with a reduced cell turnover. However, with the exception of spermatogonial stem cells, some stem cell compartments have been shown to suffer telomere erosion as a function of age.

Telomerase activity is likely to be limiting in most cells that express telomerase at physiological levels. Over expression of the RNA and protein components of telomerase individually does not result in significant telomere lengthening [60]. However when both components are over expressed simultaneously, these super-telomerase cells exhibit ongoing telomere lengthening with no apparent length control. These data imply that in cells where telomere length is stable telomerase levels are limiting; it is the shortest telomeres in the cells that are preferentially lengthened [60,61]. This must require a fine balance in the levels of telomerase activity to maintain telomere length; therefore subtly lower levels, such as may be observed in stem cell compartments, could lead to gradual telomere erosion. Whether telomere length would become limiting in this situation is unclear; telomerase preferentially extends shorter telomeres [61] and thus telomeres could erode to a new shorter, but stable state, as observed in many cancer derived cell lines [60]. Furthermore mice that are haploinsufficient for either the RNA or protein components of telomerase maintain telomeres at a shorter length but do not exhibit obvious direct telomere defects, such as telomere fusion [62,63].

3. Telomere instability

Telomeres shorten primarily as a consequence of gradual end-replication losses with ongoing cell division [64,65]. However more detailed analysis of telomere dynamics in human cells has indicated the existence of additional mechanisms that generate sporadic large-scale changes in telomere length [66]. In normal human cells, rare telomeres have been observed that lack signals from fluorescently labelled telomere repeat containing probes; these may arise as consequence of sporadic large-scale telomeric deletion events [67–69]. Using an experimentally transformed cell line carrying a tagged chromosome end, Murnane et al. observed changes in telomere length which appeared to have resulted from large-scale telomere deletion events [70]; other types of events included the deletion of the entire telomere and adjacent DNA coupled with healing of the end, possibly by de novo telomere addition, or chromosomal fusion events [71]. Interestingly these events occur in cells that express telomerase and can maintain telomere length for extended periods in culture, yet they can still suffer telomeric instability. Single molecule PCR analysis of telomere length has shown that in the absence of telomerase, gradual telomere erosion results in a decrease in the mean and an increase in the variance of the distribution [65,72]. These data are consistent with the telomere dynamics predicted as a consequence of end-replication losses, together with a putative C-strand resection [64,65]. However, superimposed on the gradual erosion of the bulk of the telomere length distribution, were large-scale, apparently sporadic, telomere deletion events. These events have been observed both in the presence or absence of telomerase and can result in telomeres containing less than 20 TTAGGG repeats [72,73]. The occurrence of these deleted telomeres is sporadic, and they do not accumulate with ongoing cell division. This indicates that, either the cell in which the deletion event occurred exited the cell cycle, or alternatively the deleted telomere was not long enough to confer telomeric function and was subjected to further processing. Such telomeres could be repaired to full length or subjected to telomere fusion. Indeed fusion analysis has revealed that telomeres that had suffered a deletion event are subjected to fusion [74]. Importantly in normal cells that contain a complement of telomeres that are long and fully functional, rare fusion events are detected that involve very short telomeres. These events are consistent with the concept that normal cells can be subjected to stochastic telomeric deletion, and these deleted telomeres can undergo fusion. This implies that cells within normal tissues *in vivo* may be subjected to telomeric deletion events, that can lead, via anaphase bridging, breakage and fusion events, to large-scale, potentially oncogenic mutation. This cell intrinsic mutational load

