Inflammatory ROS promote and cooperate with the Fanconi anemia mutation for hematopoietic senescence

Xiaoling Zhang[1], Daniel P. Sejas[1], Yuhui Qiu[1], David A. Williams[1,2] and Qishen Pang[1,2,*]

[1]Division of Experimental Hematology, Cincinnati Children's Hospital Medical Center and [2]Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH 45229, USA

*Author for correspondence (e-mail: qishen.pang@cchmc.org)

Accepted 1 March 2007
Journal of Cell Science 120, 1572-1583 Published by The Company of Biologists 2007
doi:10.1242/jcs.003152

Summary

The proinflammatory cytokine tumor necrosis factor α (TNFα) inhibits hematopoietic stem cell (HSC) expansion, interferes with HSC self-renewal and compromises the ability of HSC to reconstitute hematopoiesis. We have investigated mechanisms by which TNFα suppresses hematopoiesis using the genomic instability syndrome Fanconi anemia mouse model deficient for the complementation-group-C gene (*Fancc*). Examination of senescence makers, such as senescence-associated β-galactosidase, HP1-γ, p53 and p16^{INK4A} shows that TNFα induces premature senescence in bone marrow HSCs and progenitor cells as well as other tissues of *Fancc*$^{-/-}$ mice. TNFα-induced senescence correlates with the accumulation of reactive oxygen species (ROS) and oxidative DNA damage. Neutralization of TNFα or deletion of the TNF receptor in *Fancc*$^{-/-}$ mice (*Fancc*$^{-/-}$;*Tnfr1*$^{-/-}$) prevents excessive ROS production and hematopoietic senescence. Pretreatment of TNFα-injected *Fancc*$^{-/-}$ mice with a ROS scavenger significantly reduces oxidative base damage, DNA strand breaks and senescence. Furthermore, HSCs and progenitor cells from TNFα-treated *Fancc*$^{-/-}$ mice show increased chromosomal aberrations and have an impaired oxidative DNA-damage repair. These results indicate an intimate link between inflammatory reactive oxygen species and DNA-damage-induced premature senescence in HSCs and progenitor cells, which may play an important role in aging and anemia.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/120/9/1572/DC1

Key words: DNA damage and repair, Fanconi anemia, Genomic instability, Hematopoietic stem cells, Inflammation, Reactive oxygen species

Introduction

The genomic instability syndrome Fanconi anemia (FA) shares the common feature of having defective DNA damage response/repair processes with other premature aging syndromes such as Bloom (Bischof et al., 2001) and Werner (Chen et al., 2003), ataxia telangiectasia (Ito et al., 2004) and *BRCA1*-deficient breast cancer (Cao et al., 2003). The FA pathway has been shown to functionally interact with the proteins responsible for Bloom syndrome (BLM) (Meetei et al., 2003), ataxia telangiectasia (ATM) (Taniguchi et al., 2002)] and breast cancer (BRCA1) (Seal et al., 2003). In fact, one of the FA genes, *FANCD1*, turned out to be the breast cancer gene *BRCA2* (Howlett et al., 2002). This suggests a biological link between the FA disease and organismal aging. The FA disease model is unique in that defects are especially profound in the rapidly proliferating hematopoietic system. Thus far, aging studies have not yet focused on hematopoietic stem cells (HSCs) in FA or these other premature aging syndromes. Nevertheless, HSCs are capable of self-renewing, have a high risk of accumulating mutations and, thus, may require fewer events to become senescent or transformed (Pelicci, 2004). In addition, HSC aging can be assessed in a quantitative fashion with well-established assays. Since senescence can cause stem cell depletion, which can conceivably lead to organismal aging, such studies may yield important insights to the mechanisms of aging.

Functional failure of HSCs can result in the decrease in number and function of HSCs and progenitor cells leading to the development of anemia, neutropenia and thrombocytopenia, and has been implicated in hematologic diseases such as bone marrow (BM)-failure diseases such as FA, myelodysplastic syndromes (MDS) and aplastic anemia (Young, 2002; Bagby, Jr, 2003; Chen, 2005). Indeed, it has been shown that FA HSCs and progenitor cells have high rates of stress-induced apoptosis and reduced repopulating ability (Haneline et al., 1998; Haneline et al., 1999; Haneline et al., 2003). The FA proteins are thus believed to play important roles in the maintenance of hematopoiesis. Consistent with the observations that cells derived from FA patients are intolerant of oxidative stress, it has been reported that FA proteins, particularly the complementation group C (FANCC) protein, play a crucial role in oxidative-stress signaling in a variety of cell types including hematopoietic cells (Kruyt et al., 1998; Cumming et al., 2001; Hadjur et al., 2001; Futaki et al., 2002; Park et al., 2004; Saadatzadeh et al., 2004; Pagano et al., 2005). More recently, cytokine hypersensitivity of FA hematopoietic cells to apoptotic cues has also been proposed as a major factor in the pathogenesis of BM failure in three FA mouse models

Journal of Cell Science

(*Fanca*$^{-/-}$, *Fancc*$^{-/-}$ and *Fancg*$^{-/-}$) (Li et al., 2004; Si et al., 2006).

It is believed that cellular and organismal senescence occurs as a result of chronic exposure to extrinsic environmental factors, primarily of oxidative stress (Sohal and Weindruch, 1996). The degree of oxidative damage has been found to increase exponentially with senescence and aging in a variety of cells, and tissues of different species (Chen et al., 1995). Endogenously formed reactive oxygen species (ROS) continuously damage cellular constituents including DNA. These challenges, coupled with exogenous exposure to agents that generate ROS, are both associated with normal aging processes and linked to cardiovascular disease, age-related anemia and cancer (Maccio et al., 2005; Sablina et al., 2005; Wallace, 2005). In the aged and certain disease states, ROS produced by proinflammatory cytokines – such as tumor necrosis factor-α (TNFα) – at inflammatory sites often cause DNA damage (Goossens et al., 1999; Suematsu et al., 2003; Wheelhouse et al., 2003). However, the mechanism through which ROS mediate their effects on the aging process is unclear.

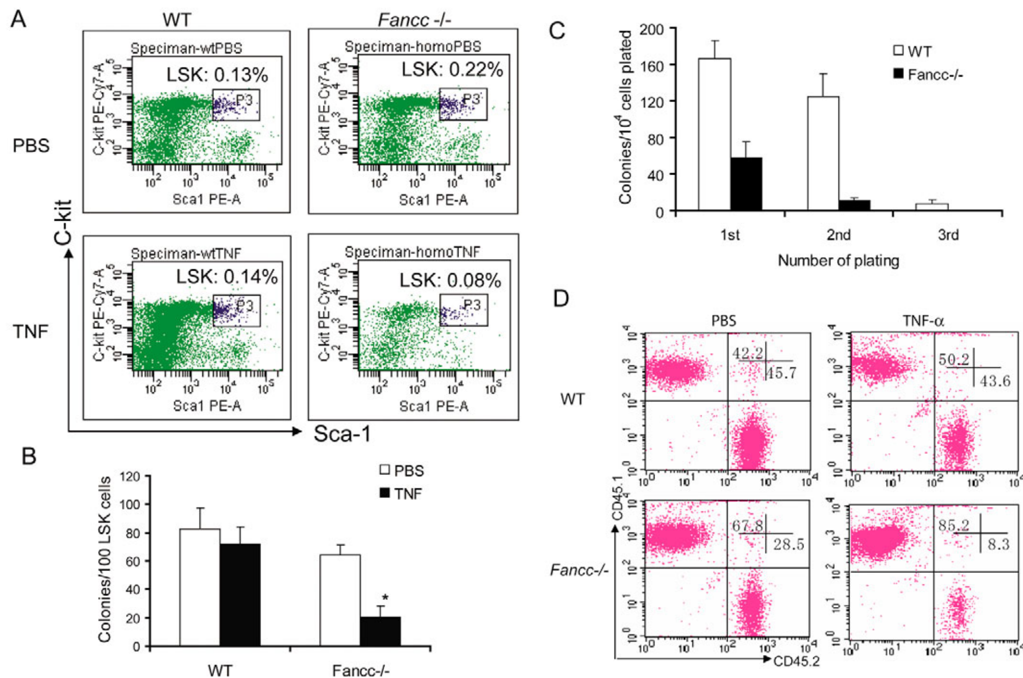

Fig. 1. TNFα suppresses HSC/progenitor cell activity. (A) Effect of TNFα on HSC frequency. Wild-type (WT) or *Fancc*$^{-/-}$ mice were injected intraperitoneally (i.p.) with mouse recombinant TNFα in PBS at a dose of 0.1 mg/kg per day for two consecutive days. The mice were then sacrificed 24 hours later and BM mononuclear cells were prepared and stained with antibodies against lineage markers (Lin) and Sca-1-PE and c-Kit-PE-Cy7. Cells were then analyzed by flow cytometry to obtain fractions representing HSCs. The frequencies of LSK cells as a percentage of total BM mononuclear cells are indicated. (B) The effect of TNFα on colony-forming activity of WT and *Fancc*$^{-/-}$ BM progenitor cells was evaluated using LSK cells isolated from control (PBS-injected) or TNFα-injected mice. Data shown represent the mean ± s.d. of colony numbers from three independent experiments; **P*<0.05. (C) TNFα inhibited BM-progenitor proliferation. LSK cells isolated from TNFα-injected mice were analyzed for series-plating efficiency of hematopoietic progenitor cells. Data represents the mean ± s.d. of three experiments. (D) TNFα inhibited HSC renewal. 2×10^6 BM cells isolated from control (PBS-injected) or TNFα-injected mice (CD45.2$^+$) were transplanted together with 1×10^6 competitor cells from B6.BoyJ mice (CD45.1$^+$) into lethally irradiated recipient (B6.BoyJ) mice and long-term engraftment was evaluated 16 weeks after transplantation. Shown are representative flow cytometric data of two independent experiments (*n*=6). Numbers in the corners indicate percent of events in that quadrant.

Overproduction of the proinflammatory cytokine TNFα has been implicated in pathological conditions related to anemia and aging that is characteristic of HSC function failure (Oster et al., 1989; Kitagawa et al., 1997; Young, 2000; Dufour et al., 2003). Here, we have employed the disease model of genomic instability syndrome FA to investigate the cellular mechanism by which TNFα-induced inflammatory ROS influence hematopoiesis. We demonstrate that ROS induce premature senescence in BM cells enriched for HSCs and progenitor cells through prolonged oxidative DNA-damage response and increased genomic instability.

