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Accelerated hematopoietic stem cell aging in a mouse model of dyskeratosis congenita responds to antioxidant treatment

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Summary

Mutations in *DKC1*, encoding telomerase associated protein dyskerin, cause X-linked dyskeratosis congenita (DC), a bone marrow failure and cancer susceptibility syndrome. Decreased accumulation of telomerase RNA resulting in excessive telomere shortening and premature cellular senescence is thought to be the primary cause of disease in X-linked DC. Affected tissues are those that require constant renewal by stem cell activity. We previously showed that in *Dkc1*$^{\Delta 15}$ mice, which contain a mutation that is a copy of a human mutation causing DC, mutant cells have a telomerase dependent proliferative defect and increased accumulation of DNA damage in the first generation before the telomeres are short. We now demonstrate the presence of the growth defect in *Dkc1*$^{\Delta 15}$ mouse embryonic fibroblasts *in vitro* and show that accumulation of DNA damage and levels of reactive oxygen species increase with increasing population doublings. Treatment with the antioxidant, N-acetyl cysteine, partially rescued the growth disadvantage of mutant cells *in vitro* and *in vivo*. Competitive bone marrow repopulation experiments showed that the *Dkc1*$^{\Delta 15}$ mutation is associated with a functional stem cell defect that becomes more severe with increasing age, consistent with accelerated senescence, a hallmark of DC hematopoiesis. This stem cell phenotype was partially corrected by N-acetyl cysteine treatment. These results suggest that a pathogenic *Dkc1* mutation, accelerates stem cell aging, that increased oxidative stress might play a role in the pathogenesis of X-linked DC, and that some manifestations of DC may be prevented or delayed by antioxidant treatment.

Keywords

dyskeratosis congenita; N-acetyl cysteine; Stem cell aging; telomerase; oxidative stress; ROS

Introduction

Dyskeratosis congenita (DC) is a disorder of telomere maintenance and offers a unique opportunity to study the consequences of telomere dysfunction (Bessler *et al.* 2008). The disease is heterogeneous in presentation and mutations in 6 different genes, all encoding components of telomerase (Vulliamy & Dokal 2008) or shelterin, (de Lange 2005; Savage *et*

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Author Contributions

B-W G, MB and PM conceived the study. B-W G and J-M F did the experimental work. B-W G, MB and PM analyzed and interpreted the data and wrote the manuscript.

al. 2008) the protein complex that caps and protects telomeres, have been implicated. Experiments using telomerase null mice showed that excessively short telomeres activate a DNA damage response causing cellular senescence and cell death leading to a mouse phenotype that mimics many aspects of the clinical disease seen in patients with DC (Blasco *et al.* 1997; Marciniak & Guarente 2001). However, in these mice, due to their long telomeres, a disease phenotype only becomes evident after several generations of inbreeding causing the telomeres to become very short (Blasco *et al.* 1997). It has therefore been postulated that excessively short telomeres are the primary cause of disease in DC (Hao *et al.* 2005; Mason *et al.* 2005; Kirwan & Dokal 2009). In fact, in patients with DC the tissues primarily affected are those that require constant renewal through stem cell activity, such as skin, bone marrow, lung and gut endothelium and at the time of disease manifestation, all patients with DC have very short telomeres (Mitchell *et al.* 1999; Vulliamy *et al.* 2001; Du *et al.* 2009).

We have previously reported a genetic DC mouse model, in which mice (Gu *et al.* 2008), carry a *Dkc1* exon 15 gene deletion, encoding a truncated dyskerin protein with a C-terminal deletion of 21 amino acids, previously found in a DC family (Vulliamy *et al.* 1999). We demonstrated that in these mice the $Dkc1^{\Delta 15}$ cells had a growth disadvantage compared to wild type (WT) cells, which in heterozygous $Dkc1^{\Delta 15/+}$ female mice, due to the localization of *Dkc1* on the X chromosome, led to progressive disparity favoring wild type over mutant cells. This was due to the growth advantage of cells expressing the wild type *Dkc1* allele over those expressing the mutant allele after random X-chromosome inactivation in early embryogenesis (Lyon 1961), a phenotype universally seen in women who are *DKC1* mutation carriers (Vulliamy *et al.* 1997; Gu *et al.* 2008). We demonstrated that the growth disadvantage of $Dkc1^{\Delta 15}$ mutant cells in part is mediated by the p53 pathway, dependent on telomerase activity, but does not require telomere shortening (Gu *et al.* 2008), and that $Dkc^{\Delta 15}$ mutant cells accumulate increased levels of DNA damage. These findings challenged the current idea that *DKC1* mutations solely cause disease through destabilizing the telomerase RNA (*TERC*)(Mitchell *et al.* 1999) which quantitatively reduces telomerase enzymatic activity, and leads to the critically short telomeres that ultimately are responsible for disease.

We now demonstrate that the reduced growth rate of $Dkc1^{\Delta 15}$ cells correlates with the accumulation of DNA damage foci that is dependent on and increases with proliferation *in vitro*, and that *in vivo* the $Dkc1^{\Delta 15}$ mutation leads to an age dependent decrease of hematopoietic stem cell (HSC) function consistent with the accelerated aging of HSC characteristic of DC. Furthermore we find that $Dkc1^{\Delta 15}$ mutant cells are hypersensitive to oxygen and show that the decreased growth rate is associated with an increased accumulation of reactive oxygen species (ROS). Finally, we demonstrate that the growth disadvantage of $Dkc1^{\Delta 15}$ cells can be overcome in part by treatment with the antioxidant N-acetyl cysteine (NAC) not only *in vitro* but more importantly also *in vivo*. Thereby our investigations in $Dkc1^{\Delta 15}$ mice identify oxidative stress as a potential new player in the pathogenesis of DC and thus uncover a new drugable target that may be utilized to prevent or delay disease in patients with DC.