may be particularly significant in the context of cell cycle checkpoint mutations, where a telomere that had suffered a deletion event, can be subjected to fusion, rather than causing the cell to exit the cell cycle. Indeed there are clonal patches of keratinocytes within the epidermis, that exhibit p53 mutations [75–77] and thus short dysfunctional telomeres in these cells may exhibit a greater propensity to undergo fusion than in keratinocytes with functional p53. These data indicate that it will be informative to understand the dynamics of telomere length in stem cell populations. To this end, we have some preliminary data to suggest that at least some stem cell populations may exhibit a greater degree of telomere stability compared to other cell types that have been analysed. Fig. 1 shows an example of this data, where human embryonic stem (hES) cells have been subjected to a large scale single telomere length analysis. At the 17p and XpYp telomeres the mean telomere length at both ends was broadly similar at 13.0 and12.8 kb respectively, this is within the range observed in the male germline [56]. A bimodal distribution was apparent at the XpYp telomere, which is consistent with the presence of two telomeric alleles that are maintained at different lengths of 6.9 and 15 kb; a telomere length differential of 8.1 kb. These data are consistent with the concept that the telomere length setting of specific alleles, in the presence of telomerase, may be determined by *cis* acting elements or allele specific epigenetic modifications [73,78,79]. Telomere length of the hES was heterogeneous with telomeres ranging from 3.0 kb to over 25 kb. However it was striking that we could not observe a single telomere out of a total sample of 753 molecules that was less than 3.0 kb (Fig. 1). In fibroblast cell cultures sporadic telomere deletion results in telomeres of less than 10 TTAGGG repeats at a frequency of 9×10^{-3}; these are telomeres that have been shown to undergo fusion [74]. Compared to these cells, the paucity of stochastic telomeric deletion events in our sample of human embryonic stem cells is significant ($P<0.001$, Chi sq.). These data indicate the possibility that human embryonic stem cells may exhibit enhanced telomere stability. Consistent with this view, whilst some human ES cells display karyotypic changes following prolonged periods in culture, the predominant aberrations are aneuploidy, specifically gains of chromosomes 17, 12 and X, with less evidence of non-reciprocal translocations that occur as a consequence of telomere dysfunction [80]. The mechanisms underlying telomere deletion events is not clear [81], but may include mechanisms such as T-loop recombination [68,82], unequal sister chromatid exchange [83], replication slippage, or resolution of stalled replication forks at sites of unrepaired damage or DNA structures, such as G-quartets, that cannot be replicated [84]. Thus the mechanism by which human embryonic stem cells may exhibit enhanced telomeric stability is also not clear, however in this context it is pertinent to note that these cells exhibit enhanced genomic maintenance in humans [85], and lower levels of reactive oxygen species in mice [86,87]. DNA damage, including that generated by oxidative stress, may provide an obstacle to telomeric replication, the resolution of which could initiate large-scale changes in telomere length. Adult stem cells may not exhibit the same levels of genomic stability compared to embryonic stem cells; it will therefore be informative to understand how levels of telomeric stability differ between these cell types.

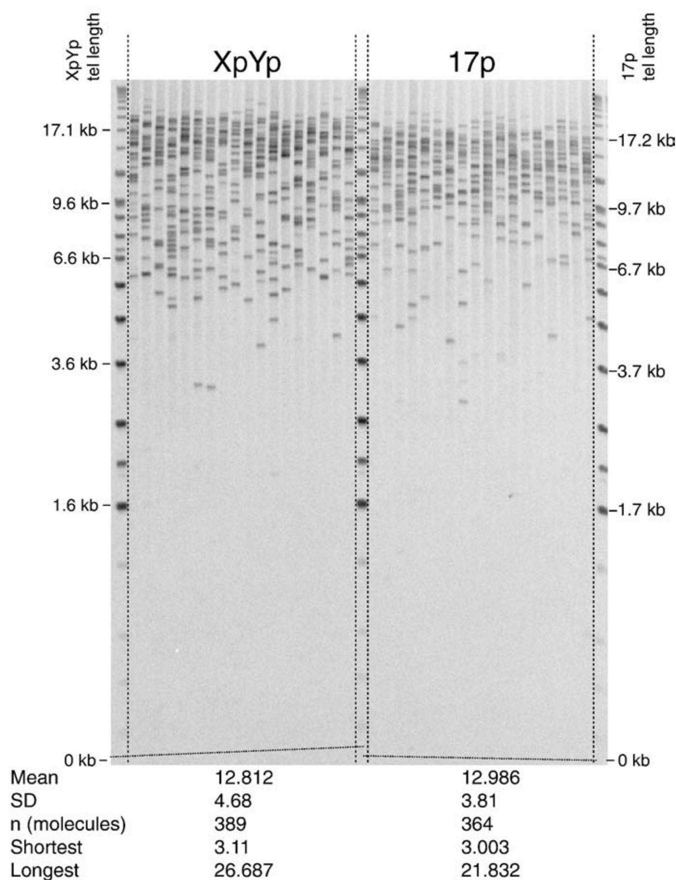

Fig. 1. Telomere length analysis of the human embryonic stem cell line, HUES9. Large scale analysis of telomere length at the XpYp and 17p telomeres using single telomere length analysis (STELA). Telomere length statistics are shown below.