Results

TNFα suppresses HSC/progenitor cell activity

To examine the effect of TNFα on HSC/progenitor cell activity, we injected intraperitoneally (i.p.) wild-type (WT) or *Fancc*$^{-/-}$ mice with mouse recombinant TNFα in PBS or PBS alone. Seven days later, mice were sacrificed, and peripheral blood (PB) and BM were analyzed for hematopoietic functions. TNFα-treated *Fancc*$^{-/-}$ mice exhibited mild cytopenia, as evidenced by a decrease in red cell counts, hemoglobin and hematocrit values (see supplementary material Fig. S1). Consistent with this, BM analysis of TNFα-treated *Fancc*$^{-/-}$ mice revealed a decrease in the number of cells compared with PBS-injected *Fancc*$^{-/-}$ mice (see supplementary material Table S1). In another set of experiments we analyzed hematopoietic recovery in mice injected with TNFα. We found that *Fancc*$^{-/-}$ mice recovered slowly from TNFα-induced hemo-suppression compared with WT mice (see supplementary material Fig. S2). Furthermore, *Fancc*$^{-/-}$ mice showed reduced rates of multi-lineage recovery (see supplementary material Table S2).

The frequencies of BM HSC/progenitor [lineage-negative, Sca-1-positive, c-Kit-positive (Lin$^-$-Sca-1$^+$-c-Kit$^+$)] cells (LSK cells) were reduced by more than twofold in TNFα-treated *Fancc*$^{-/-}$ mice compared with PBS controls (Fig. 1A). We thus asked whether TNFα suppressed clonogenic progenitor activity. As shown in Fig. 1B, the total number of colonies formed by LSK cells from TNFα-treated *Fancc*$^{-/-}$ mice was

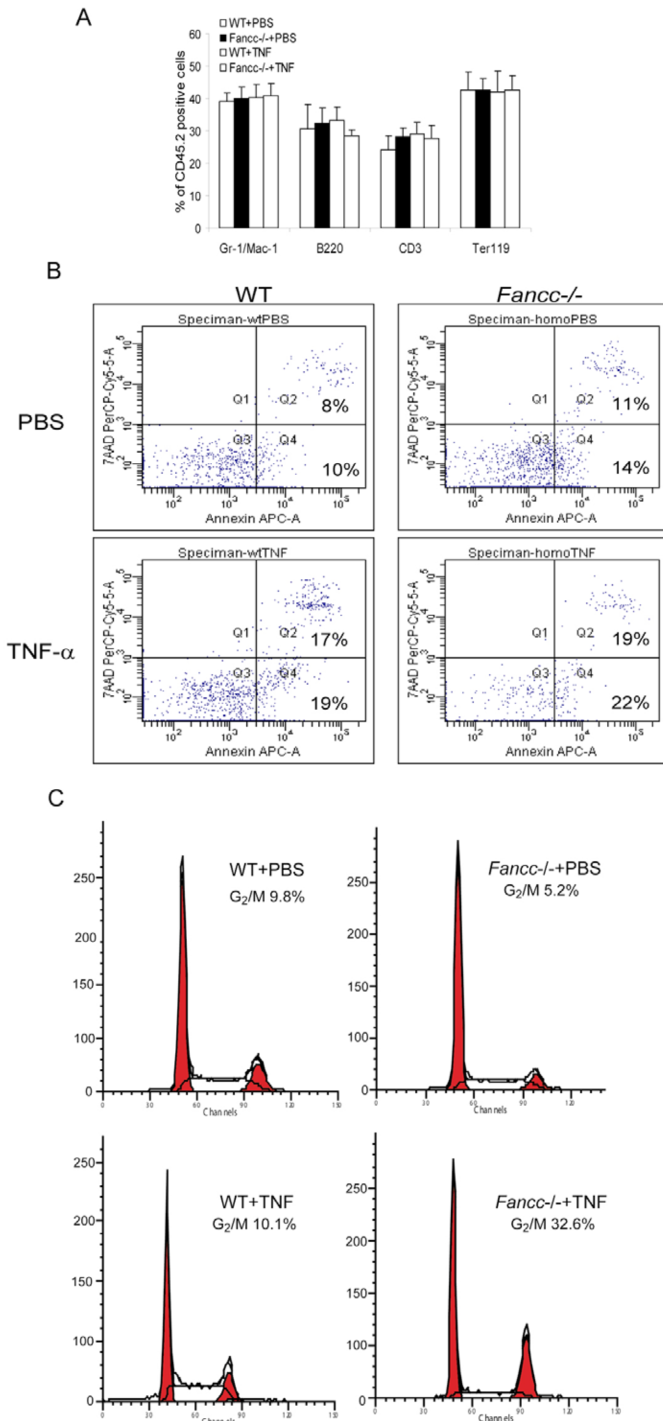

Fig. 2. Analysis of differentiation, apoptosis and cell cycle of HSCs and progenitor cells from TNFα-treated mice. (A) TNFα did not affect the differentiation of long-term repopulating HSCs. Donor-derived ($CD45.2^+$) leukocytes in peripheral blood of recipient mice transplanted with the indicated BM cells were stained with antibodies that recognize Gr-1 and Mac-1 or B220 and CD3 or Ter119. Data are expressed as mean ± s.d. of three independent experiments, each with three recipients (nine mice per group). (B) TNFα induced apoptosis in BM LSK cells of mice. BM cells from PBS- or TNFα-treated WT and *Fancc*$^{-/-}$ mice were stained with lineage maker antibodies together with Sca-1 and c-Kit antibodies, and then with annexin V. Percentages of apoptosis in the LSK population were analyzed by flow cytometry. Numbers in the quadrants indicate percent of cells labeled for 7-AAD^{low}annexin V$^+$ or 7-AAD^{high}annexin V$^+$. (C) BM LSK cells from TNFα-injected *Fancc*$^{-/-}$ mice show increased G2-M arrest. BM cells from PBS- or TNFα-treated WT and *Fancc*$^{-/-}$ mice were gated for LSK cells and stained with propidium iodide (PI) followed by analysis for cell cycle distribution. Shown are representative flow cytometric presentations of three independent experiments. Numbers in plots indicate percent of cells in G2-M phases.

more than threefold lower than that of WT mice. We also observed significantly decreased series-plating efficiency with BM LSK cells of TNFα-treated *Fancc*$^{-/-}$ mice compared with WT cells (Fig. 1C). To evaluate the in vivo effect of TNFα on HSC/progenitor cell activity, we performed BM-stem-cell transplantation. Long-term engraftment analysis shows that *Fancc*$^{-/-}$ BM HSCs were decreased in their ability to repopulate compared with WT cells (Fig. 1D), which is consistent with previous reports (Haneline et al., 1998; Haneline et al., 1999). Significantly, BM HSCs from TNFα-treated *Fancc*$^{-/-}$ mice constituted less than 10% of the peripheral blood cells at 16 weeks after transplantation compared with more than 40% reconstitution by those from TNFα-treated WT mice (Fig. 1D), indicating that TNFα impaired long-term hematopoietic reconstitution of *Fancc*$^{-/-}$ HSCs. Collectively, these results suggest that TNFα suppresses hematopoietic functions by inhibiting the self-renewal or proliferative potential of HSCs and progenitor cells.

TNFα induces premature senescence in HSC/progenitor cells

Given that TNFα suppressed the proliferative potential of HSCs and progenitor cells, we wondered whether TNFα affected differentiation or apoptosis of these primitive cells. Fig. 2A shows that BM HSCs from TNFα-injected WT or *Fancc*$^{-/-}$ donors had the ability of multilineage reconstitution, suggesting that TNFα does not affect the differentiation of long-term repopulating HSCs. Whereas TNFα induced apoptosis in BM LSK cells of treated mice, no statistically significant difference was observed between WT and *Fancc*$^{-/-}$ mice (Fig. 2B). Thus, apoptosis was not the major consequence of TNFα-mediated inhibitory effect on the self-renewal or proliferative potential of *Fancc*$^{-/-}$ HSCs and progenitor cells. However, BM LSK cells from TNFα-injected *Fancc*$^{-/-}$ mice clearly exhibited a prolonged G2-M accumulation with decreased cells in S phase (Fig. 2C), suggesting that the G2 checkpoint is compromised in these BM HSC/progenitor cells. This result reminisces of DNA-damage-induced G2-M arrest, a hallmark of FA (Bagby, Jr, 2003; Kennedy and D'Andrea, 2005).

It has been suggested that HSC senescence plays an important role in the pathophysiology of hematologic diseases, such as aplastic anemia and MDS (Maciejewski and Risitano, 2003; Zhang et al., 2005a; Zhang et al., 2005b). To determine whether HSC senescence is a defining feature of TNFα-mediated hematopoietic suppression, we examined two established senescence markers: senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995) and senescence-associated heterochromatin foci (Narita et al., 2003). We found that approximately 10% of LSK cells from TNFα-injected WT mice and more than 20% of those from TNFα-injected *Fancc*$^{-/-}$