Results

$Dkc1^{\Delta 15}$ MEF cells have a growth disadvantage that is associated with increasing ROS levels and a proliferation dependent enhanced accumulation of DNA damage

In heterozygous $Dkc1^{\Delta 15/+}$ female mice, $Dkc1^{\Delta 15}$ mutant cells have a growth disadvantage, when compared to normal cells, which is in part mediated by the p53 pathway, dependent on telomerase activity but apparently independent of telomere length (Gu *et al.* 2008). To investigate the growth phenotype conferred by the $Dkc1^{\Delta 15}$ mutation in cell culture we

compared the growth rate of male *Dkc1*$^{\Delta 15}$ (Δ15) and *Dkc1*$^{+}$ (WT) mouse embryonic fibroblasts (MEFs) (Figure 1). The growth rate of Δ15 MEF cells was lower when cultured at both ambient oxygen (21%) and low (3%) oxygen. In 21% oxygen both types of cells stopped growing and entered senescence after 8-10 population doublings, with the Δ15 cells growing more slowly than the WT cells. In 3% oxygen Δ15 cells grew more slowly and entered senescence earlier than WT cells (Figure 1A,B).

Recent studies implicate ROS in mediating cell senescence and genomic instability (Colavitti & Finkel 2005; Rassool *et al.* 2007). We thus measured the accumulation of ROS using the oxidation sensitive dye 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). We found that there was a significantly higher level of ROS accumulation in Δ15 cells and WT cells when cultured in high oxygen compared to low oxygen and in both types of cells ROS levels increased with increasing population doublings. (Figure 2). Interestingly, Δ15 cells accumulated more ROS than WT cells and the difference became very significant with more population doublings, even when cells were cultured in low oxygen.

To further investigate the basis of impaired proliferation we examined the appearance of phosphorylated histone H2AX (γ-H2AX) foci, indicators of DNA damage (Rogakou *et al.* 1998), in Δ15 and WT MEFs. There was a significantly higher number of foci in Δ15 cells and the difference was greater in high oxygen (Figure 1C and Figure S1). γ-H2AX does not accumulate when cells are maintained in a quiescent state in a confluent culture, suggesting DNA replication is required (Figure S2). Since no significant differences in overall telomere lengths were observed between Δ15 and WT MEFs after 3 and 18 population doublings (Figure 1D) these data suggest the structure of the telomeres in the mutant cells, rather than their length, leads to the DNA damage accumulation and growth impairment. An alternative explanation is that increased ROS levels may result from ribosomal stress caused by the presence of mutant dyskerin in H/ACA snoRNAs and disrupted ribosome biogenesis.

These findings show the *Dkc1*$^{\Delta 15}$ mutation causes slower cell growth associated with increased DNA damage and increased levels of ROS.

Increased DNA damage in aging *Dkc1*$^{\Delta 15}$ mice

The presence of DNA damage accumulation in untreated *Dkc1*$^{\Delta 15}$ cells, and particularly its increase with increasing population doublings in culture, led us to ask if a significant amount of DNA damage accumulated in aging *Dkc1*$^{\Delta 15}$ mice. We found that indeed, while protein extracted from the spleen of a 4 month old mouse contained almost undetectable levels of γ-H2AX the levels were dramatically increased in spleens from older mice (Figure 3). In *Dkc1*$^{\Delta 15}$ mice levels of γ-H2AX were consistently higher than in WT mice in either young or old mice and in liver, spleen and bone marrow. An increase in the steady state levels of p53 was also detected in these tissues in *Dkc1*$^{\Delta 15}$ mice, suggesting chronically increased activity of the p53 DNA damage response. In agreement with these results accumulation of DNA damage in aging HSC (Rossi *et al.* 2007) and formation of γ-H2AX foci in a number of cell types in older mice (Wang *et al.* 2009) has been reported recently.

The *Dkc1*$^{\Delta 15}$ mutation causes decay of stem cell function with age

In patients with X-linked DC the clinical manifestations, including progressive bone marrow failure, are generally not present at birth but become apparent during childhood and adolescence and affect mainly tissues with a high cell turnover. Male *Dkc1*$^{\Delta 15}$ mice have no phenotype either during the first 3 months of life, but interestingly show a decrease in the proportion of B and T lymphocytes with age along with a reduction in body weight (Figure 6C and D). We were therefore interested in further investigating the effect of the *Dkc1*$^{\Delta 15}$

mutation on hematopoiesis during aging. The number of BM cells, erythroid committed progenitor cells, and c-Kit$^+$, Sca-1$^+$ and Lineage$^-$ (KLS) cells (Rossi*et al.* 2005), a BM cell fraction enriched in functional stem cells, were not significantly different between *Dkc1*$^{\Delta 15}$ and *Dkc1*$^+$ mice or when comparing young and old mice (Figure S3), showing the absence of overt BM cell depletion in these mice. To study the effect of the mutation on stem cell function during aging we carried out competitive repopulation and serial BM transplantation studies using the CD45.1/CD45.2 congenic system (Spangrude *et al.* 1988) to identify the origin of BM cells (Figure S4). In these experiments all donors were male and in the competitive experiments the *Dkc1*$^{\Delta 15}$ and *Dkc1*$^+$ donors were the same age, either young (10 weeks) or old (77-88 weeks). First we determined whether the growth disadvantage is intrinsic to BM cells and whether the growth disadvantage is similar for BM cells from young and old *Dkc1*$^{\Delta 15}$ mice. For this we performed competitive repopulation experiments with a 1:1 mixture of *Dkc1*$^{\Delta 15}$ and WT BM cells derived from either young or old mice (Figure 4). In these experiments *Dkc1*$^{\Delta 15}$ BM cells from old mice were more compromised in the competition with WT cells in primary recipients, making up only 20% of peripheral blood cells 12 weeks after transplant compared with 40% of *Dkc1*$^{\Delta 15}$ peripheral blood cells in mice receiving *Dkc1*$^{\Delta 15}$ and WT BM cells from young mice (Figure 4). Twelve weeks after a second round of BM transplantation, 10-30% peripheral blood cells in the secondary recipients were derived from *Dkc1*$^{\Delta 15}$ BM cells when the original donors were young. In contrast no *Dkc1*$^{\Delta 15}$ cells were detectable in recipients of BM cells from old mice (Figure 5A). These results indicated that the growth defect is intrinsic to BM cells and is accentuated in BM cells from old versus young *Dkc1*$^{\Delta 15}$ mice.

