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Short communication

Replicative senescence is distinguishable from DNA damage-induced senescence by increased methylation of promoter of rDNA and reduced expression of rRNA

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ABSTRACT

Human fibroblasts become senescent after a limited number of replications or by diverse stresses, such as DNA damage. However, replicative and damage induced senescence are indistinguishable in respect to proliferation cessation and expression of senescence markers, senescence-associated β-galactosidase, *p16* and *p21*. Here, we show that senescence types can be distinguished by reduced levels of 18S, 5.8S and 28S rRNA, in replicative but not induced senescence. We also demonstrate that promoter region of rRNA is hypermethylated in replicative senescence. The findings show that expression level of rRNA or methylation of its promoter can be used to distinguish between senescence types.

Hayflick et al. (Hayflick and Moorhead, 1961) discovered that, after a limited number of divisions, fibroblasts experience a permanent loss of cell proliferation and enter a state of replicative senescence. Besides replicative senescence which occurs in aging tissues, mitogenic signals, oxidative stress or other types of damage including DNA damage lead to senescence which remains largely indistinguishable from replicative senescence (Bielak-Zmijewska et al., 2018; Hewitt et al., 2012). Senescent cells acquire multiple phenotypic changes including morphological changes leading to the expansion of their cytoplasm (Scharffetter-Kochanek et al., 2000), telomere shortening (Hewitt et al., 2012), increased expression of senescence markers including senescence-associated β-galactosidase (SA-β-Gal) (Dimri et al., 1995) and of cyclin-dependent kinase inhibitors including p16 and p21 (Ritschka et al., 2017). Although *p16 and p21*, are well established senescence-associated markers, they cannot be used to discriminate replicative senescence from induced senescence (Nelson et al., 2014).

There are two highly repetitive regions in genome, namely the telomeres and rRNA genes (rDNA). The finding that stability within rDNA regulates life span led to the rDNA theory of aging. Recent studies have confirmed that the rDNA copy and repeats stability play a critical role in the control of aging and cellular senescence (Kobayashi, 2011). Recently, it was shown that tissue-specific methylation of rDNA promoter strongly correlates with a lower expression of rRNA (D'Aquila et al., 2017).

Based on the available evidence, it is thought that the DNA damage engages a senescence program that cannot be distinguished from replicative senescence (Suram et al., 2012). Current markers of senescence are limited in terms of their specificity. In order to better understand molecular basis of senescence and identify markers to distinguish between different modes of senescence, we have investigated the differential senescence response in human adult dermal fibroblasts (aHDF). Replicative senescence was induced by extended passages and DNA damage-induced senescence was induced by treatment with 5 μg/ml bleomycin for 3 days. Successful induction of senescence was confirmed by reduction of proliferation rate and SA-β-Gal positivity in at least 60% of the cells (Supplementary Fig. S1B–C and Fig. S2A–B). Consistent with other reports, expression levels of *p16* and *p21* were significantly up-regulated in both replicative and bleomycin-induced senescent aHDF (Fig. 1A–B). These data demonstrate that these markers cannot reliably distinguish replicative from DNA-damage induced senescence. Nelson et al, reported that substantially a higher number of the p16-linked genes are upregulated in replicative senescence as compared to those found in mitogen-induced senescence indicating of existence of sustained differences in cell function, phenotype and effector programs in different types of senescence (Nelson et al., 2014)

Since repetitive nature of rDNA is prone to age–associated instability and loss of copy number (Kobayashi, 2014), we tested whether the expression levels of rRNA might differ in replicative versus damage induced senescence. Each repeat, which encompasses a region that