Journal of Cell Science

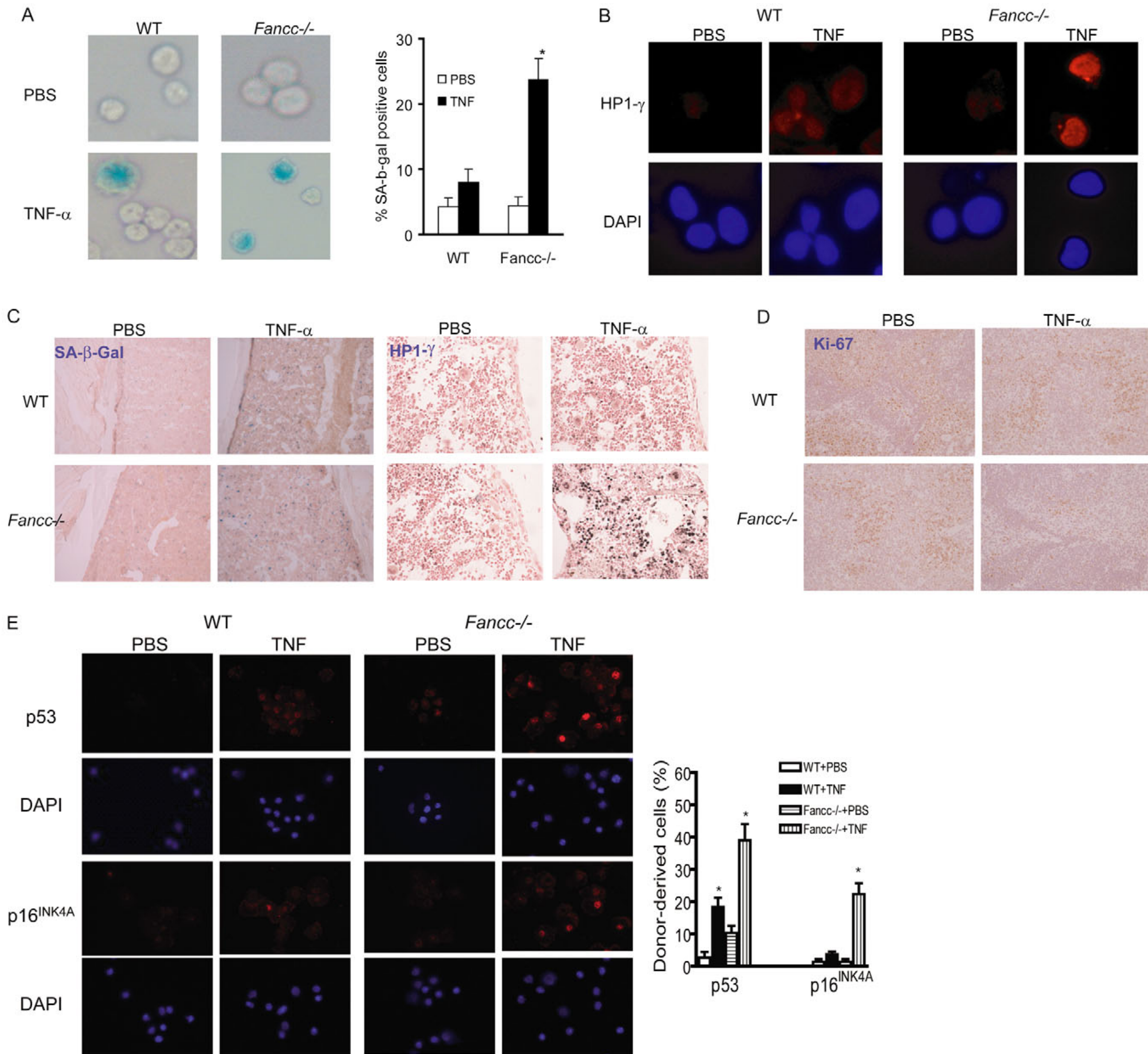

Fig. 3. TNFα induces premature senescence in HSC/progenitor cells. (A) WT or *Fancc*$^{-/-}$ mice were injected i.p. with two doses of TNFα (100 μg/kg per day) for 2 consecutive days. The mice were then sacrificed 24 hours later and BM LSK cells were isolated and stained for SA-β-gal. The bar graph shows the percentages of the cells stained positive for SA-β-gal; cells were quantified by counting >100 cells in random fields on a slide for each of three independent experiments. The data represent the mean ± s.d. of three independent experiments; *P<.05. (B) Senescence HP1-γ staining. BM LSK cells isolated from PBS- or TNFα-treated WT and *Fancc*$^{-/-}$ mice were stained for HP1-γ. DNA was then labeled with DAPI. (C) Frozen (SA-β-gal; magnification, 20×) or paraffin-embedded (HP1-γ; magnification, 40×) BM sections of PBS- or TNFα-treated WT and *Fancc*$^{-/-}$ mice were subjected to SA-β-gal (left) or HP1-γ (right) staining. (D) Paraffin-embedded spleen sections of PBS- or TNFα-treated WT and *Fancc*$^{-/-}$ mice, stained with antibody against the proliferation marker Ki-67 (magnification, 20×). (E) BM LSK cells of TNFα-treated *Fancc*$^{-/-}$ mice showed strong immunostaining for p53 and p16^{Ink4a}. The BM LSK cells isolated from PBS- or TNFα-treated WT and *Fancc*$^{-/-}$ mice were stained with the antibodies against p53 and p16^{Ink4a} and then counterstained with DAPI. The bar graph shows the percentages of the cells stained positive for p53 and p16^{Ink4a} ; cells were quantified by counting >100 cells in random fields on a slide for each of three independent experiments; *P<0.05.

animals stained positive for SA-β-gal activity (Fig. 3A). LSK cells from TNFα-treated *Fancc*$^{-/-}$ mice also stained strongly positive for HP1-γ, indicating the formation of senescence-associated heterochromatin foci (Fig. 3B) (Narita et al., 2003). In addition, analysis of these two senescence markers in BM sections from TNFα-injected *Fancc*$^{-/-}$ mice revealed abundant positive staining in those cells (Fig. 3C) accompanied with a weak proliferation index, as identified by the proliferation marker Ki-67 (Fig. 3D). We also examined two established effectors of stress-induced senescence, p53 and p16^{INK4A} (Randle et al., 2001; Serrano and Blasco, 2001). We observed that the BM LSK cells of TNFα-injected *Fancc*$^{-/-}$ mice stained

