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The effect of aging on the DNA damage and repair capacity in 2BS cells undergoing oxidative stress

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Abstract Aging is associated with a reduction in the DNA repair capacity under oxidative stress. However, whether the DNA damage and repair capacity can be a biomarker of aging remains controversial. In this study, we demonstrated two cause-and-effect relationships, the one is between the DNA damage and repair capacity and the cellular age, another is between DNA damage and repair capacity and the level of oxidative stress in human embryonic lung fibroblasts (2BS) exposed to different doses of hydrogen peroxide (H_2O_2). To clarify the mechanisms of the age-related reduction in DNA damage and repair capacity, we preliminarily evaluated the expressions of six kinds of pivotal enzymes involved in the two classical DNA repair pathways. The DNA repair capacity was observed in human fibroblasts cells using the comet assay; the age-related DNA repair enzymes were selected by RT-PCR and then verified by Western blot in vitro. Results showed that the DNA repair capacity was negatively and linearly correlated with (i) cumulative population doubling (PD) levels only in the group of low concentration of hydrogen peroxide treatment, (ii) with the level of oxidative stress only in the group of young PD cells. The mRNA expression of DNA polymerase δ1 decreased substantially in senescent cells and showed negative linear-correlation with PD levels; the protein expression level was well consistent with the mRNA level. Taken together, DNA damage and repair capacity can be a biomarker of aging. Reduced expression of DNA polymerase δ1 may be responsible for the decrease of DNA repair capacity in senescent cells.

Keywords 2BS cells · Oxidative stress · Comet assay · DNA repair · DNA polymerase δ1

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Introduction

Aging is a universal, intrinsic, and deleterious process. Although the mechanisms of aging have not been well elucidated, the free radical theory of aging, which has been universally acknowledged as one of the most prominent and well studied theories among more than 300 theories postulated, hypothesizes that free radicals are responsible for the age-related damage at cellular and tissue levels [1]. Excess generation of free radicals may overwhelm natural antioxidant defense, which lead to oxidation and further contribute to cellular functional impairment [2]. The comet assay is a versatile and sensitive method for measuring single-and double-strand breaks (DSB) as well as alkali-labile sites in DNA. All three categories of DNA damage are detected in the alkaline version of the technique (pH > 13) as used in this study [3, 4]. The extent of DNA damage can be assessed by comet assay and the chromosomal DNA migration distance correlates with the extent of DNA damage. DNA damage and repair capacity was observed and quantified by measuring comet tail lengths of various cells with time after oxidative stress [5]. Various researches at the molecular and organ levels have indicated that the repair capacity of oxidatively damaged DNA declines during aging [6] and the oxidative DNA damage and repair capacity may be exploited as a reliable marker of cell senescence [7]. The suggested biomarker of cellular aging must satisfy the criterion that it must show significant

age-related changes with a relatively brief period of time [8–10]. However, our work found that there were no differences in comet images between young and senescence cells cultured under conventional conditions using comet assay (Fig. 1). Did it mean that the DNA damage and repair capacity of the young cells was identical with that of the senescence cells? In fact, the finite DNA damage and repair capacity of senescent cells may be still sufficient to repair cumulative lesions during aging process. For better understanding of the correlations between the DNA repair capacity and population doubling (PD) levels of human fibroblasts under the state of additional oxidative stress, we tried to elucidate the two cause-and-effect relationships (i) between the DNA damage and repair capacity and PD levels of human fibroblasts, and (ii) between the DNA damage and repair capacity and the levels of oxidative stress under the pressure of oxidative stress.

Previous studies have validated the hypothesis that DNA damage observed in senescent cells reflects more a decline in repair mechanisms rather than a prolonged oxidative attack [4]. DSB in the genomic DNA arise predominantly from oxidative DNA damage endogenously but are also induced by a variety of genotoxic agents exogenously, a process which is believed to be one of the most severe types of DNA damage [11, 12]. Accurate repair of DNA strand breaks is essential to life. Indeed, defective DNA strand break repair can lead to toxicity and large scale sequence rearrangements that promote premature aging. The nonhomologous end-joining (NHEJ) and homologous recombination (HR) repair pathways are the two major mechanisms employed by mammalian cells to repair DNA DSB [13]. The NHEJ pathway comprises several components: Ku70/80, DNA-PKcs, DNA ligase IV, its cofactor XRCC4, as well as at least two DNA polymerases: polδ and polε, and it is predominant over the HR pathway which mostly includes the ataxia telangiectasia mutated (ATM) protein. Both the two mechanisms complement each other and are involved in an intricate network of DNA repair systems. The DNA repair capacity depends primarily on the amounts and activities of repair proteins [14]. Unfortunately, little is understood regarding the relationship between the DNA repair enzymes and the replicative senescence of human fibroblasts cells. In this study, we used reverse-transcription and polymerase chain reaction (RT-PCR) to select the genes expressing discrepantly among DNA repair enzymes involved in the two major repair pathways. According to the results, we further verified the candidate protein by Western blotting.