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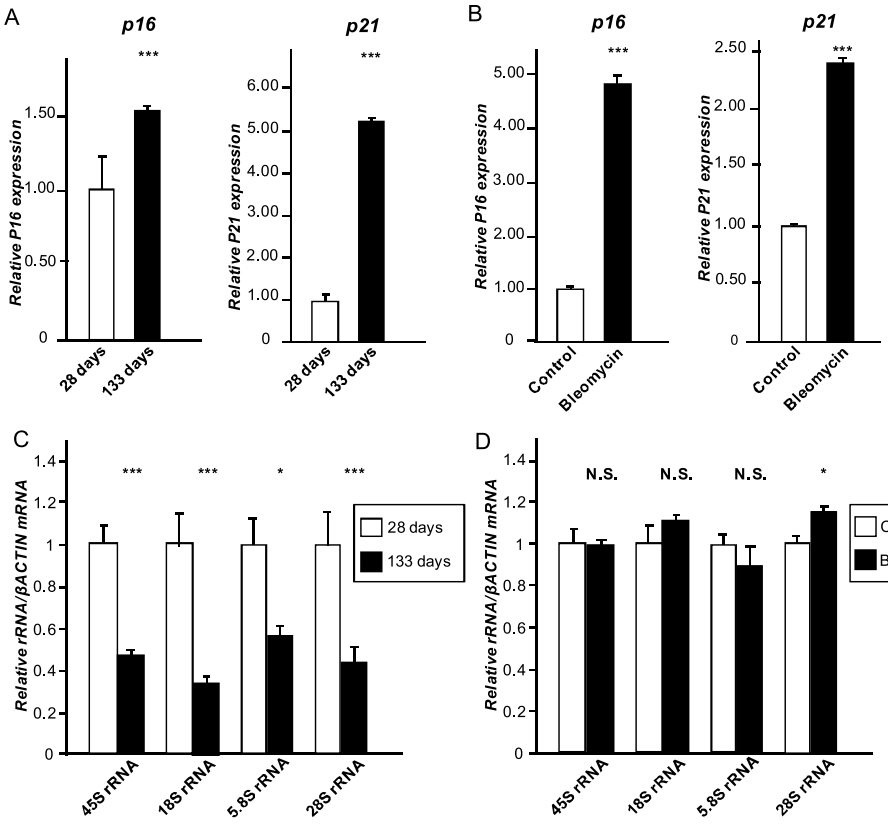

Fig. 1. Expression of senescence markers and pre-45S, 18S, 5.8S and 28S in replicative and damage induced senescence. The expression levels of *p16* and *p21* and rRNA genes were assessed by real-time PCR analysis. A,C. Replicative senescence, B,D. Damage induced senescence. Mean values are shown with error bars. *; $p < 0.05$, ***; $p < 0.0005$, N.S.; Statistically not significant.