Journal of Cell Science

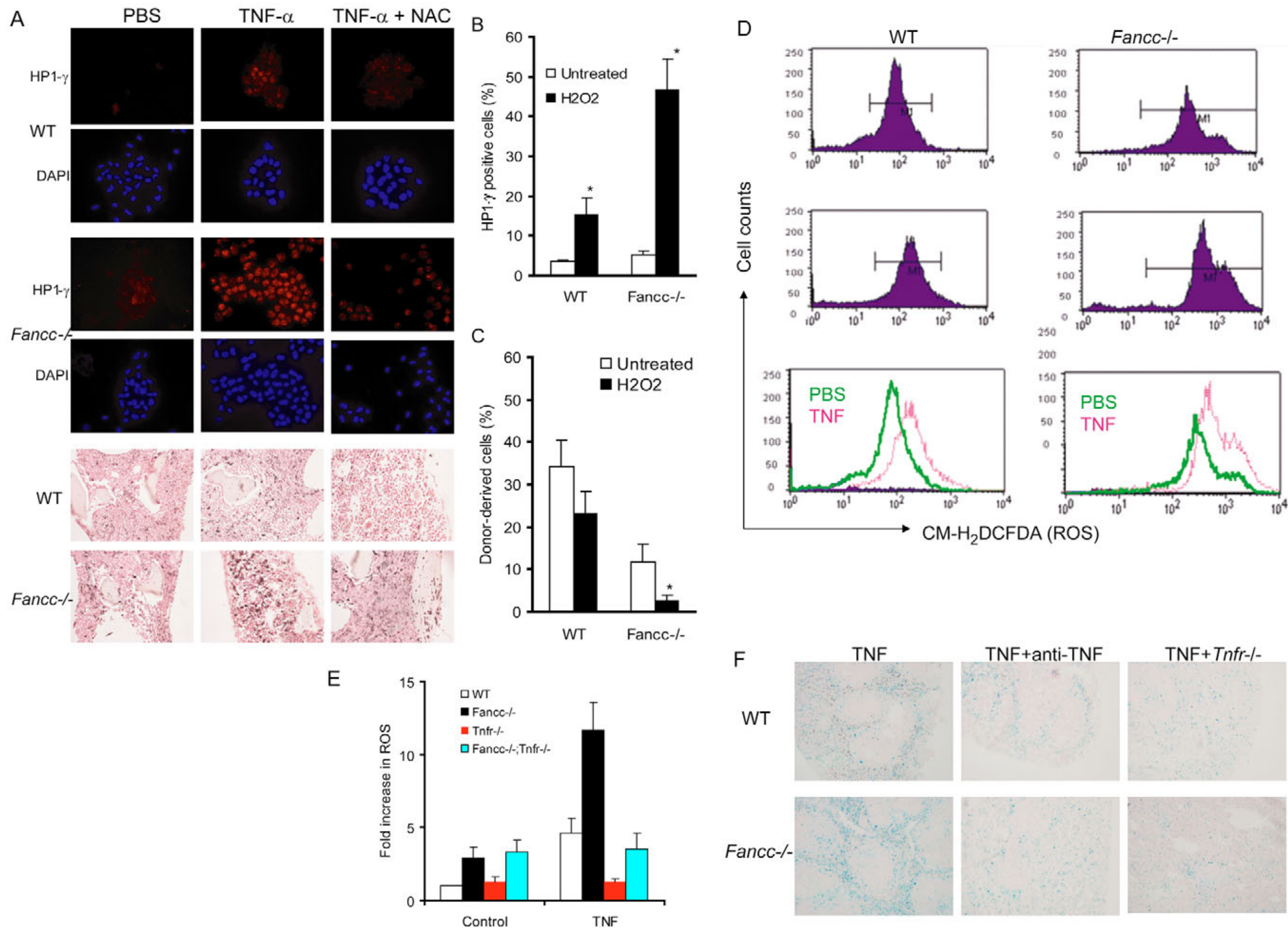

Fig. 4. TNFα-induced senescence is mediated by oxidative stress in *Fancc*-deficient HSCs and progenitor cells. (A) The ROS scavenger NAC mitigated TNFα-induced senescence in HSCs and progenitor cells. WT and *Fancc*$^{-/-}$ mice were injected i.p. with TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse per day) was administered 30 minutes before and after each TNFα injection. The isolated BM LSK cells (top and middle groups of panels) or paraffin-embedded BM sections (bottom group of panels; magnification, 40×) were stained with the antibody against the senescence marker HP1-γ. The LSK cell slides were also counterstained with DAPI. (B) BM LSK cells from WT and *Fancc*$^{-/-}$ mice were cultured in the absence or presence of H_2O_2 (100 μM) for 45 minutes, washed and further cultured in fresh medium for 24 hours. Cells were then stained with the antibody against the senescence marker HP1-γ. Shown is quantification of HP1-γ-positive cells by scoring >100 cells in random fields on a slide for each of three independent experiments; *P<0.05. (C) BM LSK cells from WT and *Fancc*$^{-/-}$ mice were cultured in the absence or presence of H_2O_2 (100 μM) for 45 minutes, washed and further cultured in fresh medium for 24 hours. 1000 cells were mixed with 1×10^6 competitor cells were transplanted into lethally irradiated recipient mice and long-term engraftment was evaluated 16 weeks after transplantation. Data are expressed as mean ± s.d. of two independent experiments, each with six recipients (12 mice per group). P<0.05 between untreated and treated *Fancc*$^{-/-}$ samples. (D) ROS production. BM cells from PBS- or TNFα-treated WT and *Fancc*$^{-/-}$ mice were labeled with CM-H2DCFDA followed by flow cytometry. (E) ROS production was mediated by TNFα. WT, *Fancc*$^{-/-}$ mice or their littermates lacking the *Tnfr1* gene were injected with PBS or TNFα at 0.1 mg/kg per day for 2 consecutive days. 24 hours after TNFα injection, BM cells from individual animals were isolated and labeled with CM-H2DCFDA followed by flow cytometry. Data represent the mean ± s.d. of two independent experiments, each with six mice (total 12 mice per group). (F) WT, *Fancc*$^{-/-}$ mice or their littermates lacking the *Tnfr1* gene were injected with TNFα (0.1 mg/kg per day) for 2 consecutive days. TNFα-treated mice were injected with 20 μg of a TNFα-neutralizing antibody 30 minutes after each TNFα injection. At 24 hours after TNFα injection, frozen spleen sections were prepared and subjected to SA-β-gal staining.

positive for p53 and p16^{INK4A}, whereas those from untreated mice stained essentially negative (Fig. 3E). These results suggest that senescence is a defining feature of TNFα-mediated hematopoietic suppression in *Fancc*$^{-/-}$ mice.

Premature senescence of HSC/progenitor cells is linked to TNFα-induced ROS production

Telomere shorting has been proposed to be a cause of HSC senescence (Lansdorp, 2005). The telomere length of BM cells from TNFα-injected mice was comparable to that of untreated mice, regardless of genotype (see supplementary material Fig. S3A). Overexpression of the mouse telomere reverse transcriptase (mTERT) in TNFα-treated *Fancc*$^{-/-}$ BM cells enriched for HSCs and progenitor (Lin$^-$) cells failed to abrogate senescence (see supplementary material Fig. S3B) or to restore long-term repopulating ability of these HSCs and progenitor cells (see supplementary material Fig. S3C). The production of reactive oxygen species (ROS) has been

implicated as a mechanism of TNFα-induced cell death (Sakon et al., 2003; Ventura et al., 2004). To address whether ROS might be relevant for TNFα-induced hematopoietic senescence, we pretreated the TNFα-injected mice with the ROS scavenger N-acetyl-L-cysteine (NAC). Remarkably, NAC almost completely abrogated the induction of senescence-associated heterochromatin foci (HP1-γ) by TNFα in both BM LSK cells and BM sections of *Fancc*$^{-/-}$ mice (Fig. 4A), indicating that TNFα-induced senescence was mediated by oxidative stress. Examination of the effect of exogenous peroxide (H_2O_2; a potent ROS producer) on BM LSK cells demonstrated that, indeed, *Fancc*$^{-/-}$ HSC/progenitor cells were vulnerable to oxidative-stress-induced senescence (Fig. 4B) and inhibition of hematopoietic reconstitution (Fig. 4C). Analysis of ROS production by flow cytometry revealed that TNFα did not cause substantial ROS production in the BM of WT mice (Fig. 4D). By contrast, there was a significant ROS accumulation in the *Fancc*$^{-/-}$ BM. In vitro culture of isolated BM LSK cells in the presence of TNFα further demonstrated increased ROS production in *Fancc*$^{-/-}$ HSC/progenitor cells (see supplementary material Fig. S4).

To ascertain that it was TNFα that induced ROS production in hematopoietic cells, we took two approaches: pretreatment of mice with neutralizing anti-TNFα antibody and inactivation of TNFα signaling in *Fancc*$^{-/-}$ mice. For the latter, *Fancc*$^{-/-}$ mice were crossed with mice carrying a null mutation in the type 1 TNF receptor (*Tnfr1*$^{-/-}$ mice). Nearly complete prevention of TNFα-induced ROS accumulation (Fig. 4E) and senescence induction (Fig. 4F) was obtained with TNFα-injected mice deficient for *Tnfr1*. Whereas treatment of mice with anti-TNFα antibody 30 minutes after each TNFα injection did not completely neutralize serum TNFα [mean ELISA values for anti-TNFα and vehicle-injected animals were 78.6±12.4 pg/ml and 245±21.5 pg/ml, respectively, in TNFα-treated WT mice (*n*=6) compared with 63.9±17.2 pg/ml and 227±23.8 pg/ml, respectively, in TNFα-treated *Fancc*$^{-/-}$ mice (*n*=6); the basal level of serum TNFα in either WT or *Fancc*$^{-/-}$ mice was approximately 20 pg/ml], administration of the anti-TNF monoclonal antibody resulted in significant reduction of senescence in the spleen (Fig. 4F). In vitro assays further demonstrated that *Fancc*$^{-/-}$;*Tnfr1*$^{-/-}$ BM HSC/progenitor cells were resistant to TNFα-induced ROS production and senescence (see supplementary material Fig. S5). Thus, oxidative stress is responsible for TNFα-induced hematopoietic senescence in *Fancc*-deficient mice.

To specifically assess TNFα-induced oxidative damage in hematopoietic organs, we immunostained for 4-hydroxy-2-nonenal (HNE), an established marker of ROS-induced tissue damage and an aldehyde product of polyunsaturated fatty acid oxidation (Cauwels et al., 2003). HNE staining was evident in the spleen of WT mice treated with TNFα (see supplementary material Fig. S6), in which we did not observe significant induction of senescence (Figs 3, 4). We found further increase in HNE immunoreactivity in the treated *Fancc*$^{-/-}$ mice (see supplementary material Fig. S6). This increase in HNE immunoreactivity was largely prevented by anti-TNFα antibody or NAC treatment (see supplementary material Fig. S6). Thus, oxidative organ damage induced by TNFα, as detected by fatty acid oxidation, appears to be proportional to the level of ROS production but not the extent of induction of senescence.

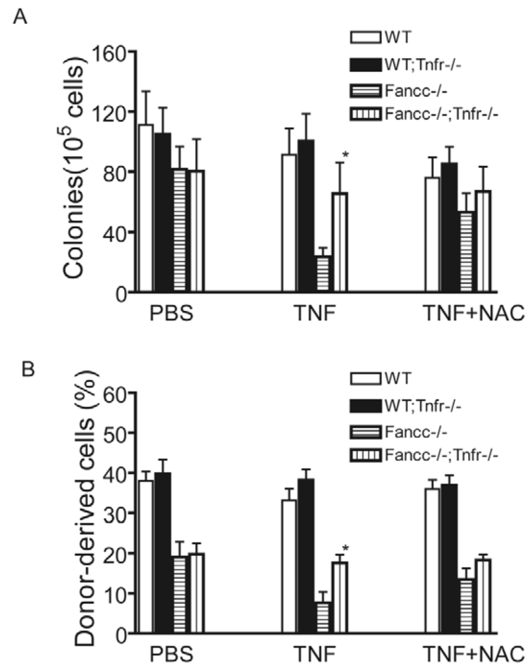

Fig. 5. Role of TNFα and ROS in hematopoietic suppression. (A) The ROS scavenger NAC or deletion of *Tnfr1* gene rescued progenitor growth. WT, *Fancc*$^{-/-}$ mice or their littermates lacking *Tnfr1* were injected with PBS or TNFα (0.1 mg/kg per day) for 2 consecutive days. Mice were injected with NAC (1 mg/mouse per day) 30 minutes before and after TNFα injection. Twenty-four hours later, BM cells were isolated and subjected to clonogenic assay. Data shown represent the mean ± s.d. of the total number of colonies from three independent experiments; **P*<0.05. (B) Anti-oxidant NAC or deletion of the *Tnfr1* gene restored HSC self-renewal ability. 2×10^6 BM mononuclear cells isolated from the mice described in A were transplanted together with 1×10^6 competitor cells from B6.BoyJ mice (CD45.1$^+$) into lethally irradiated recipient mice; long-term engraftment was evaluated 16 weeks after transplantation. Data represent the mean ± s.d. of three independent experiments with three recipients per group for each experiment. **P*<0.05.

To determine the effect of TNFα-induced ROS on *Fancc*$^{-/-}$ hematopoietic function, we performed two established assays: clonogenic progenitor assay and competitive hematopoietic repopulation assay using BM cells from double-knockout (*Fancc*$^{-/-}$;*Tnfr1*$^{-/-}$) mice. Inactivation of TNFα signaling in *Fancc*$^{-/-}$ (*Fancc*$^{-/-}$;*Tnfr1*$^{-/-}$) mice rescued progenitor growth (Fig. 5A) and stem cell repopulating ability (Fig. 5B). Pretreatment of TNFα-injected mice with the ROS scavenger NAC also significantly reduced the inhibitory effect of TNFα in progenitor growth and hematopoietic reconstitution. Interestingly, NAC did not further improve progenitor proliferation or hematopoietic reconstitution of BM cells from mice deficient for the *Tnfr1* gene (Fig. 5A,B).