Next, we were interested in whether this age dependent growth defect occurs during hematopoietic differentiation or occurs at the stem cell level resulting in successive depletion of functional hematopoietic stem cells. For this, serial BM transplant experiments were performed using BM cells from either young or old *Dkc1*$^{\Delta 15}$ mice (Figure 5). In the primary transplant experiments both young and old cells were about equally capable of populating the recipient's BM as suggested by the about equal proportion of *Dkc1*$^{\Delta 15}$ circulating blood cells (Figure 4). In secondary transplant recipients young *Dkc1*$^{\Delta 15}$ BM cells fully restored hematopoiesis reaching levels of 90% after 16 weeks, comparable to the results obtained with secondary transplants of WT BM cells from young and old animals. In contrast, secondary transplants of BM cells from old *Dkc1*$^{\Delta 15}$ mice contributed only little to peripheral blood and their numbers decreased with time in the secondary recipients, consistent with an increasing failure of stem cell function in aging *Dkc1*$^{\Delta 15}$ BM cells. Finally, BM cells from secondary recipients were transplanted a third time. In the tertiary recipients less than 10% of peripheral blood cells were derived from the originally transplanted *Dkc1*$^{\Delta 15}$ BM cells obtained from old mice, whereas in recipients receiving BM cells obtained from young *Dkc1*$^{\Delta 15}$ mice 60% were derived from the originally transplanted BM cells. In recipient mice receiving either young or old WT BM cells about 80% of cells in tertiary recipients were derived from the original donors (Figure 5B).

The results from competitive and serial BM transplant experiments indicate that *Dkc1*$^{\Delta 15}$ BM cells have an intrinsic proliferative defect that worsens with age and is associated with an accelerated decline in stem cell function when compared with that to BM from WT mice.

Growth of *Dkc1*$^{\Delta 15}$ cells is improved by antioxidant treatment *in vitro* and *in vivo*

Two findings implicated ROS in the growth defect we observed in *Dkc1*$^{\Delta 15}$ cells and mice. First, Δ15 MEF cells were more sensitive than WT MEF cells to culture in 21% oxygen (Figure 1A and C). Second, Δ15 MEF cells accumulated more ROS during normal cell growth, in 21% or 3% oxygen than did WT MEFs (Figure 2). In addition regulation of oxidative stress has been shown to be an important factor in HSC aging (Ito *et al.* 2004; Ito *et al.* 2006; Diehn *et al.* 2009; Abbas et al. 2010; Li and Marban, 2010). We therefore asked

if oxidative stress plays a role in the growth disadvantage of *Dkc1*$^{\Delta 15}$ cells compared with WT cells by testing whether treatment with an antioxidant could rescue the growth disadvantage of *Dkc1*$^{\Delta 15}$ cells. First, we tested primary MEF cells from *Dkc1*$^{\Delta 15/+}$ female mice, in the presence or absence of 100μM N-acetyl cysteine (NAC), a clinically approved antioxidant (Figure 6A) (Atkuri *et al.* 2007). MEF cells from *Dkc1*$^{\Delta 15/+}$ female mice in early passages consist of 50% expressing WT and 50% expressing truncated Δ15 dyskerin, reflecting random X-chromosome inactivation. While without NAC the WT cells have almost completely outgrown the *Dkc1*$^{\Delta 15}$ cells after 11 population doublings, in the presence of NAC the *Dkc1*$^{\Delta 15}$ cells are still clearly present after 15 population doublings, suggesting that NAC at least partially rescues the growth disadvantage of dyskerin mutant cells. We next tested whether the NAC treatment would rescue the growth disadvantage of *Dkc1*$^{\Delta 15}$ cells *in vivo* and administered NAC to *Dkc1*$^{\Delta 15/+}$ female mice from the age of 3 weeks by adding 1mg/ml NAC into their drinking water. Analysis of the spleens from these mice compared with untreated *Dkc1*$^{15/+}$ mice showed that the *Dkc1*$^{\Delta 15}$ cells persist in the NAC treated mice for much longer than in the untreated animals, being still detectable at 24 weeks of age while in untreated mice the mutant protein, and therefore mutant cells, are barely detectable after 12 weeks (Figure 6B), indicating that NAC treatment partially rescues the growth disadvantage of *Dkc1*$^{\Delta 15}$ cells.

Decayed stem cell function rescued by antioxidant treatment

To determine if NAC treatment could be useful therapeutically, it is important to investigate its effect on stem cell function, which is defective in DC. To address this issue, we established a cohort of mice that were given NAC in their drinking water (1mg/ml) from 3 weeks of age and maintained on NAC for 1 year. We found that long term NAC treatment did not show significant side effects on the mice. They had slightly increased neutrophils, but no difference in mortality and body weight compared with the untreated group (data not shown). Impressively, old (52-55 weeks) male *Dkc1*$^{\Delta 15}$ mice from the NAC cohort did not show the decreased B and T cell proportions in peripheral blood observed in untreated *Dkc1*$^{\Delta 15}$ mice (Figure 6C and D). Competitive bone marrow transplantation experiments were carried out in which a 1:1 mixture of BM cells from mutant and WT mice was used to repopulate lethally irradiated recipient mice. These experiments showed that, when taken from NAC treated animals, old *Dkc1*$^{\Delta 15}$ BM cells could compete with age matched WT cells with 40-45% of *Dkc1*$^{\Delta 15}$ cells in primary recipients compared with only 20% for the untreated group. Moreover, after secondary transplantation, cells from the NAC treated group still represent 15-20% of *Dkc1*$^{\Delta 15}$ cells in recipients while those from the untreated group could not be detected (Figure 6E).

