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SHORT TAKE

Cytogenetic analysis of human cells reveals specific patterns of DNA damage in replicative and oncogene-induced senescence

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Summary

Senescence is thought to be triggered by DNA damage, usually indirectly assessed as activation of the DNA damage response (DDR), but direct surveys of genetic damage are lacking. Here, we mitotically reactivate senescent human fibroblasts to evaluate their cytogenetic damage. We show that replicative senescence is generally characterized by telomeric fusions. However, both telomeric and extratelomeric aberrations are prevented by hTERT, indicating that even non-telomeric damage descends from the lack of telomerase. Compared with replicative senescent cells, oncogene-induced senescent fibroblasts display significantly higher levels of DNA damage, depicting how oncogene activation can catalyze the generation of further, potentially tumorigenic, genetic damage.

Key words: cell cycle inhibitors; cytogenetic analysis; DNA damage; oncogene-induced senescence; replicative senescence; telomerase.

Introduction

Cellular senescence is a state of permanent cell cycle arrest, resulting from the limited ability of cells to proliferate *in vitro* (Hayflick & Moorhead, 1961). This phenomenon, called replicative senescence (RS) results, at least in part, from damaged or shortening telomeres. However, other initiators of senescence have been identified (Rodier & Campisi, 2011), such as sustained cell stress, high oxygen levels, or expression of activated oncogenes, this last condition causing oncogene-induced senescence (OIS) (Gorgoulis & Halazonetis, 2010). These apparently disparate triggers can be conceptually unified as generally resulting in DNA damage and consequent DDR, which in turn engenders permanent growth arrest (Bartkova *et al.*, 2006; Di Micco *et al.*, 2006). However, DNA damage has never been assessed directly in senescent cells, having been largely inferred from the activation of the DDR itself. Thus, a comprehensive picture of genetic damage in the different varieties of senescence is lacking.

The cytogenetic approach allows to survey DNA damage globally and directly. Although this approach has been taken with presenescent fibroblasts (Nakamura *et al.*, 2008), it has never been applied to fully senescent cells because their very proliferation arrest forbids metaphase analysis. Here, we investigate DNA damage in replicative senescent fibroblasts and compare it to that found in OIS cells by exploiting our finding that senescent cells can be induced to proliferate by ablation of the CDNK1A (p21) or INK4a (p16) cell cycle inhibitor (Pajalunga *et al.*, 2007).

Primary human C3PV fibroblasts were serially passaged until they attained senescence (BrdU incorporation $\leq$10% over 24 h). The cells were then transduced with a lentiviral vector, LV-TH-shp21, expressing a p21 shRNA. Ablation of p21 induced resumption of DNA synthesis, which lasted several passages, although it decreased with time (Fig. S1A). Senescence-associated β-galactosidase (SA-β-gal) expression mirrored BrdU incorporation, being lowest at the peak of proliferation and progressively resuming when DNA synthesis abated. To determine whether long-term proliferation remained dependent on p21 suppression, we then co-transduced senescent C3PV cells with LV-TH-shp21 and a second lentiviral vector expressing the tTR-KRAB repressor, to establish a doxycycline (dox)-inducible expression system (Wiznerowicz & Trono, 2003). As shown in Fig. S1B,C, proliferation remained dependent on p21 suppression even after 1 month in culture.

Two more primary human fibroblast strains, FB1329 and BJ, were cultured until they reached RS. Senescence in BJ cells was confirmed by lack of BrdU incorporation, expression of SA-β-gal (not shown), pRb dephosphorylation, and secretion of high levels of IL-6 and IL-8 (Fig. S2). To establish a Ras-dependent OIS model, BJ cells were transduced with a BabePuro retroviral vector carrying a Tamoxifen (OHT)-inducible V12ras-ER chimera (BP-Ras-ER) or an empty BabePuro vector (BP). Upon OHT treatment, BP-ras-ER reached a virtual proliferation standstill (BrdU incorporation <10% in 24 h) within 15–20 days and displayed intense SA-β-gal activity (Fig. S3A,B,D), while control cells continued to proliferate.

To characterize the DNA damage that accumulates in the senescent state, RS and OIS cell cultures were subjected to p21 or p16 siRNA transfection to transiently reactivate their cell cycle (Pajalunga *et al.*, 2007). In addition, p21 RNAi was applied to low-passage (LP), proliferating fibroblasts and LP, serum-starved, quiescent cells, which were induced to reenter the cell cycle by the RNAi itself. Metaphase spreads were prepared from the transfected fibroblasts treated with colcemid between 34 and 37 h post transfection, a time window

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ensuring that the first wave of mitoses following reactivation is harvested. To assess the cytogenetic status of the cells shortly before RS, metaphases were also prepared from the small portion of spontaneously proliferating fibroblasts in presenescent cultures (24-h BrdU incorporation $\leq$20%). Finally, analysis of LP, proliferating fibroblasts established the baseline for each of the strains considered.