TNFα induced senescence of HSC/progenitor cells as a result of accumulation of oxidative DNA damage and increased genomic instability

Oxidative stress can induce DNA damage that causes growth arrest and cellular senescence (Ames et al., 1993; Chen, 2000). To determine whether TNFα-generated ROS induced DNA damage in HSC/progenitor cells that appeared to have

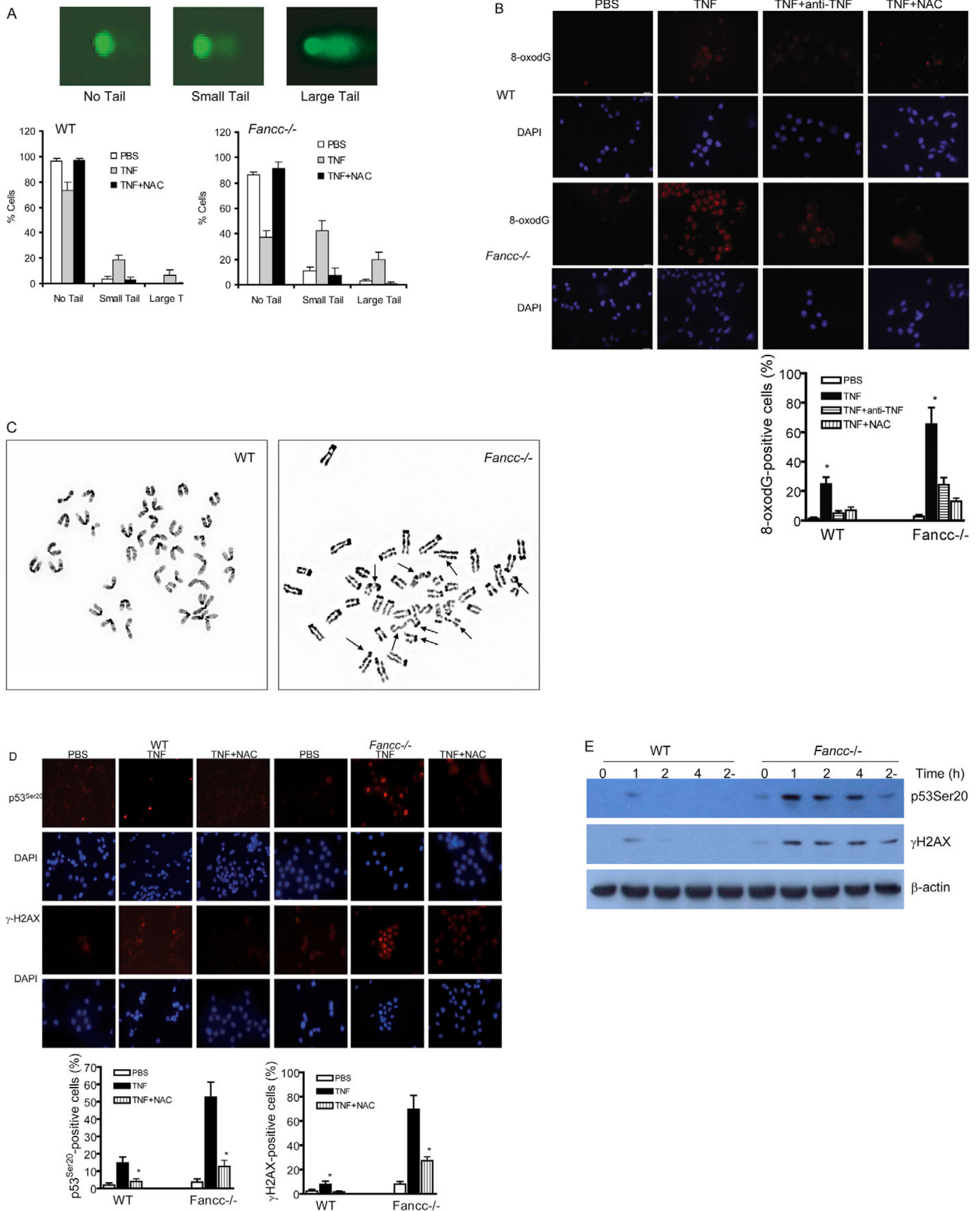

Fig. 6. See next page for legend.

Journal of Cell Science

Journal of Cell Science

Fig. 6. TNFα-induced senescence of HSCs and progenitor cells is associated with oxidative DNA damage and genomic instability. (A) WT or *Fancc*$^{-/-}$ mice were injected i.p. with two doses of TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse per day) was administered 30 minutes before and after each TNFα injection. The mice were then sacrificed 24 hours later and BM cells from individual mice were analyzed for DNA strand breaks in a the comet assay. Larger tails represents higher levels of DNA damage. For each treatment, at least 100 cells were scored from random sampling. Data are expressed as the mean ± s.d. of two independent experiments, each with three mice (six mice per group). (B) WT or *Fancc*$^{-/-}$ mice were injected i.p. with two doses of TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse/day) was administered 30 minutes before and after each TNFα injection. The mice were then sacrificed 24 hours later and BM LSK cells were isolated and stained for the oxidative DNA damage marker 8-oxo-deoxyguanosin (8-oxodG). The bar graph shows the percentages of the cells stained positive for 8-oxodG and quantified by counting >100 cells in random fields on a slide for each of two experiments with total 6 mice. (C) Examples of metaphase chromosomes prepared from TNFα-treated WT and *Fancc*$^{-/-}$ BM cells. Arrows indicate aberrant chromosomes. (D) WT or *Fancc*$^{-/-}$ mice were injected i.p. with two doses of TNFα (100 μg/kg per day) for 2 consecutive days. NAC (1 mg/mouse/day) was administered 30 minutes before and after each TNFα injection. Mice were sacrificed 24 hours later and BM LSK cells were then isolated and stained for p53^{Ser20} and γH2AX. Bar graphs show the percentages of the cells stained positive for p53^{Ser20} and γH2AX. Cells were quantified by counting >100 cells in random fields on a slide for each of two experiments with total six mice. *$P<0.05$. (E) Kinetics of DNA repair of oxidative DNA damage and DNA strand breaks. BM cells from WT and *Fancc*$^{-/-}$ mice were treated with or without H_2O_2 (100 μM) for the indicated time periods, and protein extracts were prepared and analyzed by immunoblotting with antibody against phosphorylated p53^{Ser20} (p53^{Ser20}) and anti-γH2AX and anti-actin antibodies. Extracts were also prepared from cells 2 hours (2-) after H_2O_2 withdrawal after the cells had been treated with H_2O_2 for 4 hours.

undergone ROS-dependent senescence (Fig. 4), we employed the comet assay (Fairbairn et al., 1995) using a Fpg-FLARE (fragment length analysis using repair enzymes) assay kit. This assay measures specifically oxidative DNA damage including single- and double-strand DNA breaks. There was significant accumulation of DNA damage in BM LSK cells freshly isolated from TNFα-treated WT mice and, to a much greater degree, in TNFα-treated *Fancc*$^{-/-}$ mice (Fig. 6A). Moreover, there was a significant difference between WT and *Fancc*$^{-/-}$ cells in terms of kinetics of DNA damage repair, as measured by the remaining amounts of DNA strand breaks over a period of 8 hours of TNFα treatment (see supplementary material Fig. S7). The levels of TNFα-induced DNA strand breaks remained high in *Fancc*$^{-/-}$ cells at each time point compared with those in WT cells. A similar increase in 8-oxo-deoxyguanosin (8-oxodG), an established marker of oxidative DNA damage, was also demonstrated in BM LSK cells from TNFα-injected *Fancc*$^{-/-}$ mice compared with treated WT mice (Fig. 6B). Treatment of TNFα-injected *Fancc*$^{-/-}$ mice with neutralizing anti-TNFα antibody or NAC reduced the accumulation of both DNA strand breaks and 8-oxodG (Fig. 6A,B). Thus, TNFα-induced senescence in HSC/progenitor cells appears to involve oxidative DNA damage.

Genomic instability is the cellular hallmark of FA (Bagby, Jr, 2003; Kennedy and D'Andrea, 2005), and unrepaired TNFα-induced oxidative DNA damage may increase genomic instability leading to premature senescence of HSC/progenitor cells. To test this possibility, we conducted karyotype analysis of BM Lin$^-$ (enriched for HSC/progenitor) cells from TNFα-injected WT and *Fancc*$^{-/-}$ mice. Chromosomal aberrations were rare in cells from treated WT mice, with fewer than 10% of the cells showing one aberration and almost no cells containing two or more aberrations (Table 1). By contrast, BM HSC/progenitor cells from TNFα-injected *Fancc*$^{-/-}$ mice showed significant increase in chromosomal aberrations (56% of the cells contained at least one aberration, 35% had two or more aberrations, and 23% had three or more aberrations; Table 1). FISH analysis of the chromosomal damages indicated that the TNFα-induced aberrations in *Fancc*$^{-/-}$ cells consisted mostly of chromatid breaks, gaps, chromosomal breaks, dicentric chromosomes, double minutes, chromosome fragments and fusions (Fig. 6C; Table 2), suggesting that the efficiency and accuracy of DNA strand break/repair may have been compromised in these cells.

Table 1. Chromosome aberrations in Lin$^-$ BM cells from TNF-treated mice

	Frequency (%)			
	WT		*Fancc*$^{-/-}$	
Aberrations	PBS	TNF	PBS	TNF
⩽1 aberration	3	8	3	56
⩽2 aberrations	0	1	1	35
<3 aberrations	0	0	0	23
<39 chromosomes	1	2	0	5
>40 chromosomes	1	4	1	9

A biochemical consequence of genomic damage is the activation of DNA-damage response markers, which include phosphorylated kinases ATM and Chk2, and phosphorylated histone H2AX (γH2AX) and p53 (Shiloh, 2003; Bartkova et al., 2005). Phosphorylation of p53 at Ser20 (p53^{Ser20}) is considered a specific indicator of oxidative DNA damage (Shieh et al., 1999; d'Adda di Fagagna et al., 2003); whereas the formation of γH2AX foci constitutes a robust marker of DNA strand breaks (Banin et al., 1998; Celeste et al., 2003). In HSC/progenitor cells from TNFα-injected WT mice, about 15% of cells stained positive for p53^{Ser20} and less than 10% stained positive for γH2AX (Fig. 6D). However, a majority of the cells from TNFα-injected *Fancc*$^{-/-}$ mice were intensively stained for p53^{Ser20} (62%) or γH2AX (80%). Treatment of the mice with NAC reduced these damage responses to near basal levels (Fig. 6D).

The persistent high levels of oxidative DNA damage observed in HSC/progenitor cells from TNFα-injected *Fancc*$^{-/-}$ mice suggest that a deficiency in the FA pathway renders chromosomal DNA susceptible to ROS attack, thereby increasing oxidative DNA damage. Alternatively, a defect in the FA function might compromise the damage response/repair process. To distinguish between these possibilities, we treated BM cells from WT and *Fancc*$^{-/-}$ mice with H_2O_2, and conducted a time-course study to assess DNA-repair kinetics by examining the levels of p53^{Ser20} and γH2AX. Compared with WT cells, *Fancc*$^{-/-}$ cells consistently showed a significant delay in the kinetics of DNA-damage response/repair as evidenced by the much slower clearance of the markers of oxidative DNA damage (p53^{Ser20}) and DNA strand break

Journal of Cell Science

(γH2AX) (Fig. 6E). This suggests that *Fancc*$^{-/-}$ cells accumulated high levels of oxidative DNA damage due to impairment of DNA-damage response/repair rather than to an increase in the susceptibility of their DNA to oxidative damage.