Methods

Cell culture

Human embryonic lung diploid fibroblasts (2BS cells) were originally established at the National Institute of Biological

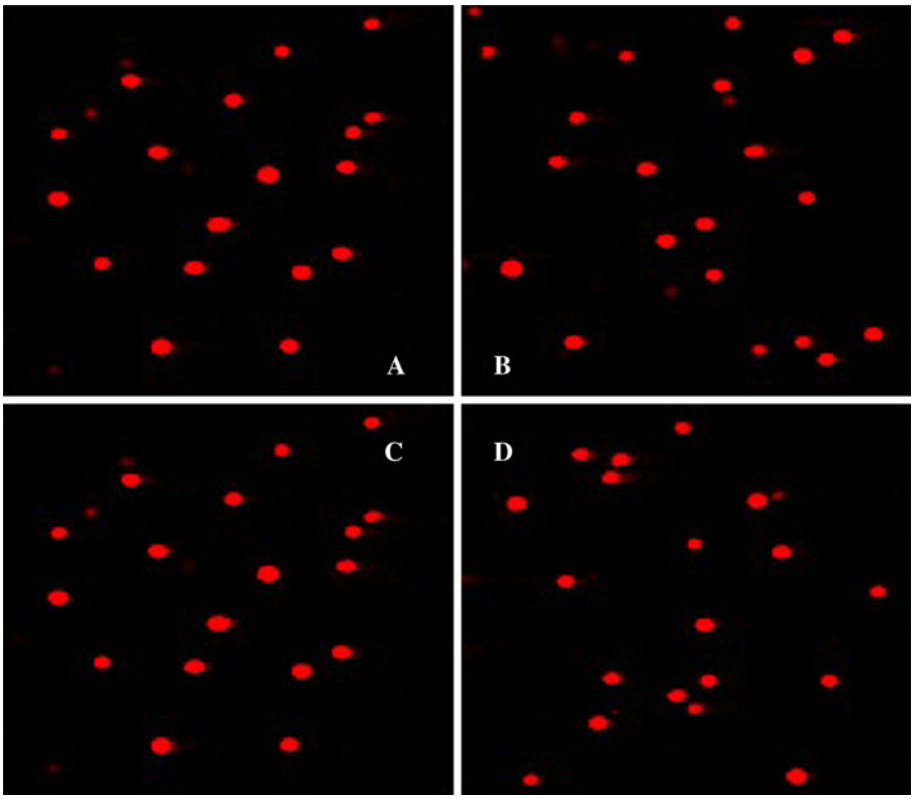

Fig. 1 Comet assay of 2BS cells at various PD levels cultured under conventional conditions. **a**, **b**, **c**, and **d** represent 2BS cells with PD levels of 26, 35, 45, and 56, respectively

Products (Beijing, China). The 2BS cells are considered to be "young" at PD30 or below, and to be fully "senescent" at PD56 or above. Cells are grown in Dulbecco's Modification of Eagle's Minimum Essential Medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and with 100 units/ml penicillin and 100 μg/ml streptomycin in an incubator at 37°C with 5% CO_2.

Comet assay

The comet assay was carried out under alkaline conditions, as described by Singh et al. [15]. Agarose gels were prepared on fully frosted slides coated with 1% normal melting point agarose (Sigma). The pretreated 2BS cells were mixed with 0.5% low melting point (LMP) agarose (Sigma), placed on the slides, and covered with a layer of 0.5% LMP agarose. The slides were immersed for 85 min in freshly prepared ice-cold lysis solution (2.5 mol/l NaCl, 100 mmol/l Na_2EDTA, 10 mmol/l Tris, pH 10) with 1% triton-X 100 (sigma) and 10% dimethyl sulfoxide (Sigma). Then, denaturation and electrophoresis were carried out at 4°C in the dark with freshly prepared electrophoresis buffer (300 mmol/l NaOH, 1 mmol/l Na_2EDTA, pH 13). After 20 min of denaturation, the slides were randomly placed in the horizontal gel-electrophoresis tank, facing the anode. Electrophoresis was carried out at 25 V and 300 mA for 20 min. After electrophoresis, the slides were washed with a neutralization buffer (0.4 mol/l Tris-CL, pH 7.5) three times for 5 min. Slides were stained with ethidium bromide (5 μg/ml) for 15 min and examined using laser scanning confocal microscope FV1000S (OLYMPUS, Japan) equipped with an excitation filter at 515–560 nm. Random fields were selected at a constant depth of the gel, avoiding the edges. Data were analyzed using the CASP software (CASP-1.2.2, download in http://casp.sourceforge.net/index.Php), the two parameters including the Tail DNA% (TDNA %) and the Olive tail moment (OTM) were measured.