Discussion

The data presented here show that *Dkc1*$^{\Delta 15}$ mice show an accelerated aging phenotype of the HSC similar to that presumed to occur in DC in humans. In the human disease it is generally believed that stem cell dysfunction is caused by accelerated telomere erosion, leading to critically short telomeres and cell senescence (Kirwan & Dokal 2009). This in turn would lead to increased recruitment of stem cells and eventual exhaustion of the stem cell pool (Mason *et al.* 2005). The results presented here, where an accelerated rate of stem cell aging is evident in the first generation of *Dkc1*$^{\Delta 15}$ mice, in the presence of long telomeres, suggest that in X-linked DC stem cell function worsens throughout life and that accumulation of DNA damage may contribute to the development of stem cell depletion.

Lesions in DNA repair genes have been found to affect HSC aging in a similar way to the *Dkc1* mutation studied here (Nijnik *et al.* 2007; Rossi *et al.* 2007). Interestingly these studies included later generation mice lacking telomerase RNA. (Rossi *et al.* 2007) DNA damage triggered by the excessively short telomeres was thought to be the factor that caused loss of

HSC function with aging. In our studies the same effect is seen, but in contrast to the previous study this was seen in the first generation of *Dkc1*$^{\Delta 15}$ mice before deficiency in telomerase activity is expected to significantly shorten the telomeres (Blasco *et al.* 1997). Indeed, in-gel hybridization of DNA from MEFs did not reveal significant telomere shortening (Figure 1). These findings infer that dyskerin may have an important role at the telomeric site of telomerase action, in agreement with the presence of dyskerin in purified active telomerase (Cohen *et al.* 2007). This suggests that mutant dyskerin acts in a different way to the *TERT* and *TERC* mutations, which generally act through haploinsufficiency (Vulliamy *et al.* 2001). The data are consistent with a model of X-linked DC whereby telomerase, with a mutated dyskerin, gains access to the telomeric DNA and extends the telomeres with reduced efficiency, rendering the telomere more susceptible than normal to DNA damage or failing to protect it from being recognized as damaged DNA. Interestingly, modulation of DNA damage was not seen in *Tert*-/- cells (Erdmann & Harrington 2009) suggesting that with a reduced supply of TERT and possibly *TERC*, fewer molecules of otherwise normal telomerase will be present at the telomeres leading to the extension of fewer but intact telomeres without the induction of a DNA damage response. Our finding that the DNA damage response is dependent on replication is compatible with this model.

Alternatively we cannot exclude a telomere independent role for the mutant dyskerin. Ribosomal stress caused by perturbation of ribosome biogenesis may lead to increased ROS levels which then cause DNA damage. The DNA damage, to which telomeres are particularly susceptible may then limit stem cell function. In this model the telomerase dependent growth inhibition we previously observed might arise if telomeres were hypersensitive to ROS induced damage when being extended by active telomerase. That the effect of dyskerin mutations on ribosome biogenesis may contribute to the DC phenotype is supported by the observation that another congenital bone marrow failure syndrome, Diamond Blackfan Anemia, is caused by mutations in ribosomal proteins (Lipton and Ellis, 2010).

Our *Dkc1*$^{\Delta 15}$ mouse model is distinct from a previously reported hypomorphic *Dkc1* mouse model (Ruggero *et al.* 2003) in that our mice genetically reproduce a pathogenic *DKC1* mutation previously identified in a family with X-linked DC, whereas in the previous model, a decreased in level of *Dkc1* mRNA levels was achieved through transcriptional interference after integration of the targeting vector downstream of the *Dkc1* gene. Disease causing *DKC1* mutations cluster in the N-terminal region and the RNA binding domain of dyskerin. Models of the 3-dimensional structure have shown that these regions closely associate (Rashid *et al.* 2006) suggesting that the mutations have a specific effect on dyskerin which is likely to be reproduced in our *Dkc1*$^{\Delta 15}$ model, but not reproduced in a model expressing low levels of otherwise normal dyskerin.

Interestingly, we identified an increased replication dependent accumulation of ROS in *Dkc1*$^{\Delta 15}$ mutant cells. ROS are being increasingly implicated in cellular aging and as mediators of cell senescence and apoptosis as well as in various normal physiological processes and signaling pathways (Bertram & Hass 2008). In particular, control of ROS in HSC is essential to maintain their function (Gazit *et al.* 2008). An increased accumulation of ROS has been shown to cause a decreased hematopietic stem cell function in mice deficient in ATM (Ito *et al.* 2004), a cell cycle check point regulator that is activated by DNA damage, and in mice lacking FoxO transcription factors, which are involved in the transcriptional control of genes encoding ROS scavenging enzymes (Tothova *et al.* 2007). Stem cell defects in ATM deficient mice or in mice lacking transcription factors FoxOs 1, 3 and 5 are partially corrected by antioxidant NAC treatment (Ito *et al.* 2004; Tothova *et al.* 2007). We therefore tested the effect of NAC treatment of our *Dkc1*$^{\Delta 15}$ cells *in vitro* and *in vivo* and found that indeed, NAC treatment prevented the accumulation of ROS and restored

the proliferation defect of *Dkc1*$^{\Delta 15}$ MEF cells *in vitro.* Impressively, the treatment of heterozygous *Dkc1*$^{\Delta 15/+}$ female mice by adding NAC to the drinking water, significantly improved the survival of cells expressing the truncated Δ15 protein, indicating that NAC improved the proliferation defect of mutant cells. More interestingly, after treatment with NAC for over 1 year, *Dkc1*$^{\Delta 15}$ mice showed significant improvement in bone marrow stem cell repopulation activity as well as a restoration of the wild type proportion of lymphocytes in peripheral blood. These results suggest that pathogenic *Dkc1* mutations may operate at least in part through the accumulation of ROS levels identifying oxidative stress as a novel factor in the pathogenesis of DC.