Discussion

We used the disease model of the genomic instability syndrome FA to investigate the role of TNFα-mediated inflammation in HSC senescence. We demonstrated that TNFα induced premature senescence in BM HSCs and progenitor cells as well as other tissues of mice deleted for the *Fancc* gene, which is required for the maintenance of genetic integrity (Bagby, Jr, 2003; Kennedy and D'Andrea, 2005). Importantly, TNFα-induced senescence of HSCs and progenitor cells correlated with the accumulation of inflammatory ROS, and oxidative DNA damage. ROS are involved in the induction of a senescent phenotype characterized by irreversible growth arrest (Chen et al., 1995; Sohal and Weindruch, 1996). The question naturally arises as to whether the inhibition of ROS can rescue HSCs and progenitor cells from TNFα-mediated senescence. Our findings revealed that pretreatment of TNFα-injected *Fancc*-deficient mice with antioxidant abrogated the exacerbated inflammatory phenotype, and prevented oxidative DNA damage and hematopoietic senescence. It is clear that the persistent inflammatory response, as reflected by prolonged production of inflammatory molecules, is primarily responsible for the generation of ROS and senescence, because mice deficient for both FA function and TNFα signaling (*Fancc*$^{-/-}$*;Tnfr1*$^{-/-}$) were resistant to TNFα-induced ROS production and senescence. Moreover, HSCs and progenitor cells from TNFα-treated *Fancc*-deficient mice showed increased genomic instability and an impaired repair of oxidative DNA damage. Our study thus provides a link between inflammatory ROS and HSC senescence.

Compelling evidence indicates that ROS are widely implicated in the inflammatory process (Lavrovsky et al., 2000). However, the mechanism by which ROS contribute to the proinflammatory states of the aging process is not well defined. Our results demonstrated that TNFα-induced ROS contributed to the increase in oxidative DNA damage in *Fancc*-deficient HSCs and progenitor cells. Inflammatory ROS have been associated with the initiation or aggravation of diverse pathological states including aging and cancers (Cutler, 2005; Wallace, 2005; Yoshida et al., 2005). We believe that in cells with compromised DNA repair capacity, the ability of inflammatory ROS to damage DNA is the mechanism through which ROS mediate their effects on the inflammatory and, thus, aging process. Indeed, studies have shown that the production of ROS by TNFα at inflammatory sites causes DNA damage (Goossens et al., 1999; Suematsu et al., 2003; Wheelhouse et al., 2003). The role of ROS in TNFα-induced inflammation and HSC senescence was validated by the use of the ROS scavenger NAC, which showed that inhibition of ROS accumulation reduced oxidative DNA damage and cellular senescence. In addition, mice treated with TNFα accumulated high levels of ROS, and administration of NAC significantly reduced the amounts of secreted pro-inflammatory cytokines (Y.Q. and Q.P., unpublished results), indicating that the inflammatory response is, at least in part, mediated by ROS. Thus, these results suggest that ROS serves as a link between inflammation and senescence.

Table 2. TNF-induced chromosome instability in *Fancc*$^{-/-}$ HSC/progenitors

Aberration	WT (%)	*Fancc*$^{-/-}$ (%)
Chromosome breaks	1	15
Chromosome fragment	2	8
Chromatid break	2	13
Chromatid gap	2	10
Complex rearrangement	0	3
Deletions	1	8
Dicentric chromosome	0	5
Double minute	0	1
Translocation	0	22

By investigating four well-established senescence makers (SA-β-gal, HP1-γ, p53 and p16^{INK4A}), we provided evidence that TNFα induced premature senescence in BM HSCs and progenitor cells and also other tissues of *Fancc*$^{-/-}$ mice. Recently, another senescence marker protein, SMP30, has been shown to play an important role in senescence and aging, and SPM-deficiency renders mice highly susceptible to oxidative stress (Sato et al., 2006). It would be interesting to examine the effect of TNFα on the expression pattern of this new senescence marker in HSCs and progenitor cells of *Fancc*$^{-/-}$ mice.

The persistent DNA damage response and increased genomic instability in senescent HSCs, and progenitor cells are two important features of our study. BM progenitor cells from TNFα-treated *Fancc*$^{-/-}$ mice contained high levels of DNA strand breakage and oxidative DNA damage. Moreover, studies of repair kinetics revealed much slower clearance of the oxidative DNA damage and DNA strand break markers in *Fancc*$^{-/-}$ HSCs and progenitor cells than in WT cells. Consistent with this, DNA damage response was persisted in these *Fancc*$^{-/-}$ cells. This suggests that premature senescence observed in *Fancc*$^{-/-}$ HSCs and progenitor cells may result from the prolonged activation of the oxidative DNA damage and DNA double-strand-break checkpoints. However, this prolonged checkpoint activation did not facilitate damage repair. Instead, we found dramatically increased genomic instability in *Fancc*$^{-/-}$ cells. Thus, we conclude that it is unrepaired DNA damage that results in the persistent DNA damage response in these cells.

Our study raises an important question: can inflammatory response be a link between aging and HSC function? It is long known that general inflammatory stress tends to increase with age (Chung et al., 2001). Under inflammatory conditions, HSCs must be able to produce a large number of leukocytes, which are then activated to fight against invaders or stressing agents. This, ultimately, may lead to premature exhaustion of the HSC pool. In the meantime, the HSCs become targets of the toxicity of inflammatory ROS. The ensuing consequence will depend upon the capacity of HSCs to repair ROS-induced damage, particularly DNA damage. In aged and certain disease states, inefficient repair of the oxidative damage may lead to the decrease of the HSC quality (self-renewal capacity). Hence, the toll of inflammatory stress consists in premature senescence of HSCs.

Chronic inflammation and oxidative stress are important features in the pathogenesis of BM diseases such as FA (Lavrovsky et al., 2000; Bagby, Jr, 2003; Chen, 2005). The

increased oxidative stress in FA patients may be the result of an increased burden of endogenously produced oxidants as well as increased amounts of ROS generated by various inflammatory cytokines – as suggested in our study. Therefore, understanding the relationship between ROS and inflammation in the context of HSC senescence and aging in these disease states provides a unique opportunity to mechanistically comprehend, and potentially intervene in these physiologically important processes. In addition, our results suggest that, antioxidant compounds may be of therapeutic value in monitoring disease progression, and antioxidant therapy could be used to stop the initiation and propagation of inflammatory response in these diseases.

Materials and Methods

Mice and treatments

Generation of *Fancc* knockout mice has been described by Chen et al. (Chen et al., 1996). *Fancc*$^{+/-}$ mice were intercrossed with C57Bl/6 mice for more than ten generations to develop an inbred strain. *Fancc*$^{-/-}$ mice and their WT littermate controls were generated by interbreeding the heterozygous *Fancc*$^{+/-}$ mice. The mice were maintained on a C57BL/6 (CD45.2$^+$) background. *Fancc*$^{-/-}$*;Tnfr1*$^{-/-}$ double-knockout mice were generated by mating *Fancc*$^{+/-}$ with *Tnfr1*$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME), followed by the mating of F1 heterozygous siblings. All mice were used at approximately 10-14 weeks of age. Mice were injected intraperitoneally (i.p.) with mouse recombinant TNFα (Peprotech) in PBS at 0.1 mg/kg per day for 2 consecutive days. N-acetyl-L-cysteine (NAC; Sigma) was injected i.p. at 1 mg per mouse 30 minutes before and after each TNFα injection. For anti-TNFα antibody treatment, TNFα-treated mice were injected with 20 μg of neutralizing mouse anti-TNFα antibody (R&D Systems) 30 minutes after each TNFα injection. All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Isolation of BM LSK Cells

BM mononuclear cells were depleted of lineage-committed cells using the magnetic-activated cell separation (MACS) cell separation columns (Miltenyi Biotec Inc.). Lin$^-$-Sca-1$^+$-c-Kit$^+$ (LSK) cells were then purified by staining Lin$^-$ cells with phycoerythrin (PE)-conjucated anti-Sca-1 (Sca-1–PE) and allophycocyanin (APC)-conjucated anti-c-Kit (c-Kit–APC) antibodies (BD PharMingen) followed by cell sorting using a fluorescence-activated cell sorter (FACS) FACSCalibur (Becton Dickinson).

Clonogenic progenitor assays

BM progenitor cells were cultured in a 35-mm tissue culture dish in 4 ml of semi-solid medium containing 3 ml of MethoCult M 3134 (Stem Cell Technologies) and the following growth factors: 100 ng/ml stem cell factor (SCF), 10 ng/ml interleukin-3 (IL-3), 100 ng/ml granulocyte colony-stimulating factor (G-CSF) and 4 U/ml erythropoietin (Peprotech). On day 10 after plating, the colonies were counted. Colony growth results are given expressed as the mean (of triplicate plates) ± s.d.

BM transplantation

Age-matched congenic B6.SJL-PtrcaPep3b/BoyJ (B6.BoyJ; CD45.1$^+$) mice (Jackson Laboratories, Bar Harbor, ME) were used as transplant recipients. These mice were lethally irradiated (9.5 Gy, 110 cGy/minute, ^{137}Cs γ-rays) and injected intravenously with 2×10^6 test cells (CD45.2$^+$), mixed with 1×10^6 competitor cells (BoyJ; CD45.1$^+$). Donor-derived repopulation in recipients was assessed by the proportion of leukocytes in peripheral blood that expressed the CD45.2 marker by flow cytometry. Short- and long-term engraftment and multi-lineage repopulation analysis of donor cells were performed 1 month and 4 month after transplantation, respectively.

