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Reactive oxygen species mediated DNA damage is essential for abnormal erythropoiesis in peroxiredoxin II$^{-/-}$ mice

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ABSTRACT

Erythroid cells are highly prone to oxidative damage generated during erythropoiesis and thus are well equipped with antioxidant defense systems. However, their roles have been poorly characterized. Here, we investigated the role of peroxiredoxin II in mouse erythropoiesis. Loss of Prx II significantly increased apoptosis and cell cycle arrest leading to abnormal erythropoiesis at 3 weeks of age when erythropoietin levels were almost same between wild type and Prx II$^{-/-}$. In Prx II$^{-/-}$ bone marrow cells, DNA tail length as an indicator of the oxidative damage was greatly increased and mRNAs of the molecules associated with DNA damage and repair and transcription regulators of antioxidant enzymes were also significantly increased. In addition, *N*-Acetyl-L-Cysteine treatment significantly decreased immature erythroblasts and apoptotic cells increased in Prx II$^{-/-}$ BMCs. These results strongly demonstrate that Prx II plays an essential role in maintaining normal erythropoiesis by protecting DNA damage.

1. Introduction

Peroxiredoxins (Prxs) are a family of antioxidant enzymes that scavenge hydrogen peroxide and mediate signal transduction in mammalian cells [1]. Six distinct mammalian Prx isozymes were detected in a wide range of tissues, and shown to have strong antioxidant activities *in vitro* [2]. All of the known mammalian Prx proteins use thioredoxin as an immediate electron donor, and hence were formerly known as thioredoxin peroxidases [3]. Prx II is the third most abundant protein in erythrocytes [4], and competes effectively with catalase and glutathione peroxidase to scavenge low levels of hydrogen peroxide [5].

Prx II participates in regulation of cellular functions such as cell proliferation and differentiation, and protects a number of specific proteins from oxidative damage [6]. Moreover, Prx II has an essential function for sustaining the life span of erythrocytes in mice. Our previous studies showed that Prx II$^{-/-}$ mice expressed hemolytic anemia with Heinz body formation and showed higher levels of reactive oxygen species (ROS) and abnormal red blood cells (RBCs) in their peripheral blood [7]. The following study showed a different cell population between wild type (WT) and Prx II$^{-/-}$ bone marrow cells (BMCs) [8]. The Lin$^-$ Sca-1$^+$ c-Kit$^+$ cell population slightly decreased, but TER119$^+$ and CD71$^+$ cell populations were expanded in Prx II$^{-/-}$ mice, indicating that erythropoietic recovery actively occurred in Prx II$^{-/-}$ mice [8]. Meanwhile, Prx II expression was also increased in the early stage of erythroid cell differentiation of K562 cells during *in vitro* erythropoiesis [9]. However, Prx I and other cytosolic Prxs were not changed [10].

It is well known that erythropoiesis is a multistep process that involves the differentiation of pluripotent hematopoietic stem cells through the lineage committed erythroid burst-forming unit (BFU-E) and erythroid colony-forming unit (CFU-E) progenitor cells, that give rise to a series of early and late erythroblasts, leading to the formation of reticulocytes and erythrocytes [11]. The modulation of progenitor cell amplification is thought to play a major role in increasing the erythroid output in response to the stress of tissue hypoxia [12]. Loss of erythroid antioxidant molecules or enzymes in mice (or humans) caused hemolytic anemia as a result of less antioxidant protection [13]. These studies illustrated that unbalanced accumulation of ROS limits the life span of mature RBCs. Although the direct causal effect of Prx II regulation of oxidative stress and mammalian organismal life span remains to be established, recent evidence indicated that erythroid cell life span was

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much more controlled by Prx II in host defense mechanisms under oxidative stress [14]. In addition, the findings from the Prx II$^{-/-}$ mouse studies suggested that oxidative stress leads to the deregulated coordination of cell cycle and maturation in bone marrow erythroid precursor cells, which may in turn play a significant role in the regulation of stress erythropoiesis in a disease state.