RT-PCR

The total RNA of 2BS cells of various PD levels was harvested using the RNeasy mini Kit (QIAGEN, Germany). Cells were pelleted and processed according to the animal cell protocol. Absorbance at 260 nm was used to quantify the RNA preparation, which was then analyzed on native agarose gels to assess the RNA integrity. An equal amount of each RNA was converted into double-stranded cDNA using the Thermoscript RT-PCR system (Invitrogen, Carlsbad, CA). The sequences of the primers were as follows: 5′-TCAACGGATTTGGTCGTATTG-3′ (forward) and 5′-TGGAAGATGGTGATGGGATT-3′ (reverse) for glyceraldehyde 3-phosphate dehydrogenase (GAPDH); 5′-GTGCCCATCAACCAAAGAAC-3′ (forward) and 5′-TGTGGCGTCGAAACATACTG-3′ (reverse) for DNA ligase IV; 5′-AAGCGGTTTATTCTGGTGTT-3′ (forward) and 5′-ATCTCGGTCAGCAGTCATTT-3′ (reverse) for XRCC4; 5′-ACGAGCGATGAGAAATGA-3′ (forward) and 5′-AAGGACTCCATCCCACAA-3′ (reverse) for DNA-PKcs; 5′-CTCAAGGACCTCATCAACGA-3′ (forward) and 5′-CTTCAAATACTAGCGCCAAG-3′ (reverse) for PCNA; 5′-GTCTTGTGCTGCTCACTTTA-3′ (forward) and 5′-TACTCTGGCTTCCTTCTTCA-3′ (reverse) for ATM; 5′-GCTCCGCTCCTACACGCTCAA-3′ (forward) and 5′-GTCTGGTCGTTCCCATTCTGC-3′ (reverse) for DNA polymerase δ1.

All the primers were verified by sequencing. The linear range of amplification of the GAPDH control gene was observed at cycle 30. One percent of the reverse transcription reaction was amplified for 30 s at 95°C, 30 s at 55.2°C for GAPDH, PCNA, DNA LigIV; 50°C for ATM; 52.5°C for XRCC4, DNA-PKcs; 62.5°C for DNA polymerase δ1, and then 30 s at 72°C for 30 cycles. The products were examined by 1% agarose gel electrophoresis and quantified by computerized gel documentation (Bio-Rad).

Western blotting

Cells were lysed in radioimmune precipitation assay buffer (1 × PBS, 1% NonidetP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 50 kallikrein-inactivating units/ml aprotinin, 1 mol/l sodium orthovanadate). The protein concentration of each sample was determined by BCA Protein Assay Reagent (Pierce). Samples (containing 60–100 μg of total proteins) were subjected to electrophoresis on a 15% SDS–polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were pre-incubated with a blocking agent and then incubated with a primary antibody at 1:1000 in 5% nonfat dry milk in TBST (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) overnight at 4°C. After washing, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase at 1:5000 in TBST for 1 h at room temperature. Proteins were visualized with a chemiluminescent substrate (Pierce) according to the manufacturer's instruction. Antibodies against the DNA polymerase δ1 were purchased from Abcam and used at 1 mg/ml.

Statistical analysis

Results were expressed as means ± standard error. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by post-hoc multiple comparison tests. Differences were considered as significant for

P values <0.05. Pearson correlation coefficient was used to analyze the correlation between gene expression level of DNA polymerase $\delta 1$ and cumulative PD levels of 2BS cells.

Results

Changes in DNA damage and repair capacity of 2BS cells during aging process under different conditions

Comet assay is a simple and sensitive method for quantitatively measuring DNA breakage and repair in individual cells. Cells with increased DNA damage display increased DNA migration from the nucleus toward the anode, which resembles the shape of a comet. The migration is observed by fluorescence microscopy after staining with a fluorescent DNA-binding dye, and the intensity of the comet tail reflects the number of DNA breaks [16]. The extent of DNA damage was measured using 2BS cells of PD26, 35, 44 and 56 at the conventional culture conditions. As showed in Fig. 1, neither young nor senescent cells appeared evident "comet tails". The difference showed no statistical significance (Statistical values were not listed in the text).