Table 1 summarizes our findings. At low passages, all three fibroblast strains showed low levels of total chromosomal damage, which was highly increased in presenescent cells. In RS fibroblasts, total aberrations per cell were further increased about threefold, compared with their presenescent counterparts. Of note, chromosomal exchanges, which included telomeric fusions, characterized nearly exclusively RS cells and were virtually absent in LP and presenescent fibroblasts (Table 1). Distal chromosomal fusions are in keeping with the notion that uncapped telomeres tend to attach to one another (Capper *et al.*, 2007). However, the abundance of extratelomeric damage, in the form of both chromosome exchanges and breaks (examples in Fig. 1D), suggests that telomere erosion is not the sole determinant of RS. Strikingly, nearly 100% of FB1329 and C3PV cells became aneuploid before reaching senescence (presenescent cells, Table 1). To investigate whether or not aneuploidy involved specific chromosomes, comparative genomic hybridization array analysis was performed. However, no statistically significant chromosome imbalance was detected (negative data not shown).

To determine whether extratelomeric damage in RS cells is due to the absence of telomerase function or unrelated mechanisms, we transduced LP C3PV cells with an hTERT-carrying (C3PV-TERT) or a control LX (C3PV-LX) retroviral vector, selected them with puromycin, and verified hTERT expression (Fig. S4). These fibroblasts were passaged until C3PV-LX reached replicative senescence, seven population doublings (PD) after selection. At the same time point, C3PV(TERT) had undergone 14 PD, but continued to proliferate vigorously**.** Cytogenetic analysis of both cell populations (Table 1) shows that hTERT expression not only abolished telomeric exchanges but prevented accumulation of all kinds of chromosomal aberrations. Thus, even extratelomeric cytogenetic damage in RS cells results, directly or indirectly, from the lack of telomerase.

BJ cells that had undergone OIS showed a dramatically different pattern of chromosomal aberrations. Upon p21 RNAi and cell cycle reactivation, two thirds of their metaphases displayed extremely fragmented chromosomes. Also frequent were highly micronucleated cells (examples and quantitation in Fig. 1A,B), not present before cell cycle reactivation, thus likely resulting from fibroblasts undergoing mitosis with numerous DSBs. Table 1 shows that OIS BJ have about fivefold more total chromosomal aberrations than RS BJ fibroblasts. However, even this number vastly underestimates aberrations because the number of abnormalities in extremely fragmented metaphases could not be cytogenetically quantitated. Nonetheless, chromosome breaks represent

Table 1 Cytogenetic alterations in human fibroblasts in different proliferation states

STRAIN									
Age in culture	Initial proliferating state	Treatment	Percent aneuploid	Chromosomes average ± SD	Total aberrations/ cell	Gaps/ cell	Breaks/ cell	Exchanges/ cell[1]	Tel./non-tel. exchanges[2]
FB1329									
LP	Proliferating	–	4	46.0 ± 0.3	0.05	0.05	ND	ND	ND
LP	Proliferating	p21 RNAi	8	46.0 ± 0.4	0.08	0.08	ND	ND	ND
LP	Quiescent	Serum	4	46.0 ± 0.2	0.03	0.02	0.01	ND	ND
LP	Quiescent	p21 RNAi	2	46.0 ± 0.2	0.03	0.02	0.01	ND	ND
Presenescent	Proliferating	–	94	51.2 ± 7.2	[a] 0.52	0.37	0.13	0.02	0/2
RS	Perm. arrest	p21 RNAi	100	53.1 ± 1.1	[b]1.61	0.97	0.43	0.21	9/12
RS	Perm. arrest	p16 RNAi	100	53.6 ± 1.3	[b]1.96	1.22	0.62	0.12	6/6
C3PV									
LP	Proliferating	–	3	46.0 ± 0.2	0.07	0.07	ND	ND	ND
LP	Proliferating	p21 RNAi	6	46.0 ± 0.3	0.09	0.08	0.01	ND	ND
Presenescent	Proliferating	–	92	50.0 ± 6.2	[a]0.38	0.29	0.09	ND	ND
RS	Perm. arrest	p21 RNAi	97	51.4 ± 8.6	[b]1.16	0.68	0.32	0.16	7/9
TERT HP	Proliferating		5	46.0 ± 0.4	0.06	0.06	ND	ND	ND
TERT HP	Proliferating	p21 RNAi	9	46.0 ± 0.4	0.11	0.11	ND	ND	ND
LX RS	Perm. arrest	p21 RNAi	95	52.1 ± 10.6	[c,a]1.23	0.76	0.33	0.14	7/7
BJ									
LP	Proliferating	–	4	46.0 ± 0.3	0.04	0.04	ND	ND	ND
RS	Perm. arrest	p21 RNAi	26	46.3 ± 2.2	[d,a]0.57	0.48	0.09	ND	ND
OIS	Perm. arrest	p21 RNAi	82	49.2 ± 6.1	[a]2.73	0.77	1.71	0.25	1/24