4. Chromatin structure and genomic organisation in stem cells

If the apparent stability of telomeres in hES cells, and indeed other stem cell types, is borne out by further analysis, the mechanistic basis of telomeric stability would warrant investigation. In addition to the enhanced levels of DNA repair one could speculate that telomeric stability could be related to differences in chromatin distributions and/or differences in nuclear organisation. The organisation of loci within the nucleus can regulate transcription, with different cell types displaying distinct nuclear organisation patterns [88,89]. Stem cells are no exception, as they display a nuclear architecture, such that loci required for the maintenance of pluripotency, display specific nuclear locations; for example NANOG on 12p has a more central location in ES cells compared to differentiated cells, or 6p that maintains its chromosome position but the OCT4 locus on that chromosome is outside of its usual position [90]. It is known from studies in yeast that the tethering of telomeres at the nuclear periphery is important to regulate transcription of telomere associated genes such as the mating-type loci [91], which may be mediated by regulating the telomeric chromatin [91]. It also appears that embryonic stem cells exhibit a distinct chromatin structure, that is important for controlling the expression profiles required to maintain pluripotency [92–95]. ES cells are uniquely characterised by a prevalence of a bivalent chromatin state, in which many developmentally regulated genes simultaneously possess both activating and silencing epigenetic chromatin modifications [96]. Whether these differences in chromatin extend to the telomeres is not clear. It is becoming apparent that the chromatin status of telomeres, which exhibit epigenetic marks of compacted heterochromatin, can control telomeric stability [78,79,97,98]. For example in mice the subtelomeric regions are heavily methylated, but mammalian telomere repeats lack the CpG sites for methylation. Interestingly the knockout of DNA methyltransferases results in large-scale changes in telomere length, that may be associated with increased recombinational activity including sister chromatid exchange [98]. Thus chromatin structure contributes to telomeric stability, and specific chromatin patterns in stem cells may confer enhanced telomere length stability.

In summary, telomere length in stem cell populations is an important determinant of the replicative potential that is required for tissue homeostasis, yet telomere length may decline as a function of

age. The maintenance of telomere length is determined by a combination of stem cell turnover and limiting levels of telomerase, possibly also with enhanced telomere stability. More studies of telomere length dynamics in human stem cells are required for us to fully understand the role that telomeres may play in stabilising the genomes of these cells and how this may relate to the age-related deterioration in stem cell function.

5. Human ES cell culture

The hESC line HUES9 (hES facility; Harvard University, Cambridge, MA, USA) derived in accordance with local and national guidelines was used. hESCs were cultured and passaged on a layer of irradiated mouse embryonic fibroblasts at a density 1×10^6 cells/60 mm dish and passaged by enzymatic and mechanical means [99]. To remove contaminating feeder cells prior to DNA extraction, hESCs were first passaged twice onto matrigel coated plates (BD Biosciences; Oxford UK; http://www.bdeurope.com) with feeder conditioned culture medium. Culture medium (KSR) consisted of Knockout Dulbecco's Modified Eagle's Medium (KO-DMEM) supplemented with 20% Serum Replacement, 1% Non-Essential Amino Acids, 1 mM L-Glutamine, 0.1 mM β-mercaptoethanol (all from Invitrogen; Carlsbad, CA, USA; http://www.invitrogen.com) and 10 ng/ml human FGF2 (R and D systems; Minneapolis, MN, USA; http://www.rndsystems.com).

6. DNA extraction and single telomere length analysis

DNA was extracted by standard phenol chloroform proteinase K method as described previously [72]. Single telomere length analysis was undertaken at the XpYp and 17p telomeres as described previously [74]. Each telomere was analysed using 18 reactions each containing approximately 21 telomeric molecules.

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