Telomeres are thought to be particularly vulnerable to oxidative DNA damage and oxidative stress causes telomere shortening (Passos *et al.* 2007; Richter & von Zglinicki 2007). The exact mechanism whereby oxidative stress affects the growth of *Dkc1*$^{\Delta 15}$ mutant cells and the aging of HSC in *Dkc1*$^{\Delta 15}$ mice is not entirely clear since ROS can act as agents of DNA damage as well as being part of the response to DNA damage. (Macip*et al.* 2002; Rai *et al.* 2009; Abbas et al. 2010; Li and Marban, 2010) Whether the increase in ROS that we observe accumulating in mutant cells is a consequence of the DNA damage response mediated by ATM/p53/p21 (Macip *et al.* 2002) or due to an unknown pathway linking mutant dyskerin, through the telomerase or ribosome biogenesis pathways, to the regulation of ROS accumulation is unknown (Passos *et al.* 2007) (Perez-Rivero *et al.* 2008) and the subject of current investigations.

Interestingly, NAC has been shown to improve pulmonary function in patients with pulmonary fibrosis (Sharma *et al.* 2003), and for this purpose is also empirically used in patients with DC who develop pulmonary fibrosis. Telomere shortening has been thought to be the major contributor to pulmonary fibrosis in these patients (Alder *et al.* 2008) but perhaps increased sensitivity to oxidative stress plays an additional role in pathogenesis. The therapeutic effect of antioxidants or NAC treatment on hematopoietic stem cell function and whether these agents may ameliorate, delay or prevent the occurrence of bone marrow failure or pulmonary fibrosis in these patients if given early in the course of the disease remains to be determined.

Experimental Procedures

Mice

The generation of *Dkc1*$^{\Delta 15}$ mice was as previously described. (Gu *et al.* 2008) These mice were backcrossed with wild type C57BL/6 mice (CD45.2) at least 7 times. C57BL/6 mice (CD45.1) were from the Jackson Laboratory (Bar Harbor, Me).

Measurement of telomere length

MEF cells were embedded in agarose plugs by using CHEF agarose plug kit according to the manufacturer's instructions (Bio-Rad). DNA embedded in the plug was extracted, digested with Mbo I and electrophoresed through a 1% agarose gel for 20 hours at 6V/cm, 1-6seconds switch time using CHEF DR-III pulse-field system (Bio-Rad). ^{32}P-γ-ATP labeled $(CCCTAA)_4$ probe was used in the in-gel hybridization procedure (Dionne & Wellinger 1996).

Western Blot analysis

Total protein from cells and mouse tissues was prepared by using RIPA lysis buffer (1×TBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide and 1× protease inhibitor cocktail). Protein concentration was measured by using the Bio-Rad protein assay (Bio-Rad, Hercules CA).

Immunofluorescence

Immunofluorescence was performed with a standard paraformaldehyde technique (slides were fixed in PBS buffered 4% paraformaldehyde for 10 minutes, permeabilised with 0.5% Triton-PBS for 15 minutes, blocked with 30% normal goat serum for one hour). Primary antibody was used at 1/500 in 1.5% normal goat serum for two hours. After washing with PBS, cells were incubated with a secondary goat anti-rabbit IgG conjugated with FITC and/or Alexa 568 at 1/1000 in 1.5% normal goat serum for 45 minutes. All blocking and incubation steps were carried out at room temperature. Finally, slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and covered by mounting media. The cells were examined at 1000× magnification using a fluorescence microscope (Nikon, Melville, NY, USA). FITC, Alexa 568 and DAPI images were overlapped by using ISIS FISH imaging software (Metasystems).

Measurement of intracellular ROS

After being trypsinized and washed with PBS, 5×10^5 MEF cells were resuspended in pre-warmed PBS loaded with 10 μM 5,6-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA, Invitrogen) in the dark for 30 minutes at 37°C, 5 % CO_2. After one time washing with pre-warmed PBS, the oxidative conversion of CM-H_2DCFDA to its fluorescent product was measured immediately by Flowcytometry using 488nm FL1 channel.

Antibodies

The sources of antibodies were as follows: anti-γ-H2AX-S139 (Abcam, ab2893), anti-p53 (Abcam, ab26), anti-dyskerin was as previously described (Mochizuki *et al.* 2004), anti-β-Actin was used as total protein loading control (Abcam, ab20272).

Establishment of Primary Mouse Embryonic Fibroblasts (MEFs)

Dkc1$^{\Delta 15}$, control WT male and *Dkc1*$^{\Delta 15/+}$ female MEF cells were prepared from a cross of *Dkc1*$^{\Delta 15/+}$ females with WT male mice. Primary MEF cells were isolated from 13.5-day mouse embryos and harvested for analysis after 2 to 3 passages. Cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37°C in a humidified atmosphere of 3% O_2, 10% CO_2.

MEF cells lifespan analysis

Population doublings (PDs) were counted by subculturing a 90% confluent culture 1:4 for 2 population doublings.

Senescence associated β-galactosidase (SA-β-gal) staining

MEF cells were plated in a 6-well plate and 24 hrs later, the cells were washed twice in PBS for 5 min. The cells were fixed for 10 min in 4% formaldehyde and 0.2% glutaraldehyde in PBS and washed 3 times in PBS for 5 min each. The staining reaction was performed with 2 ml staining solution (1mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), 40mM citric acid/sodium phosphate, pH 6.0, 5mM potassium ferrocyanide, 5mM potassium ferricyanide, 150mM NaCl and 2mM $MgCl_2$) at 37°C for overnight in the dark. Cells were washed twice with PBS and photographed.