2BS cells of PD26, 35, 44 and PD56 were respectively pre-treated with acute sublethal dose of H_2O_2, the concentration gradient of which included 50, 100, and 200 μmol/l. After 5 min, comet assay was used to assess the extent of DNA damage. The results listed in Figs. 2, 3 and 4 showed that there were significant differences in the late PD cells including obviously longer tail lengths and bigger tail areas compared with the young 2BS cells. The senescence cells of PD56 treated with high dose of H_2O_2 showed dispersive tails. The comet assay results confirmed that the extent of DNA damage increased gradually during aging process in cultured fibroblasts.

The data were summarized in Table 1. The TDNA % and the OTM are the two effective parameters to estimate the extent of DNA damage, of which the later prevailed over the former [17]. Values of the two parameters were significantly higher in the group of late PD cells as compared to the young and middle (cells with an intermediate PD levels) counterparts regardless of which doses of H_2O_2 treatment ($P < 0.01$, ANOVA). Regression analysis showed the two significant linear-correlations (i) between the DNA damage-and-repair capacity and PD levels only in cells treated with low dose of H_2O_2 (50 μmol/l); and (ii) between the DNA damage-and-repair capacity and H_2O_2 concentrations only in the group of young PD cells (PD26) as showed in Fig. 5a and b.

Changes in DNA repair ability of 2BS cells during aging process after oxidative stress with another 1 h repair

To determine the relationship between DNA repair capacity and repair time under oxidative stress, 2BS cells of PD26, 35, 45, and PD56 were exposed to 200 μM H_2O_2

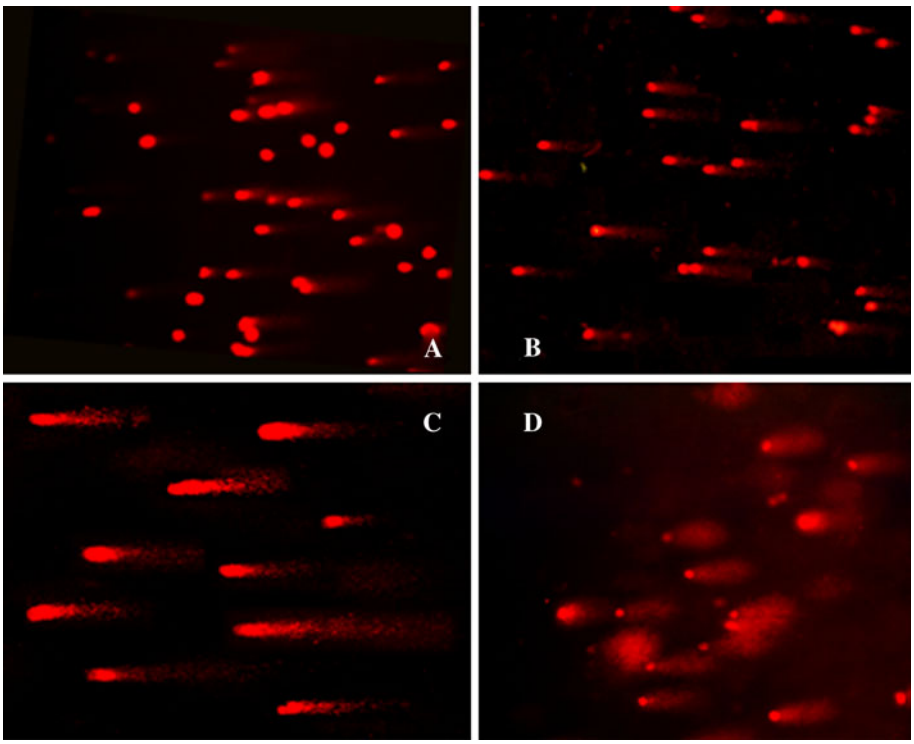

Fig. 2 Comet assay of 2BS cells at various PD levels exposed to 50 μM H_2O_2 for 5 min. 2BS cells were exposed to 50 μM H_2O_2 for 5 min at 4°C in the dark, washed with PBS, and immediately analyzed using the comet assay. **a** PD26, **b** PD35, **c** PD44, **d** PD56