Here we examined the detailed physiological function of Prx II in erythropoiesis. The Prx II$^{-/-}$ mice significantly increased apoptosis and cell cycle arrest leading to abnormal erythropoiesis with significantly increased erythroid precursor cells and significantly decreased erythroid cell maturation. In addition, double strand DNA break and mRNAs of the related signaling molecules were remarkably increased compared to WT mice. Our findings suggest that Prx II plays a key role in prevention of ROS mediated DNA damage inducing cell death and cell cycle arrest via the p53 signaling pathway, which ultimately leads to abnormal erythropoiesis in mice.

2. Materials and methods

2.1. Mice

WT and Prx II gene knockout mice with 129/SvJ background were maintained in a specific pathogen-free authorized facility in the Korea Research Institute of Bioscience and Biotechnology (KRIBB) [7]. All animal procedures were conducted in accordance with the guidelines of the institutional Animal Care and Use Committee, KRIBB. The genotyping of animals was performed as described [7].

2.2. Fluorescence activated cell sorting (FACS) and cell cycle analyses

The mouse BMCs and splenocytes (1×10^6 cells) at 3 weeks of age were stained with a biotin conjugated antibody CD71-FITC (Biolegend) and TER 119-PE (Biolegend) and sorted using a FACSAria (Becton Dickinson). The stimulated cells were washed once with PBS. Cell pellets were fixed with 70% ethanol in PBS, stained with propidium iodide (PI, BD Biosciences) for 20 min on ice, and stained with a biotin conjugated antibody CD71-FITC (Biolegend), and TER 119-cy^5 (Biolegend). Cells were analyzed by CELLQueste software in FACSCalibur (Becton Dickinson).

2.3. Measurement of ROS

The intracellular ROS of BMCs were determined using 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA; Invitrogen). BMCs were incubated with 20 μM of DCF-DA for 15 min at 37 °C, and then washed with PBS. Fluorescent intensity was read using a FACSCalibur (Becton Dickinson).

2.4. Measurement of apoptosis in BMCs

BMCs were washed in PBS, and stained with Annexin V Binding Buffer (BD Biosciences), as recommended by the manufacturer's instruction, labeled with Annexin V-FITC (BD Biosciences) and analyzed by FACSCalibur (Beton Dickinson).

2.5. Colony-forming assays

Methylcellulose colony-forming assays were performed in MethoCult M3434 complete medium with cytokines (Stem Cell Technologies, USA) as previously described [15].

2.6. Immunocytochemistry

Immunocytochemistry was performed as previously described [16].

2.7. Comet assay

Comet assay was performed using the Comet assay kit (Trevigen) as previously described [16].

2.8. QRT-PCR

RNA was isolated from BMCs at each passage using TRIZOL (Invitrogen). cDNA was synthesized from 1 mg of total RNA, using oligo dT18 primers and Superscript reverse transcriptase (Bioneer; Korea) in a final volume of 20 μl. For standard PCR, 1 μl of the first strand cDNA product was used as a template for PCR amplification with Taq DNA polymerase (Fermentas). QRT-PCR reactions were performed using SYBR Green JumpStart Taq ReadyMix (Takara) in an Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer; Korea). Relative quantification was achieved by normalization to endogenous β-actin. Primers used are shown in Supplementary Table S3.

2.9. Data analysis

Statistical analysis was performed using an ANOVA test. A p value of less than 0.05 was considered significant.