Flow cytometry

Bone marrow and blood leukocytes were processed for flow cytometry analysis as previously described (Keller *et al.* 2001). The following anti-mouse monoclonal antibody were used: FITC antibodies against TCRβH57-597), Gr1(RB6-8C5), CD45.2(104),

CD4(GK1.5), PE antibodies against B220(RA3-6B2),CD11b(M1/70), CD8(53-6.7), CD45.1(A20). Unless otherwise indicated, all antibodies were obtained from BD Pharmingen, San Diego, CA. The dates were collected with a FACScan.

To analyze KLS cells, lineage+ cells were first depleted by incubating with PE-Cy7 conjugated lineage markers: iL7, CD3, CD4, CD8, B220, CD19, Gr1 and ter-119. Following incubation with anti-PE-Cy7 colloid, lineage+ cells were depleted using the AutoMacs System (Miltenyi Biotec, Auburn, CA). The lineage depleted cells were then incubated with APC anti-Sca-1(D7), APC-AlexaFluor-750 anti-c-Kit (2B8), Biotin-CD34 (RAM34). All of these antibodies were purchased from e-Bioscience. Finally, cells were sorted using a MoFlo high speed flow cytometer (Dako Cytomation, Fort Collins, CO)

Competitive bone marrow repopulation assay

CD45.1 C57/BL6 recipient mice were γ-irradiated with two 5-Gy doses 4 hours apart, and injected intravenously with 5×10^6 CD45.2-*Dkc1*$^{\Delta15}$ bone marrow cells, either separately or mixed in a 1:1 ratio with CD45.1-WT cells. As a control, age matched CD45.2-WT bone marrow cells were injected separately. The recipients were kept on trimethoprim-sulfamethoxazole antibiotic drinking water during the first 2 weeks following transplantation. Mice were analyzed at 8 and 12 weeks after transplantation.

Serial bone marrow transplantation analysis

We collected 5 million CD45.2-WT or CD45.2-*Dkc1*$^{\Delta15}$ bone marrow cells and transplanted them into lethally irradiated mice (CD45.1) as described for the competitive transplantation assay. 16 weeks after the first or second transplantation, we collected 5 million bone marrow cells from transplanted mice and injected them into the secondary or tertiary recipient.

N-Acetyl-l-Cysteine (NAC) treatment

NAC (Sigma) was dissolved in PBS buffer, and was added to culture media to 100μM for culture of MEF cells. We added NAC to mouse drinking water at 1mg/ml concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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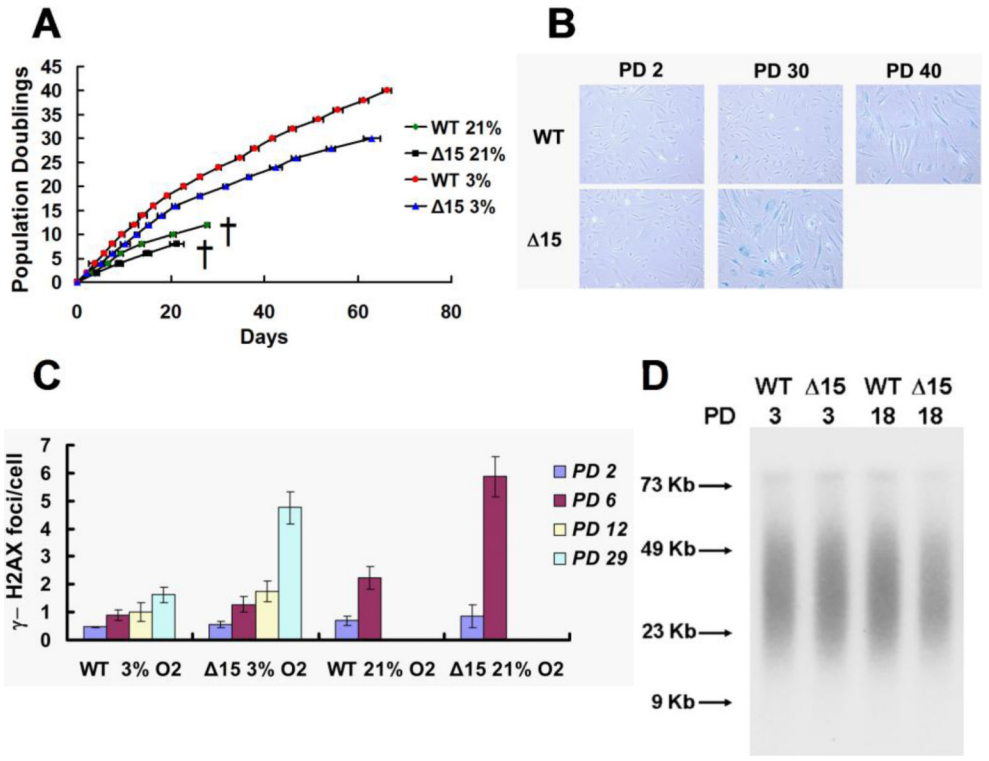

Figure 1.

Impaired proliferation and increased DNA damage in *Dkc1*$^{\Delta 15}$ MEFs.

A: Cumulative population doubling in WT and *Dkc1*$^{\Delta 15}$ (Δ15) MEFs growing in 21% or 3% O_2. The results shown represent a single experiment but similar results were obtained in three independent experiments.

B: Senescence-associated β-galactosidase (*SA-β-Gal*) staining of WT and *Dkc1*$^{\Delta 15}$ (Δ15) MEFs after different population doublings (PD). The blue staining indicates the senescent cells (Dimri *et al.* 1995).

C: Quantititation of γ-H2AX foci between WT and *Dkc1*$^{\Delta 15}$ (Δ15) MEF cells after different population doublings (PD) in different O_2 concentrations from Supplementary Figure 2. More than 200 cells were analyzed.

D. Telomere length of male WT and *Dkc1*$^{\Delta 15}$ (Δ15) MEF cells after different population doublings.

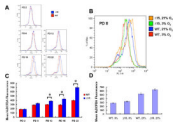

Figure 2.

Increased intracellular ROS level in *Dkc1*$^{\Delta 15}$ MEF cells.