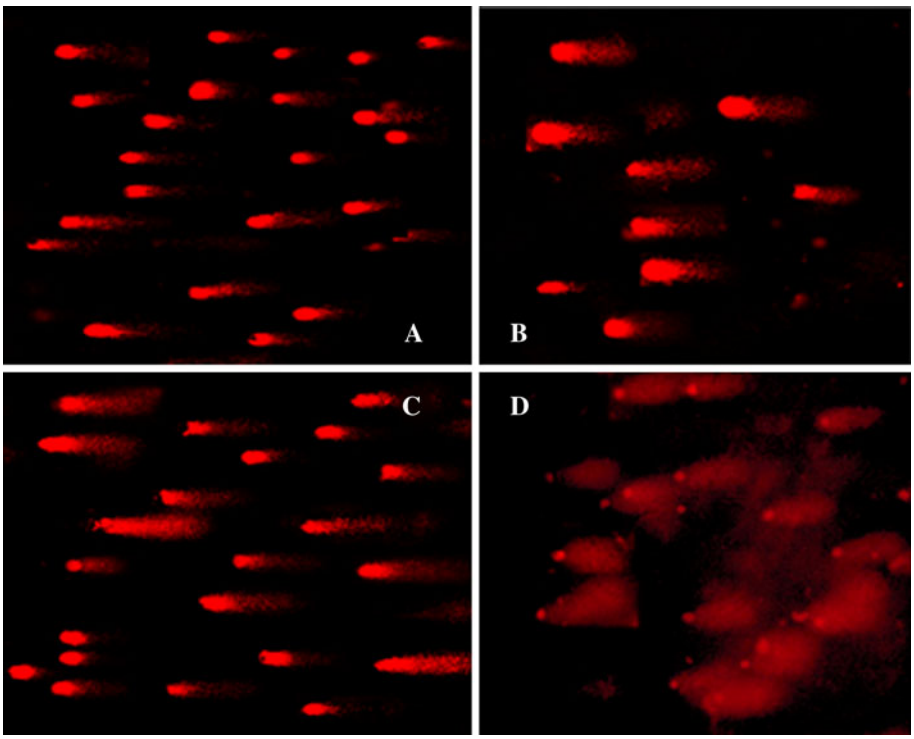

Fig. 3 Comet assay of 2BS cells at various PD levels exposed to 100 μM H_2O_2 for 5 min. 2BS cells were exposed to 100 μM H_2O_2 for 5 min at 4°C in the dark, washed with PBS, and immediately analyzed using the comet assay. **a** PD26, **b** PD35, **c** PD44, **d** PD56

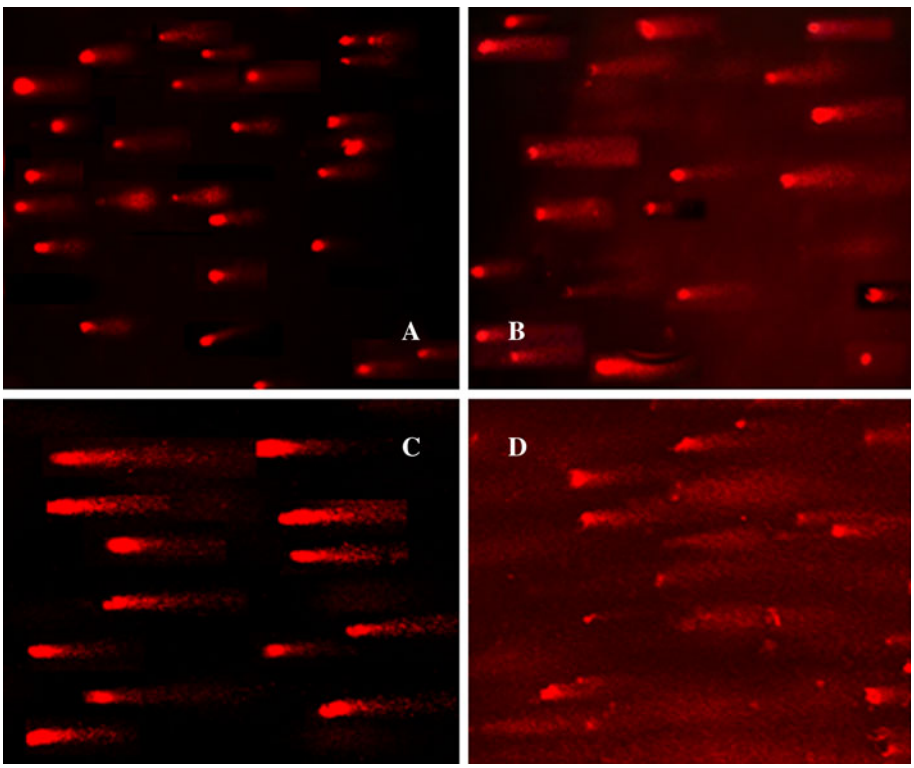

Fig. 4 Comet assay of 2BS cells at various PD levels exposed to 200 μM H_2O_2 for 5 min. 2BS cells were exposed to 200 μM H_2O_2 for 5 min at 4°C in the dark, washed with PBS, and immediately analyzed with the comet assay. **a** PD26, **b** PD35, **c** PD44, **d** PD56

for 5 min, subsequently placed in the H_2O_2-free culture medium to recover for 1 h, and then examined by comet assay. The result listed in Fig. 6 showed that the senescent fibroblasts displayed much longer comet tails compared with the young and middle-aged cells in which no or only sporadic comet cells were observed ($P < 0.01$, ANOVA). There was no statistical significance between neighboring groups below 56. Whereas, additional experiments found that the cells of no matter which PD levels showed no "comet tails" even treated with high dose of sub-lethal