3. Results

3.1. Aberrant erythropoiesis in three weeks old Prx II$^{-/-}$ mice

Adult Prx II$^{-/-}$ mice showed hemolytic anemia with Heinz body formation in their peripheral blood [7]. The percentage of RBCs with Heinz bodies was over 30% in Prx II$^{-/-}$ mice after 4 weeks of age, and the erythropoietin (EPO) level of Prx II$^{-/-}$ mice was twice that of WT mice at 8 weeks of age, indicating that EPO could play a role in maintaining hematologic homeostasis in Prx II$^{-/-}$ mice [7]. Based on these results, to further investigate the role of Prx II in hematologic homeostasis without the effect of EPO, we checked blood parameter of Prx II$^{-/-}$ mice at 3 weeks of age when the EPO level in blood had not increased compared to WT mice. Hematocrit (HCT), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were significantly decreased in Prx II$^{-/-}$ compared to WT. However, RBC and White blood cell (WBC) were not changed and the red cell distribution width (RDW) increased (Fig. 1A; Supplementary Table S1). Consistent with these results, reticulocytes were more significantly increased in peripheral blood of Prx II$^{-/-}$ mice (Fig. 1B), associated with splenomegaly (Fig. 1C). Flow-cytometric analysis showed that the percentage of the TER119$^+$ splenocytes in Prx II$^{-/-}$ mice was ~23% higher than in WT (Fig. 1D). In addition, a higher population of CD71$^+$ and TER119$^+$ splenocytes was also detected in 8 week old Prx II$^{-/-}$ mice (Supplementary Fig. 1). These results indicate that Prx II may be involved in the protection of oxidative damage inducing aberrant erythropoiesis, regardless of EPO level in mice.

3.2. Abundant immature erythroblasts in Prx II$^{-/-}$ BMCs are associated with increased apoptosis

To examine the colony formation potential of hematopoietic progenitors in Prx II$^{-/-}$ BMCs, a conventional set of CFU assays were performed. The result of CFU assays showed no significant

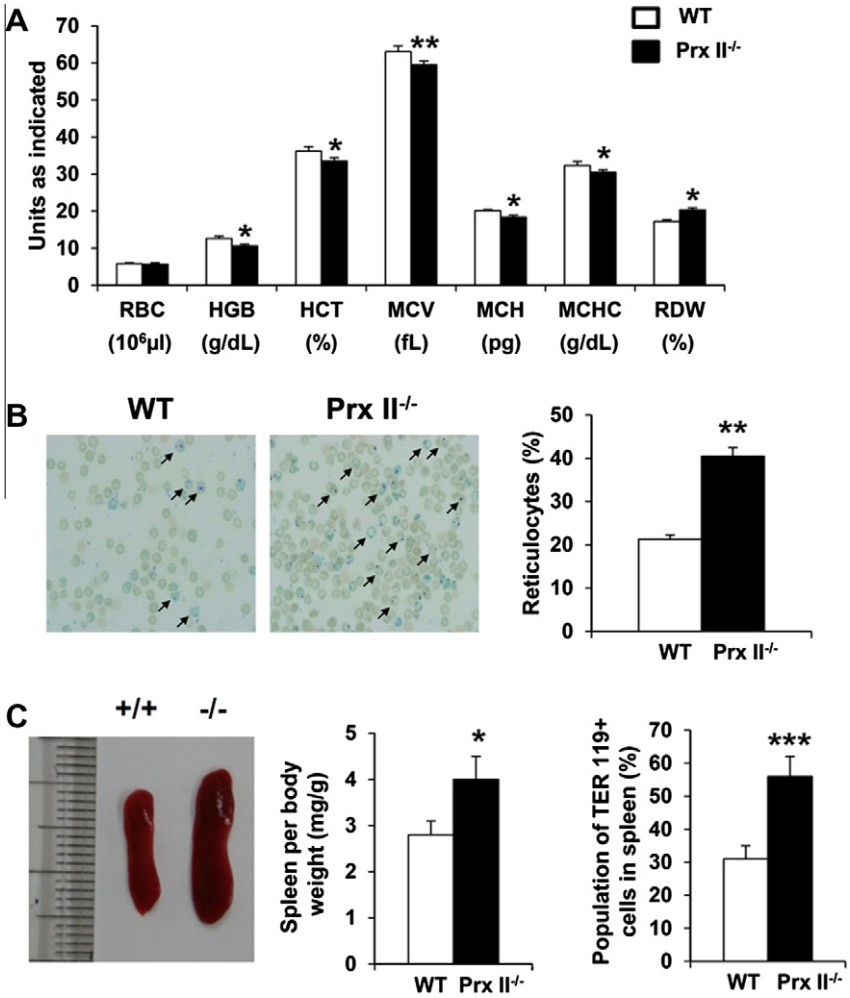

Fig. 1. Anemia, splenic erythroblastosis and erythrocyte abnormalities in Prx II$^{-/-}$ mice. (A) The hematologic parameters of Prx II$^{-/-}$ mice, Counts of RBC, HGB, and HCT concentration and MCV, MCH, MCHC, and RDW. (B) Peripheral blood smears were stained with Wright Giemsa. Reticulocytes (arrow) were detected in WT and Prx II$^{-/-}$ mice. (C) Splenomegaly and ratios of spleen/body weight of WT and Prx II$^{-/-}$ mice. (D) TER119^{+} splenocytes in WT and Prx II$^{-/-}$ mice. Data means ± SEM (n = 6) $^{*}P$ < .05, $^{**}P$ < .01, $^{***}P$ < .001.