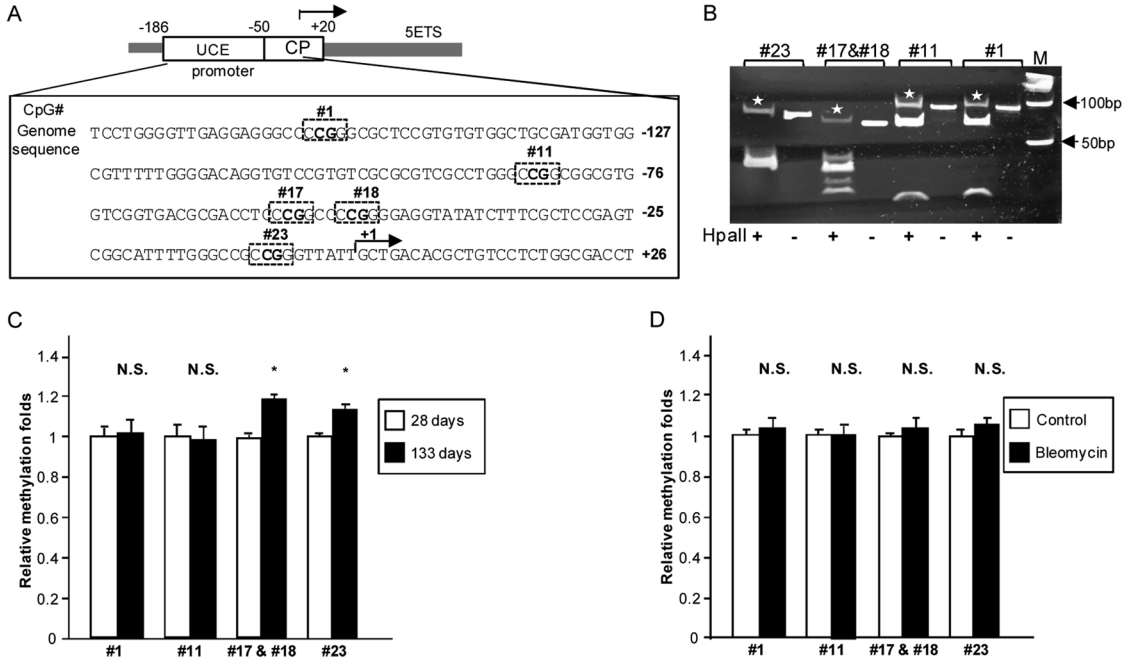

Fig. 2. DNA methylation analysis in replicative and damage induced senescence. A. HpaII restriction enzyme sites (CpG#1, #11, #17, #18 and #23) in the promoter (position -186 from the transcription start site) of rDNA are boxed. UCE: Upstream control element, CP: core promoter. ETS: External transcribed spacers. B. To verify methylation, genome DNA was digested with HpaII, undigested or digested samples were amplified, and then subjected to electrophoresis in a 10% PAGE gel. In lanes digested with HpaII, the upper band (marked by a star symbol) represents uncut methylated CpG. C–D. DNA methylation analysis was performed by real-time PCR using genomic undigested DNA or DNA digested with HpaII. Results were normalized to undigested DNA. Data are shown as relative methylation ratio (28 days and control as 1.0, respectively). In data of replicative senescence, open bars are shown as cultured for 28 days and black bars are shown as cultured for 133 days. In data of damage induced senescence, open bars represent untreated control and black bars show data of cells treated with bleomycin. Mean values are shown with errors. *; $p < 0.05$, N.S.; Statistically not significant.

encodes 18S, 5.8S and 28S rRNA, is separated by two internally transcribed spacers and flanked by two externally transcribed spacers 5′-ETS and 3′-ETS. Real-time PCR analysis revealed that expression levels of pre-45S, 18S, 5.8S and 28S decreased by about 50% in replicatively senescent cells but remained unchanged in damage induced senescent cells (Fig. 1C–D and Supplementary Fig. S3, S4, S5). Previously, it was demonstrated that rRNA production decreases with age (D'Aquila et al., 2017; Noh et al., 2017). Our results are in concordance with these studies, and show that the expression of 18S, 5.8S and 28S rRNA is significantly decreased only in replicative senescent cells. These findings suggest that stalled cell proliferation in replicative but not DNA damage-induced senescence is associated with the down-regulation of rDNA.

The transcription of rDNA genes is inhibited by methylation of CpG within their promoter region. Therefore, we compared the methylation status of the rRNA promoter in replicative and damage induced senescence in fibroblasts. As shown in Fig. 2A, there are 5 HpaII sensitive (5'-CCGG-3') sequence in promoter region (from position -186 to position +20) of rDNA, the so-called CpG#1, CpG#11, CpG#17, CpG#18 in upstream control element region and CpG#23 in the core promoter region (Pietrzak et al., 2011).

We analyzed CpG methylation in the promoter region or rDNA to assess the extent of methylation in senescent cells. Genome DNA was digested without or with HpaII, undigested or digested samples were amplified. Fig. 2B shows that each amplicon has an original band as well as lower band after digestion with HpaII. The upper band in each lane with HpaII is the band representing methylated CpG. The relative methylation level of CpG #17 & #18 and #23 was significantly increased in replicative senescent cells (Fig. 2C and Supplementary Fig. S6). However, there was no change in methylation in bleomycin-treated aHDF (Fig. 2D). Decrease in expression level of rRNA (pre45S, 18S, 5.8S and 28S rRNA) in replicative senescence coincided with, region specific, hypermethylation in the promoter region of rRNA (CpG #1, #11, #17&#18 and #23).

Currently, replicative senescence is distinguishable from DNA damage induced senescence by critical shortening of telomeres. Indeed, the length of telomeres was reduced in replicative but not in bleomycin-induced senescent aHDF (Supplementary Fig.S1D). The current methods of telomere assessment includes Southern blotting, flow FISH, or qPCR (Cawthon, 2002; Harley et al., 1990). Yet, these methods are difficult to perform, have technical limitations and require further refinement. Clearly, there is a need for the development of alternative strategies that are unique to a specific type of senescence (Kimura et al., 2010). Loss of rDNA and methylation of promoter region of rRNA is a feature of aging and age-associated neurodegenerative disorders (Hallgren et al., 2014; Kuilman et al., 2010). Downregulation of 18S, 5.8S and 28S rRNA due to increased methylation within #17, #18 and #23 CpG islands of promoter region of rDNA was observed only in replicative senescence. It is reasonable to suggest that the repression of rRNA in replicative senescence is due to increased methylation of promoter region of rDNA. Therefore, evaluation of the 18S, 5.8S and 28S rRNA expression and analysis of methylation of promoter region sets the replicative apart from damage induced senescence.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.mad.2019.111149.

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