A. WT and *Dkc1*$^{\Delta 15}$ (Δ15) MEF cells grown in 3% O_2 were treated with the oxidation sensitive dye, CM-H_2DCFDA, and the conversion to its oxidized fluorescent derivative assessed by flow cytometry.

B. WT and *Dkc1*$^{\Delta 15}$ (Δ15) MEF cells were grown in 3% or 21% O_2 for 8 population doublings and ROS measured as in A. above.

C. Quantitation of mean CM-H_2DCFDA fluorescence of A. Asterisk indicates significant difference (Student's *t-test*) P<0.01, n=6.

D. Quantitation of mean CM-H_2DCFDA fluorescence of B.

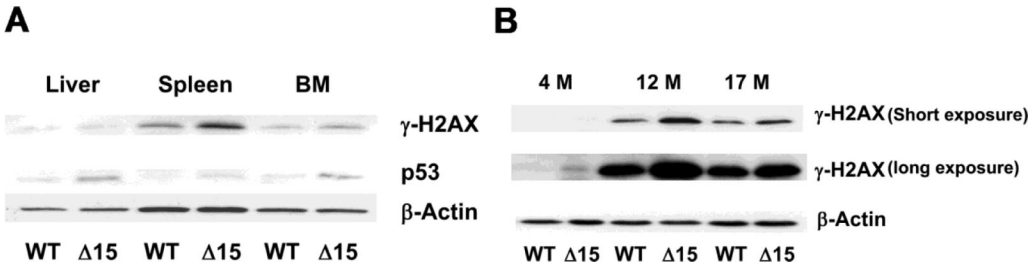

Figure 3.

DNA damage in *Dkc1*$^{\Delta 15}$ (Δ15) and WT mice.

A. A Western blot of extracted protein from mouse tissues probed with γ-H2AX, p53 and β-actin antibodies.

B. A Western blot of extracted protein from the spleens of mice of different ages probed with γ-H2AX, and β-actin antibodies.

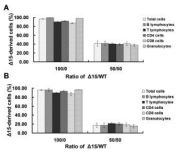

Figure 4.

Effect of aging on the competitive repopulation ability of *Dkc1*$^{\Delta 15}$ bone marrow. The percentage of total cell counts derived from *Dkc1*$^{\Delta 15}$ (Δ15) mice is plotted for B lymphocytes, T lymphocytes, CD4+ lymphocytes, CD8+ lymphocytes and granulocytes from peripheral blood of irradiated recipients 12 weeks after bone marrow transplantation with 5×10^6 *Dkc1*$^{\Delta 15}$ bone marrow cells (CD45.2) alone (100/0) or mixed with CD45.1 allotype-marked wild-type (WT) cells in 1:1 ratio (50/50). Donor ages are 10 weeks (A) or 77-88 weeks (B). In the serial transplantation experiments (100/0) *Dkc1*$^{\Delta 15}$ bone marrow cells (CD45.2) or age-matched WT cells (CD45.2) were transplanted into a CD45.1 WT recipient. In the 50/50 mixing experiments the CD45.2 *Dkc1*$^{\Delta 15}$ bone marrow cells were mixed with CD45.1 WT cells of the same age and transplanted into a CD45.1 WT recipient. N=3~5.

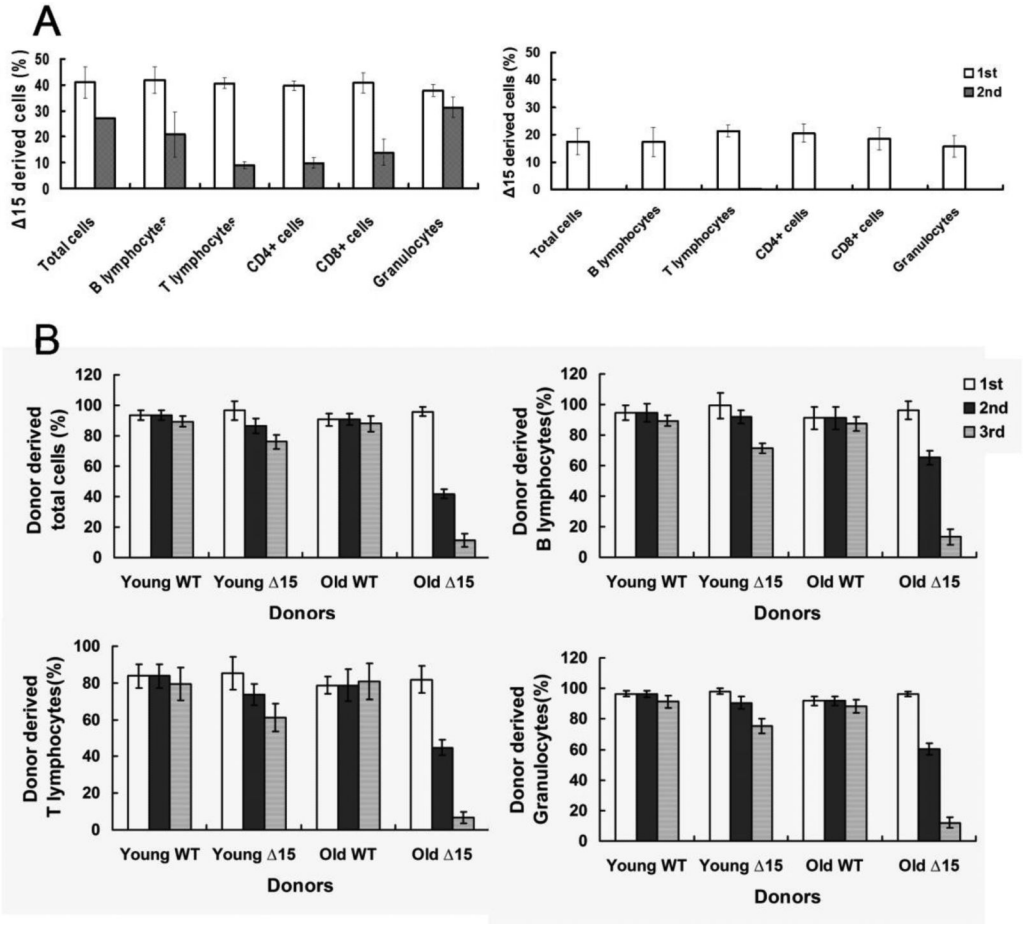

Figure 5.