differences between WT and Prx II$^{-/-}$ BMCs in the number of total CFC, CFU-GEMM, CFU-GM, CFU-G, and BFU-E derived colonies per BMCs (Fig. 2A), whereas the erythroid cell compartment was largely increased (Fig. 2D and E; Supplementary Table S2). Because the early erythroid progenitors differentiate through the basophilic, polychromatic, and orthochromatic erythroblast stages, they lose the expression of CD71 while maintaining the expression of TER119. Thus, these cell surface markers can be used to differentiate the various erythroblast stages [15]. Here, we analyzed the BMCs population between WT and Prx II$^{-/-}$ mice. Erythroblast maturation follows from pro erythroblasts (CD71^{+}, TER119^{-}; Pro E) and basophilic erythroblasts (CD71^{+}, TER119^{+}; R1) to late basophilic and chromatophilic erythroblasts (CD71^{mid+}, TER119^{+}; R2) to orthochromatic erythroblasts (CD71^{-}, TER119^{+}; R3) [17]. As shown in Fig. 2B and C, a quantitative imaging analysis demonstrated that the CD71^{+} ratio of BMCs was increased by approximately ~12% in Prx II$^{-/-}$ mice compared to WT mice. The basophilic and chromatophilic erythroblasts of Prx II$^{-/-}$ BMCs significantly increased, however the orthochromatic erythroblasts decreased compared to WT (Fig. 2D and E). To determine whether immature erythroblasts are associated with cell apoptosis, we examined externalization of phosphatidylserine by annexin V staining on the surface of TER119^{+} BMCs. As shown in Fig. 2F, annexin V labeled cells were significantly increased in total BMCs and pro erythroblast (Pro E), basophilic (R1), chromatophilic (R2) and orthochromatic (R3) erythroblasts of Prx II$^{-/-}$ BMCs compared to WT. These results indicate that the deletion of Prx II can trigger erythropoiesis in bone marrow. Therefore Prx II deletion induced accumulation of pro erythroblasts, and basophilic and chromatophilic erythroblasts, suggesting that Prx II has a function of inhibiting apoptosis of immature erythroblasts during erythroid differentiation under oxidative stress.

3.3. Double strand DNA breaks were significantly increased in erythroblasts of Prx II$^{-/-}$ mice

Stage matched immature erythroblasts and apoptosis were increased in Prx II$^{-/-}$ BMCs compared to WT (Fig. 2B–F). Consistent with this result, the ROS levels were significantly increased in Prx II$^{-/-}$ BMCs (Fig. 3A). To know the reason why Prx II deletion induced this response, we checked DNA damage in erythroblasts by comet assay, which measures DNA breakage including double strand DNA breaks through an increase in the mobility of denatured genomic DNA in single cell electrophoresis. As shown in Fig. 3B, Tail moment of Prx II$^{-/-}$ mice was greatly increased compared to WT, indicating that double strand DNA breaks were significantly increased in Prx II$^{-/-}$ mice. It is well known that DNA damage could induce cell growth arrest [18]. So, we checked cell