LP: low-passage; perm.: permanent; tel.: telomeric; HP: high passage, 14 PD after puromycin selection; ND: not detected.

[1]Exchanges include triradial chromosomes.

[2]Number of exchanges involving telomeres/number of exchanges not involving telomeres.

Symbols indicate differences' significance by Student's unpaired *t*-test; all other comparisons are not significant.

[a]$P < 0.001$ vs. LP (all types).

[b]$P < 0.001$ vs. presenescent cells and vs. LP (all types).

[c]$P < 0.001$ vs. TERT HP (both strains).

[d]$P < 0.001$ vs. OIS.

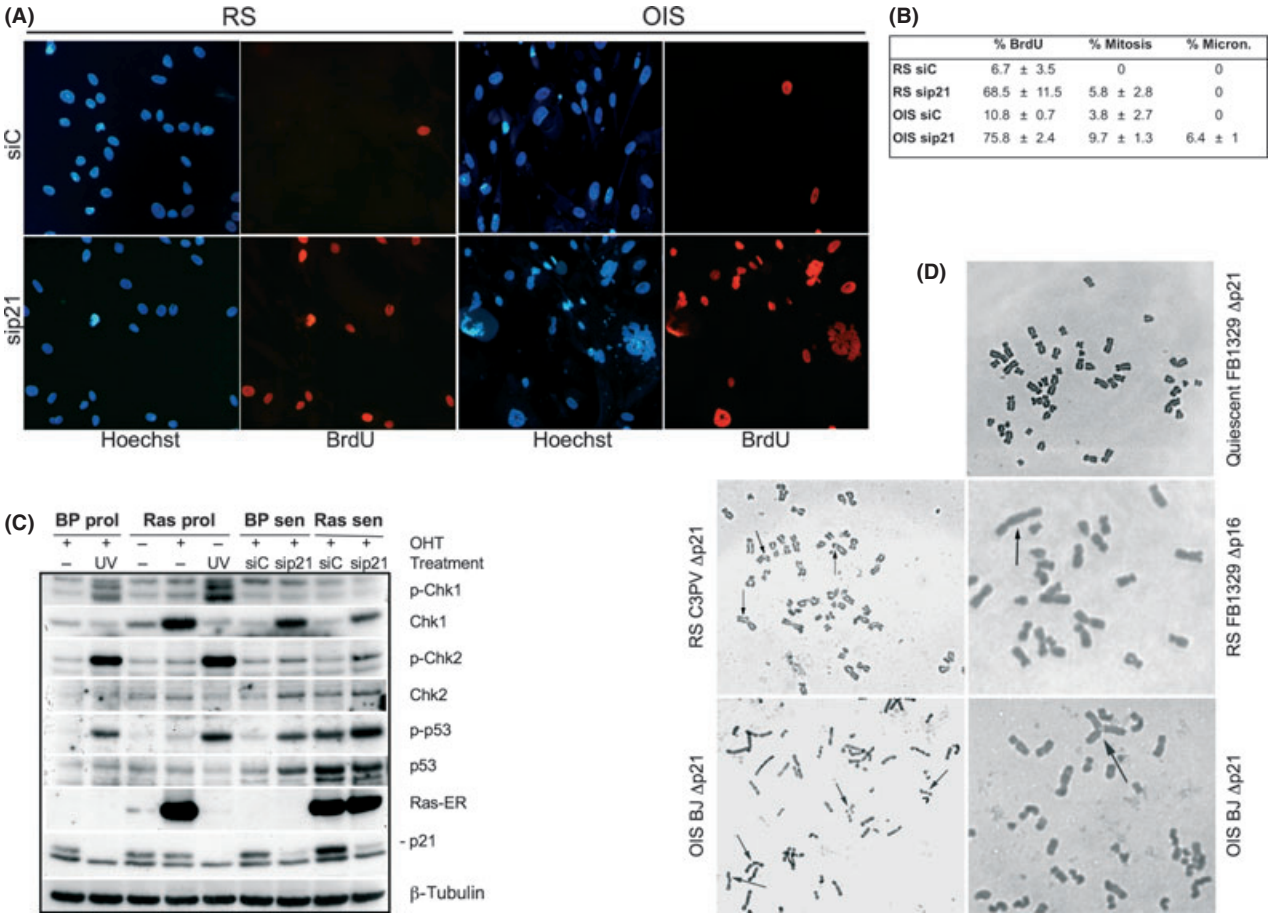

Fig. 1 DNA damage and DDR in RS and OIS cells. RS and OIS BJ fibroblasts were mitotically reactivated by p21 RNAi (sip21) or transfected with control RNAi (siC). Cells were labeled with BrdU from 24 to 48 h post transfection and fixed at 48 h. Extracts for DDR analysis were also prepared at 48 h post transfection. (A) Immunofluorescence for BrdU and nuclear staining with Hoechst. (B) Quantification of $BrdU^+$, mitotic, and micronucleated cells in three independent experiments (averages ± SE). (C) The indicated cells were transduced with empty (BP) or RasER (Ras) vector and treated with OHT for 48 h (prol) or until senescence (sen) or mock-treated; UV-irradiated cells are also shown as controls of DDR activation. The cells were analyzed by western blot for the expression and phosphorylation (p-) of the DDR-activated proteins, p53, chk1, and chk2. Note that p21 is degraded following high-dose UV-irradiation, as previously reported (Al-Khalaf *et al.*, 2011). (D) Proliferating BJ fibroblasts and reactivated RS and OIS cells were subjected to p21 or p16 RNAi as indicated. Partial metaphases showing normal (proliferating cells) and aberrant (RS and OIS cells) karyotypes are shown. Aberrations are indicated by arrows, from left to right in each photograph, as follows. RS C3PV Δp21: 1 gap, 2 breaks; RS FB1329 Δp16: telomeric fusion; OIS BJ Δp21 (left picture): 2 breaks, 1 triradial chromosome, 1 break; OIS BJ Δp21 (right picture): triradial chromosome.