Flow cytometric analysis of HSC and lineage differentiation

Cells were suspended in FACS buffer (0.1% FCS in 0.02% sodium azide) and incubated with the indicated antibodies on ice for 30 minutes, followed by two washes. Data were collected on a FACSCalibur (Becton Dickinson). Labeling was done using the following antibodies: anti-Sca-1, anti-c-Kit, anti-B220, anti-CD3e, anti-CD4, anti-CD8, anti-Gr-1, anti-Mac-1 and anti-Ter119 (all from BD PharMingen, San Diego, CA).

Apoptosis assay and cell cycle analysis

Cells were stained with annexin V and 7-AAD using BD ApoAlert Annexin V kit (BD PharMingen) in accordance with the manufacturer's instructions. Apoptosis was analyzed by quantification of annexin-V-positive cell population by flow cytometry. For cell cycle analysis, cells were permeabilized with 0.3% Nonidet P-40 (NP-40), and then stained with propidium iodide (PI) containing 1 mg/ml RNase A, followed by FACS analysis of the G0-G1, S and G2-M populations.

Analysis of HSC senescence

Cells were cytospun onto slides and fixed in ice-cold methanol for 5 minutes at –20°C. After air drying, cells were blocked for 1 hour with 5% normal serum. Then cells were incubated with antibodies against HP1-γ (07-332; Upstate Cell Signaling Solutions, Lake Placid, NY), p53 (FL-393) and p16^{INK4A} (F-12) (both from Santa Cruz Biotechnology) in PBS with 2% normal serum at room temperature for 1 hour. After extensive washes, cells were incubated with PE-conjugated secondary antibody (Jackson, Bar Harbor, ME). DNA was then labeled with 4,6 diamidino-2-phenylindole (DAPI; Sigma). Slides were finally mounted in mounting medium (Vector). For senescence-associated β galactosidase (SA-β-gal) assay, cells were stained using a SA-β-gal staining kit (#9860; Cell Signaling) according to manufacturer's instructions.

Determination of ROS production

Cells were incubated with CM-H_2DCFDA (Molecular Probe) in the dark for 15 minutes at 37°C. After washing, cells were analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data were analyzed by using the CellQuest program (BD Biosciences).

Immunohistochemistry

During necropsy, organs were removed, preserved in formalin and then embedded in paraffin blocks. Paraffin sections were deparaffinized, rehydrated, incubated in 0.1 mM sodium citrate (pH 6.0), washed and incubated with peroxidase blocking reagent (Vector Laboratories, VectaStain Elite ABC kit). After washing in PBS, slides were incubated with primary antibodies against HNE (11-S; Alpha Diagnostic International, San Antonio, TX) or HP1-γ (07-332; Upstate Cell Signaling Solutions, Lake Placid, NY). Following three PBS washes, slides were incubated with secondary antibody and then detected with the VectaStain Elite ABC reagents.

Retroviral vectors and infection

The retroviral vector expressing the mTERT was kindly provided by Fuyuki Ishkawa (Kyoto University, Japan). Retroviruses were prepared by the Vector Core at Cincinnati Children's Research Foundation. Retroviral supernatant was collected 36 hours, 48 hours and 60 hours after transfection. Cells were plated onto non-tissue culture 24-well plates coated with Retronectin (Takara-Shuzo, Shiga, Japan) and pre-stimulated for 2 days in Iscove's modified Dulbecco's medium (IMDM) containing 20% FCS, 100 ng/ml SCF, 20 ng/ml IL-6, and 50 ng/ml Flt-3L (Peprotech). Cells were then exposed to the retroviral supernatant for 3 hours at 37°C in the presence of 4 μg/ml Polybrene (Sigma). Cells were centrifuged at 600 ***g*** for 45 minutes. Infection was repeated twice and infection efficiency was assessed by the detection of green fluorescent protein (GFP)-positive cells by FACS.

Immunoblotting

Cells were solubilized in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet NP-40) containing a cocktail of protease inhibitors (Calbiochem, San Diego, CA). Equal amounts of protein were separated on 10% SDS-PAGE, transferred to a nitrocellulose membrane and blotted with antibodies against p53^{Ser20} (Santa Cruz Biotech), γ-H2AX (Upstate Biotechnologies), and β-actin (Sigma).

Serum levels of cytokines

Serum levels of inflammatory cytokines were measured using enzyme-linked immunoadsorbent assays (ELISA) kits (R&D Systems).

Comet assay

Generation of DNA strand breaks was assessed by the single-cell gel electrophoresis (comet) assay (Fairbairn et al., 1995), using a Fpg-FLARE (fragment-length analysis using repair enzymes) comet assay kit in accordance with the manufacturer's instructions (Trevigen, Gaithersberg, MO). For each experimental point at least three different cultures were analyzed, and 100 cells were evaluated for comet-tail length from each culture.

Cytogenetic analysis

Cells were incubated in 0.1 mg/ml colcemid for 30-60 minutes and then incubated in 75 mM KCl. Chromosomes were subsequently fixed in methanol:acetic acid (3:1) and dropped onto glass slides. Metaphase chromosomes were Giemsa-stained and examined for abnormalities. Chromosome aberrations were defined using the nomenclature rules from the Committee on Standard Genetic Nomenclature for Mice.

Statistics

Data were analyzed statistically using a two-tail Student's *t*-test. The level of statistical significance stated in the text was based on the *P* values. $P<0.05$ was considered statistically significant.

Journal of Cell Science

We thank Manuel Buchwald (Hospital for Sick Children, University of Toronto) for the Fancc$^{+/-}$ mice, Fuyuki Ishkawa (Kyoto University) for the mTERT-expressing retroviral vectors, Reena Rani for technical assistance, Jeff Bailey and Victoria Summey for bone marrow transplantation, and the Vector Core of the Cincinnati Children's Research Foundation (Cincinnati Children's Hospital Medical Center) for the preparation of retroviruses. Q.P. thanks Grover Bagby (Oregon Health Science University) for continued support. This work was supported in part by NIH grants R01 CA109641 and R01 HL076712.

References

Ames, B. N., Shigenaga, M. K. and Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proc. Natl. Acad. Sci. USA* **90**, 7915-7922.

Bagby, G. C., Jr (2003). Genetic basis of Fanconi anemia. *Curr. Opin. Hematol.* **10**, 68-76.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y. et al. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science* **281**, 1674-1677.

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J. M., Lukas, C. et al. (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* **434**, 864-870.

Bischof, O., Kim, S. H., Irving, J., Beresten, S., Ellis, N. A. and Campisi, J. (2001). Regulation and localization of the Bloom syndrome protein in response to DNA damage. *J. Cell Biol.* **153**, 367-380.

Cao, L., Li, W., Kim, S., Brodie, S. G. and Deng, C. X. (2003). Senescence, aging, and malignant transformation mediated by p53 in mice lacking the Brca1 full-length isoform. *Genes Dev.* **17**, 201-213.

Cauwels, A., Janssen, B., Waeytens, A., Cuvelier, C. and Brouckaert, P. (2003). Caspase inhibition causes hyperacute tumor necrosis factor-induced shock via oxidative stress and phospholipase A2. *Nat. Immunol.* **4**, 387-393.

Celeste, A., Difilippantonio, S., Difilippantonio, M. J., Fernandez-Capetillo, O., Pilch, D. R., Sedelnikova, O. A., Eckhaus, M., Ried, T., Bonner, W. M. and Nussenzweig, A. (2003). H2AX haploinsufficiency modifies genomic stability and tumor susceptibility. *Cell* **114**, 371-383.

Chen, J. (2005). Senescence of hematopoietic stem cells and bone marrow failure. *Int. J. Hematol.* **82**, 190-195.

Chen, L., Huang, S., Lee, L., Davalos, A., Schiestl, R. H., Campisi, J. and Oshima, J. (2003). WRN, the protein deficient in Werner syndrome, plays a critical structural role in optimizing DNA repair. *Aging Cell* **2**, 191-199.

Chen, M., Tomkins, D., Auerbach, W., McKerlie, C., Youssoufian, H., Liu, L., Gan, O., Carreau, M., Auerbach, A., Groves, T. et al. (1996). Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. *Nat. Genet.* **12**, 448-451.

Chen, Q. M. (2000). Replicative senescence and oxidant-induced premature senescence. Beyond the control of cell cycle checkpoints. *Ann. N. Y. Acad. Sci.* **908**, 111-125.

Chen, Q., Fischer, A., Reagan, J. D., Yan, L. J. and Ames, B. N. (1995). Oxidative DNA damage and senescence of human diploid fibroblast cells. *Proc. Natl. Acad. Sci. USA* **92**, 4337-4341.

Chung, H. Y., Kim, H. J., Kim, J. W. and Yu, B. P. (2001). The inflammation hypothesis of aging: molecular modulation by calorie restriction. *Ann. N. Y. Acad. Sci.* **928**, 327-335.

Cumming, R. C., Lightfoot, J., Beard, K., Youssoufian, H., O'Brien, P. J. and Buchwald, M. (2001). Fanconi anemia group C protein prevents apoptosis in hematopoietic cells through redox regulation of GSTP1. *Nat. Med.* **7**, 814-820.

Cutler, R. G. (2005). Oxidative stress and aging: catalase is a longevity determinant enzyme. *Rejuvenation Res.* **8**, 138-140.

d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P. and Jackson, S. P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature* **426**, 194-198.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I. and Pereira-Smith, O. A. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. USA* **92**, 9363-9367.

Dufour, C., Corcione, A., Svahn, J., Haupt, R., Poggi, V., Beka'ssy, A. N., Scime, R., Pistorio, A. and Pistoia, V. (2003). TNF-alpha and IFN-gamma are overexpressed in the bone marrow of Fanconi anemia patients and TNF-alpha suppresses erythropoiesis in vitro. *Blood* **102**, 2053-2059.

Fairbairn, D. W., Olive, P. L. and O'Neill, K. L. (1995). The comet assay: a comprehensive review. *Mutat. Res.* **339**, 37-59.

Futaki, M., Igarashi, T., Watanabe, S., Kajigaya, S., Tatsuguchi, A., Wang, J. and Liu, J. M. (2002). The FANCG Fanconi anemia protein interacts with CYP2E1: possible role in protection against oxidative DNA damage. *Carcinogenesis* **23**, 67-72.

Goossens, V., De Vos, K., Vercammen, D., Steemans, M., Vancompernolle, K., Fiers, W., Vandenabeele, P. and Grooten, J. (1999). Redox regulation of TNF signaling. *Biofactors* **10**, 145-156.