Effect of aging on the competitive repopulation ability of *Dkc1*$^{\Delta 15}$ bone marrow in secondary recipient mice. A. Secondary transplant of cells from the mice primarily transplanted with a 1:1 mixture of bone marrow cells from *Dkc1*$^{\Delta 15}$ (Δ15) and *Dkc1*$^{+}$ (WT) mice. The panel on the left shows the results for the young mice and the panel on the right shows the results from the old mice, where no Δ15 cells are detected in the secondary transplant. In this experiment the CD45.2 *Dkc1*$^{\Delta 15}$ bone marrow cells were mixed with CD45.1 WT cells of the same age and transplanted into a CD45.1 WT recipient. B. Serial transplant of cells from the mice primarily transplanted with CD45.2 bone marrow cells of the genotypes and ages shown (100%). The proportion of original donor cells (CD45.2) of different lineages is plotted at 12 weeks after first, second and third transplantation. In this experiment *Dkc1*$^{\Delta 15}$ bone marrow cells (CD45.2) or age-matched WT cells (CD45.2) were transplanted into a CD45.1 WT recipient. There were 3 mice in each group.

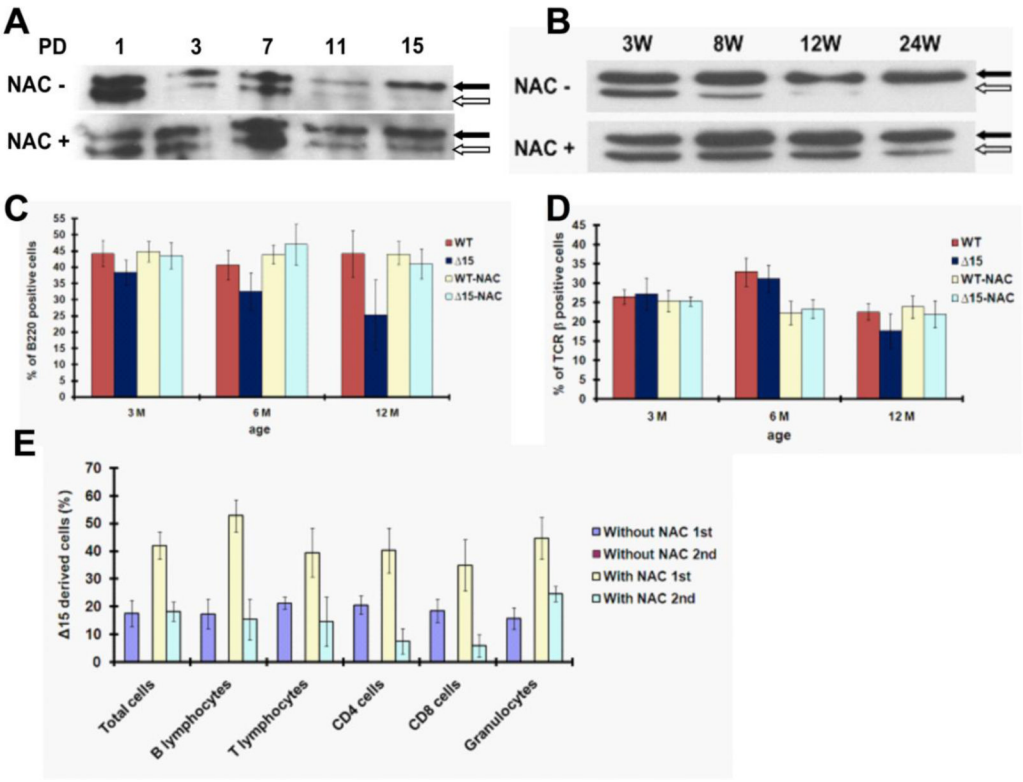

Figure 6.

N-Acetyl-l-Cysteine (NAC) can partially rescue the growth disadvantage of *Dkc1*$^{\Delta 15}$ (Δ15) cells at the stem cell level.

A. Western blot results of dyskerin expression level in MEFs from heterozygous *Dkc1*$^{\Delta 15/+}$ female mice after different population doublings (PD) with or without inclusion of 100μM NAC in the medium. The filled arrow indicates the position of WT dyskerin while the open arrow indicates the Δ15 dyskerin.

B. Western blot results of dyskerin expression level in the spleen of heterozygous *Dkc1*$^{\Delta 15/+}$ female mice at different ages with or without inclusion of 1mg/ml NAC in the drinking water after weaning. 3-5 mice were examined at each time point with similar results but the results from only one mouse are shown in the Figure.

C: The percentage of B lymphocytes in peripheral blood of WT and *Dkc1*$^{\Delta 15}$ male mice with or without NAC treatment, respectively.

D: The percentage of T lymphocytes in peripheral blood of WT and *Dkc1*$^{\Delta 15}$ male mice with or without NAC treatment, respectively.

E: In the serial transplantation experiments (50/50), *Dkc1*$^{\Delta 15}$ bone marrow cells (CD45.2) , taken from old (52-55 weeks) *Dkc1*$^{\Delta 15}$ male mice with or without NAC treatment, respectively, were mixed with CD45.1 WT cells of the same age and transplanted into a CD45.1 WT recipient. The proportion of original donor cells (*Dkc1*$^{\Delta 15}$) of different lineages is plotted at 12 weeks after first and second transplantation. N=3~5. Note that no cells from the *Dkc1*$^{\Delta 15}$ mice without NAC treatment are detected in the secondary recipients.