Table 1 Extent of DNA damage in 2BS cells during aging process exposed to 50, 100, 200 μM H_2O_2 for 5 min

Population doubling (PD) level	50 μM H_2O_2 treatment		100 μM H_2O_2		200 μM H_2O_2	
	Tail DNA% (%) ($\overline{X} \pm$ SE)	Olive tail moment (%) ($\overline{X} \pm$ SE)	Tail DNA% (%) ($\overline{X} \pm$ SE)	Olive tail moment (%) ($\overline{X} \pm$ SE)	Tail DNA% (%) ($\overline{X} \pm$ SE)	Olive tail moment (%) ($\overline{X} \pm$ SE)
26	1.38 ± 0.27*	0.36 ± 0.07*	26.69 ± 1.17*	5.96 ± 0.49*	39.59 ± 2.64*	21.66 ± 1.55*
35	22.00 ± 1.83*	6.60 ± 1.22*	36.95 ± 2.34*	13.26 ± 1.53*	52.78 ± 1.22*	37.80 ± 1.78*
44	39.71 ± 0.76*	15.88 ± 0.64*	46.17 ± 0.97*	18.96 ± 0.63*	59.98 ± 1.65*	48.06 ± 2.18*
56	56.32 ± 2.44*	38.82 ± 2.14*	63.16 ± 2.25*	53.96 ± 2.92*	74.02 ± 2.68*	73.58 ± 3.21*

Data were obtained from Fig. 2, 3, 4 and were analyzed with the SPSS statistics software

* $P < 0.05$, ANOVA, $n = 50$

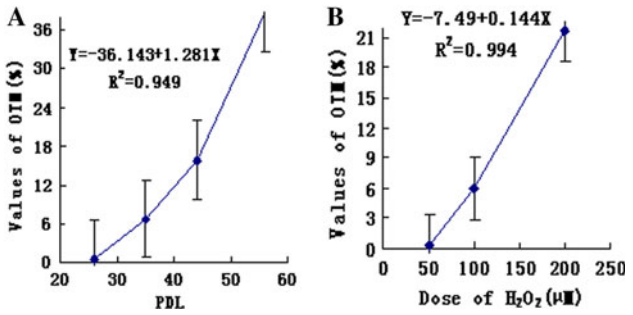

Fig. 5 **a** Relationship between the average values of Olive tail moment (OTM) and cumulative PD levels. 2BS cells at various PD levels were induced by 50 μM H_2O_2 for 5 min and then analyzed by the comet assay. The DNA damage level was linearly correlated with PD levels. **b** Relationship between the average values of OTM and doses of H_2O_2. 2BS cells at PD26 were exposed to different doses of H_2O_2 for 5 min. The DNA damage level was linearly correlated with the dose of H_2O_2

H_2O_2 (200 μM) after extending repair time to 3 h just like un-pretreated control cells (pictures not shown).

Gene expression level of DNA repair enzymes

NHEJ is the major pathway of DSB repair in mammalian cells. To determine whether the changes in the expression of some genes in the repair pathway were correlated with the age-related reduction in the DNA repair capacity, we chose six representative DNA repair genes from the repair pathways of NHEJ and HR, and then quantified their gene expressions respectively using 2BS cells of PD24, 31, 38, 46 and PD59 by RT-PCR. Data were normalized to GAPDH gene expression levels. The results listed in Fig. 7a showed no significant differences in the expression of most chosen genes, except the DNA polymerase δ1