Hadjur, S., Ung, K., Wadsworth, L., Dimmick, J., Rajcan-Separovic, E., Scott, R. W., Buchwald, M. and Jirik, F. R. (2001). Defective hematopoiesis and hepatic steatosis in mice with combined deficiencies of the genes encoding Fancc and Cu/Zn superoxide dismutase. *Blood* **98**, 1003-1011.

Haneline, L. S., Broxmeyer, H. E., Cooper, S., Hangoc, G., Carreau, M., Buchwald, M. and Clapp, D. W. (1998). Multiple inhibitory cytokines induce deregulated progenitor growth and apoptosis in hematopoietic cells from Fac$^{-/-}$ mice. *Blood* **91**, 4092-4098.

Haneline, L. S., Gobbett, T. A., Ramani, R., Carreau, M., Buchwald, M., Yoder, M. C. and Clapp, D. W. (1999). Loss of *FancC* function results in decreased hematopoietic stem cell repopulating ability. *Blood* **94**, 1-8.

Haneline, L. S., Li, X., Ciccone, S. L., Hong, P., Yang, Y., Broxmeyer, H. E., Lee, S. H., Orazi, A., Srour, E. F. and Clapp, D. W. (2003). Retroviral-mediated expression of recombinant Fancc enhances the repopulating ability of Fancc $^{-/-}$ hematopoietic stem cells and decreases the risk of clonal evolution. *Blood* **101**, 1299-1307.

Howlett, N. G., Taniguchi, T., Olson, S., Cox, B., Waisfisz, Q., De Die-Smulders, C., Persky, N., Grompe, M., Joenje, H., Pals, G. et al. (2002). Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* **297**, 606-609.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N. et al. (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* **431**, 997-1002.

Kennedy, R. D. and D'Andrea, A. D. (2005). The Fanconi Anemia/BRCA pathway: new faces in the crowd. *Genes Dev.* **19**, 2925-2940.

Kitagawa, M., Saito, I., Kuwata, T., Yoshida, S., Yamaguchi, S., Takahashi, M., Tanizawa, T., Kamiyama, R. and Hirokawa, K. (1997). Overexpression of tumor necrosis factor (TNF)-alpha and interferon (IFN)-gamma by bone marrow cells from patients with myelodysplastic syndromes. *Leukemia* **11**, 2049-2054.

Kruyt, F. A., Hoshino, T., Liu, J. M., Joseph, P., Jaiswal, A. K. and Youssoufian, H. (1998). Abnormal microsomal detoxification implicated in Fanconi anemia group C by interaction of the FAC protein with NADPH cytochrome P450 reductase. *Blood* **92**, 3050-3056.

Lansdorp, P. M. (2005). Role of telomerase in hematopoietic stem cells. *Ann. N. Y. Acad. Sci.* **1044**, 220-227.

Lavrovsky, Y., Chatterjee, B., Clark, R. A. and Roy, A. K. (2000). Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp. Gerontol.* **35**, 521-532.

Li, X., Yang, Y., Yuan, J., Hong, P., Freie, B., Orazi, A., Haneline, L. S. and Clapp, D. W. (2004). Continuous in vivo infusion of interferon-gamma (IFN-gamma) preferentially reduces myeloid progenitor numbers and enhances engraftment of syngeneic wild-type cells in Fancc$^{-/-}$ mice. *Blood* **104**, 1204-1209.

Maccio, A., Madeddu, C., Massa, D., Mudu, M. C., Lusso, M. R., Gramignano, G., Serpe, R., Melis, G. B. and Mantovani, G. (2005). Hemoglobin levels correlate with interleukin-6 levels in patients with advanced untreated epithelial ovarian cancer: role of inflammation in cancer-related anemia. *Blood* **106**, 362-367.

Maciejewski, J. P. and Risitano, A. (2003). Hematopoietic stem cells in aplastic anemia. *Arch. Med. Res.* **34**, 520-527.

Meetei, A. R., Sechi, S., Wallisch, M., Yang, D., Young, M. K., Joenje, H., Hoatlin, M. E. and Wang, W. (2003). A multiprotein nuclear complex connects Fanconi anemia and Bloom syndrome. *Mol. Cell. Biol.* **23**, 3417-3426.

Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J. and Lowe, S. W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113**, 703-716.

Oster, W., Cicco, N. A., Klein, H., Hirano, T., Kishimoto, T., Lindemann, A., Mertelsmann, R. H. and Herrmann, F. (1989). Participation of the cytokines interleukin 6, tumor necrosis factor-alpha, and interleukin 1-beta secreted by acute myelogenous leukemia blasts in autocrine and paracrine leukemia growth control. *J. Clin. Invest.* **84**, 451-457.

Pagano, G., Degan, P., d'Ischia, M., Kelly, F. J., Nobili, B., Pallardó, F. V., Youssoufian, H. and Zatterale, A. (2005). Oxidative stress as a multiple effector in Fanconi anaemia clinical phenotype. *Eur. J. Haematol.* **75**, 93-100.

Park, S. J., Ciccone, S. L., Beck, B. D., Hwang, B., Freie, B., Clapp, D. W. and Lee, S. H. (2004). Oxidative stress/damage induces multimerization and interaction of Fanconi anemia proteins. *J. Biol. Chem.* **279**, 30053-30059.

Pelicci, P. G. (2004). Do tumor-suppressive mechanisms contribute to organism aging by inducing stem cell senescence? *Clin. Invest.* **113**, 4-7.

Randle, D. H., Zindy, F., Sherr, C. J. and Roussel, M. F. (2001). Differential effects of p19(Arf) and p16(Ink4a) loss on senescence of murine bone marrow-derived preB cells and macrophages. *Proc. Natl. Acad. Sci. USA* **98**, 9654-9659.

Saadatzadeh, M. R., Bijangi-Vishehsaraei, K., Hong, P., Bergmann, H. and Haneline, L. S. (2004). Oxidant hypersensitivity of Fanconi anemia type C-deficient cells is dependent on a redox-regulated apoptotic pathway. *J. Biol. Chem.* **279**, 16805-16812.

Sablina, A. A., Budanov, A. V., Ilyinskaya, G. V., Agapova, L. S., Kravchenko, J. E. and Chumakov, P. M. (2005). The antioxidant function of the p53 tumor suppressor. *Nat. Med.* **11**, 1306-1313.

Sakon, S., Xue, X., Takekawa, M., Sasazuki, T., Okazaki, T., Kojima, Y., Piao, J. H., Yagita, H., Okumura, K., Doi, T. et al. (2003). NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death. *EMBO J.* **22**, 3898-3909.

Sato, T., Seyama, K., Sato, Y., Mori, H., Souma, S., Akiyoshi, T., Kodama, Y., Mori, T., Goto, S., Takahashi, K. et al. (2006). Senescence marker protein-30 protects mice lungs from oxidative stress, aging, and smoking. *Am. J. Respir. Crit. Care Med.* **174**, 530-537.

Seal, S., Barfoot, R., Jayatilake, H., Smith, P., Renwick, A., Bascombe, L., McGuffog, L., Evans, D. G., Eccles, D., Easton, D. F. et al. (2003). Evaluation of Fanconi Anemia genes in familial breast cancer predisposition. *Cancer Res.* **63**, 8596-8599.

Serrano, M. and Blasco, M. A. (2001). Putting the stress on senescence. *Curr. Opin. Cell Biol.* **13**, 748-753.

Shieh, S. Y., Taya, Y. and Prives, C. (1999). DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. *EMBO J.* **18**, 1815-1823.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. *Nat. Rev. Cancer* **3**, 155-168.

Si, Y., Ciccone, S., Yang, F. C., Yuan, J., Zeng, D., Chen, S., van de Vrugt, H., Critser, J., Arwert, F., Haneli, L. S. et al. (2006). Continuous in vivo infusion of interferon-gamma (IFN-{gamma}) enhances engraftment of syngeneic wild-type cells in Fanca$^{-/-}$ and Fancg$^{-/-}$ mice. *Blood* **108**, 4283-4287.

Sohal, R. S. and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. *Science* **273**, 59-63.

Suematsu, N., Tsutsui, H., Wen, J., Kang, D., Ikeuchi, M., Ide, T., Hayashidani, S., Shiomi, T., Kubota, T., Hamasaki, N. et al. (2003). Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. *Circulation* **107**, 1418-1423.

Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P. R., Gregory, R. C., Kim, S. T., Lane, W. S., Kastan, M. B. and D'Andrea, A. D. (2002). Convergence of the fanconi anemia and ataxia telangiectasia signaling pathways. *Cell* **109**, 459-472.

Ventura, J. J., Cogswell, P., Flavell, R. A., Baldwin, A. S., Jr and Davis, R. J. (2004). JNK potentiates TNF-stimulated necrosis by increasing the production of cytotoxic reactive oxygen species. *Genes Dev.* **18**, 2905-2915.

Wallace, D. C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* **39**, 359-407.

Wheelhouse, N. M., Chan, Y. S., Gillies, S. E., Caldwell, H., Ross, J. A., Harrison, D. J. and Prost, S. (2003). TNF-alpha induced DNA damage in primary murine hepatocytes. *Int. J. Mol. Med.* **12**, 889-894.

Yoshida, T., Nakamura, H., Masutani, H. and Yodoi, J. (2005). The involvement of thioredoxin and thioredoxin binding protein-2 on cellular proliferation and aging process. *Ann. N. Y. Acad. Sci.* **1055**, 1-12.

Young, N. S. (2000). Hematopoietic cell destruction by immune mechanisms in acquired aplastic anemia. *Semin Hematol.* **37**, 3-14.

Young, N. S. (2002). Acquired aplastic anemia, *Ann. Intern. Med*. **136**, 534-546.

Zhang, X., Li, J., Sejas, D. P. and Pang, Q. (2005a). Hypoxia-reoxygenation induces premature senescence in FA bone marrow hematopoietic cells. *Blood* **106**, 75-85.

Zhang, X., Li, J., Sejas, D. P. and Pang, Q. (2005b). The ATM/p53/p21 pathway influences cell fate decision between apoptosis and senescence in reoxygenated hematopoietic progenitor cells. *J. Biol. Chem.* **280**, 19635-19640.