the absolute majority of chromosomal aberrations, showing that DNA double-strand breaks (DSBs) are far more frequent and diffuse in OIS than RS. We also noticed the appearance of exchanges in OIS cells. However, while in RS cells about half of the exchanges, when present, arise from fusions between unprotected chromosomal ends, exchanges in OIS cells only rarely (1 in 25) involve telomeres (Table 1, rightmost column). This abundance of extratelomeric exchanges (examples in Fig. 1D) suggests that they represent failed attempts at repairing DNA damage. We underscore that suppression of p21 in LP, proliferating fibroblasts or reactivation of serum-starved, quiescent cells by p21 RNAi did not result in significant increases in chromosomal aberrations, compared with proliferating and serum-reactivated cells. This result shows that p21 ablation *per se* is not responsible for the observed cytogenetic abnormalities. There were no significant differences between RS FB1329 fibroblasts reactivated by p21 and p16 RNAi, in agreement with the suggestion (Pajalunga *et al.*, 2007) that the two CKIs do not suppress proliferation in distinct subsets of cells, but synergize to arrest most or all of the cells in a senescent culture. The higher level of DNA damage in OIS vs. RS cells was confirmed in the former by the greater accumulation of γ-H2AX, the generally higher expression of the DDR, and the often severe micronucleation (Fig. 1A–C and Fig. S5). Of note, the accumulation of some DDR factors increased upon p21 RNAi in RS, OIS, and quiescent (Fig. S6) fibroblasts, suggesting that the DDR is attenuated in non-proliferating cells.

Our results constitute the first cytogenetic characterization of DNA damage in senescent cells and provide a longitudinal picture of DNA damage accumulation in the process of reaching senescence. The data show that OIS, although in many ways phenotypically similar to RS, is associated with more generalized DNA damage and distinct chromosomal abnormalities. The accumulation of abundant damage in non-telomeric regions directly shows one mechanism through which, during tumor progression, early activation of a proto-oncogene might greatly facilitate the accrual of further tumorigenic alterations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Data S1. Methods.

Fig. S1. Stable and inducible p21 depletion in senescent fibroblasts.

Fig. S2. Characterization of senescent BJ fibroblasts.

Fig. S3. Characterization of OIS fibroblasts.

Fig. S4. hTERT expression in transduced C3PV fibroblasts.

Fig. S5. H2AX phosphorylation in RS and OIS cells.

Fig. S6. Expression of DDR markers in quiescent fibroblasts.