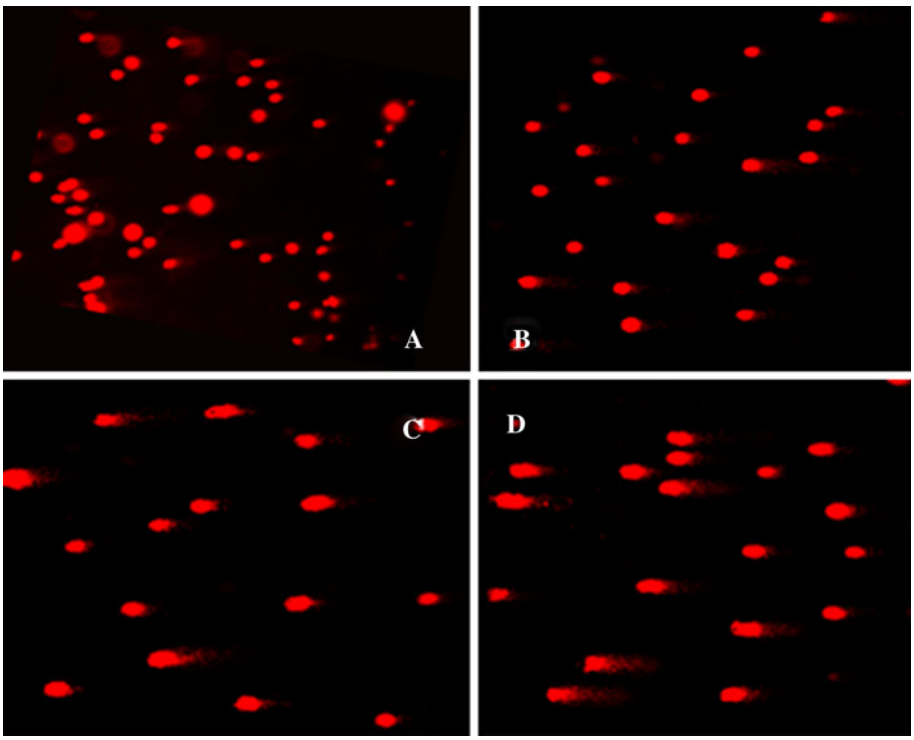

Fig. 6 Comet assay of 2BS cells at various PD levels exposed to 200 μM H_2O_2 and followed by recovery for 1 h. 2BS cells were induced by 200 μM H_2O_2 for 5 min at 4°C in the dark, washed with PBS, and then cultured with H_2O_2-free DMEM for 1 h. **a**, **b**, **c**, and **d** represent 2BS cells with PD levels of 26, 35, 45, and 56, respectively

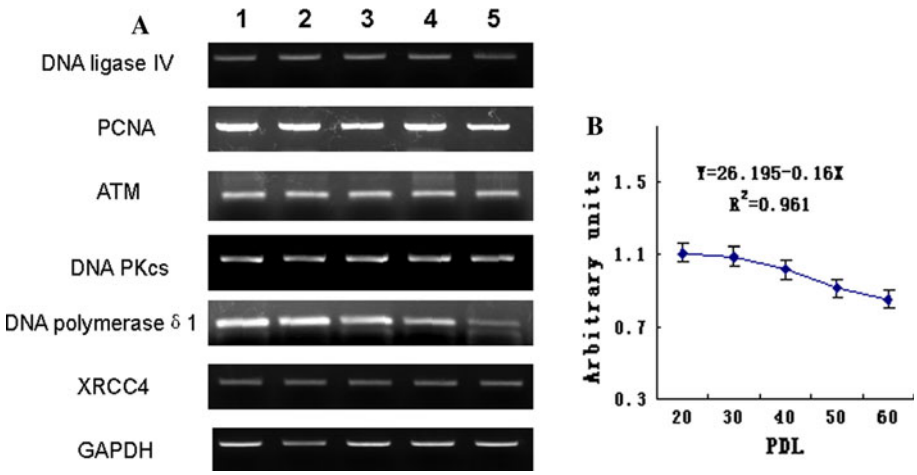

Fig. 7 **a** mRNA levels of various DNA repair genes in 2BS cells at different PD levels by RT-PCR. GAPDH served as a normalization control for RT-PCR. *1* PD24, *2* PD31, *3* PD38, *4* PD46, *5* PD59. **b** Relationship between mRNA expression level of DNA polymerase δ1 and cumulative PD levels

(pδ1), the mRNA expression level of which was substantially down-regulated during aging process. Mean mRNA level of DNA polymerase δ1 was plotted vs. cumulative PD levels, and the regression analysis in Fig. 7b showed a linear decrease in mRNA level of DNA pδ1 with cumulative PD levels as modeled by linear equation. The difference between means of all measured generations showed statistical significance.

Protein expression level of DNA polymerase δ1 in 2BS cells during aging process

The protein expression level of DNA polymerase δ1 were determined using Western blot using 2BS cells of PD25, 40 and PD50. As showed in Fig. 8a, the protein expression level of DNA polymerase δ1 was higher in the young cells but decreased significantly during aging process (2.5-fold decrease in middle-aged cells, and 6.6-fold decrease in senescent cells) (Fig. 8b). Our results suggested that the reduced mRNA expression of DNA polymerase δ1 was well paralleled with the reduction in its protein expression. These results strongly suggest that down-regulation of DNA polymerase δ1 expression may be responsible for the age-related decrease in DNA repair capacity of human fibroblasts.

Discussion

Under normal culture conditions, DNA repair systems are able to keep up with the rate of DNA damage, thereby maintaining proper cell function [18]. Our researches stemmed from a finding that there were no differences in comet images between young cells and aging cells cultured at conventional culture conditions as shown in Fig. 1, which seemingly meant no differences in DNA damage and repair capacity between young and aging cells. As a matter of fact, although senescent cells are able to maintain the capacity to counteract lesions due to oxidative stress under conventional culture conditions, they are more vulnerable than young and middle-aged cells when confronted higher oxidative stress. Here, we set up concentration gradient of sub-lethal H_2O_2 including 50, 100, and 200 μM in order to achieve the state of oxidative stress. We found that the extent of DNA damage increased during aging process and the DNA repair capacity decreased gradually as the PD levels increasing. The DNA damage and repair capacity was negatively and linearly correlated with PD levels. Moreover, the DNA repair capacity was both damage dosage-dependent and repair time-dependent. The extent of DNA damage in the young cells showed a

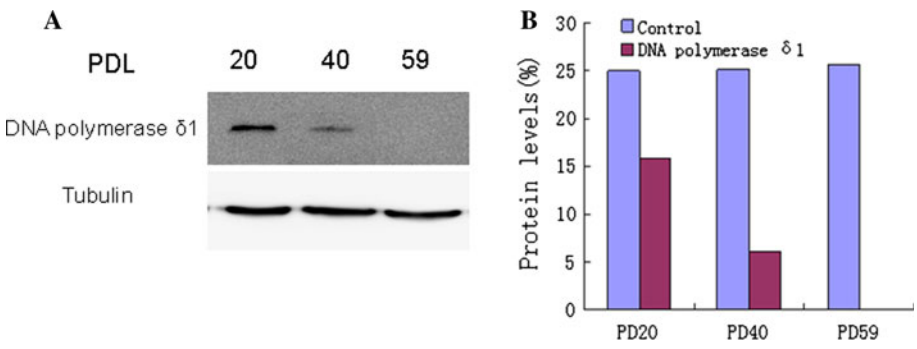

Fig. 8 **a** Protein levels of DNA polymerase δ1 in 2BS cells at different PD levels by Western blot. DNA polymerase δ1 has a molecular weight of 125 kDa. Tubulin was employed as a standard to normalize gene expression. **b** Quantitative analysis of the protein levels of DNA polymerase δ1 in cultured 2BS cells at different PD levels

positive linear relationship as the dose of H_2O_2 increasing. Our results largely confirm and enrich previous conclusions that oxidative DNA damage contributes to replicative cessation in human fibroblasts and DNA damage-and-repair capacity can be a biomarker of aging.

We next investigated the mechanism of age-related changes by tentatively screening the genes from the repair pathways of NHEJ and HR, the expression levels of which were significantly discrepant between different PD levels. The mRNA level of DNA polymerase $\delta 1$ was markedly decreased in senescent cells compared with young and middle-aged cells, and was negatively and linearly correlated with PD levels. Moreover, the mRNA level of DNA polymerase $\delta 1$ was well correlative with its protein expression level. Our result was in agreement with the previous report [19] that the protein expression of DNA polymerase $\delta 1$ was decreased in human skin fibroblasts from elderly donors.

The functional integrity of the DNA repair system should be compromised if any one of its component fails to function. Previous reports have mainly focused on the deficiency of proteins related with the NHEJ pathway at the level of organs and tissues. Mice deficient in DNA ligase IV are immunodeficient, show growth retardation, and have a progressive loss of haematopoietic stem cells and bone marrow cellularity during aging [20]. Ku mutants have an intermediate severity with accelerated senescence and severe combined immune deficiency [21, 22]. In mice, mutation in the active site of DNA polymerase δ reduces life span, increases genomic instability, and accelerates tumorigenesis [23]. Eukaryotic DNA polymerase δ has an intrinsic $3' \rightarrow 5'$ exonuclease activity and is an essential, highly conserved enzyme that plays an important role in DNA replication, DNA repair, and genetic recombination. It's believed to replicate a large portion of the genome, synthesizes most of the lagging strand and perhaps contributes to leading-strand synthesis as well [24, 25]. Decreases in DNA polymerase expression or function may lead to carcinogenesis and may accelerate the process of aging [26]. Further work will be necessary to fully elucidate the mechanism of DNA polymerase $\delta 1$ in the age-related decrease of DNA repair capacity.

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