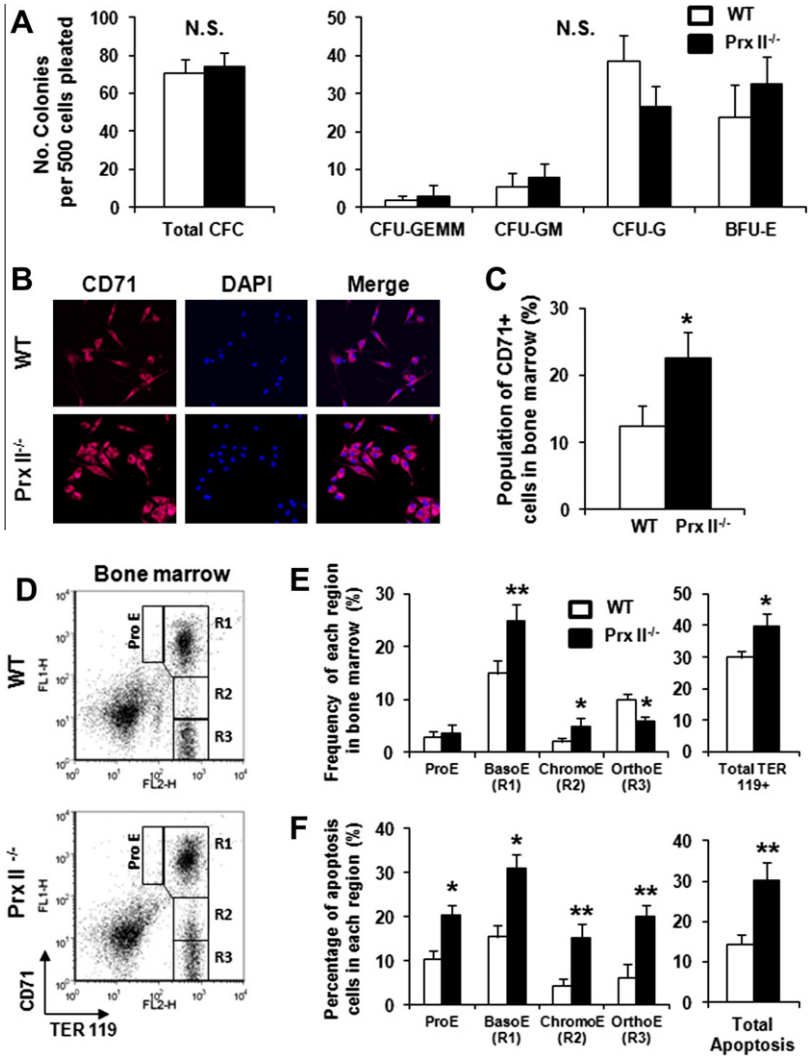

Fig. 2. Immature erythroblasts in the BMCs of Prx II$^{-/-}$ mice. (A) The numbers of total CFC, CFU-GEMM, CFU-GM, CFU-G, and BFU-E–derived colonies per BMCs are shown. No significant difference (N.S.) was found in the number of total CFC, CFU-GEMM, CFU-GM, CFU-G and BFU-E –derived colonies per BMCs in WT and Prx II$^{-/-}$ mice. (B) Progenitor cells in total BMCs of WT and Prx II$^{-/-}$ mice were stained by immature erythroid cell marker CD71 (red) and DAPI (blue). (C) Population of CD71^{+} cells in WT and Prx II$^{-/-}$ mice. (D) Flow cytometric analysis of total BMCs stained by CD71 and TER119. The gate of Pro E, Baso E (R1), Chromo E (R2), Ortho E (R3). (E) Frequencies (%) of cells found in each region are shown. (F) The percentage of annexin V positive cells in each region (R1, R2 and R3) of WT (open bars) and Prx II$^{-/-}$ (black bars) mice. Data means ± SEM (n = 6) $^{*}P < .05$, $^{**}P < .01$.

cycle by the PI staining method. Prx II$^{-/-}$ induced a significant increment in G0 and G1 cells, but a decrement in G2/M cells, suggesting that DNA damage induced by ROS increased in Prx II$^{-/-}$ BMCs is responsible for G1 cell arrest and apoptosis in erythroblasts (Fig. 3C–E). These results indicate that Prx II plays an important role in protecting DNA damage by inhibiting double strand breaks in erythroblasts.

3.4. Prx II deletion induced increment of immature erythroblasts through activation of the p53 signal pathway

In order to address how Prx II deletion induced an increment of immature erythroblast in mice, we compared transcript levels of the candidate molecules associated with DNA damage and cell cycle arrest in bone marrow precursor cells (CD71^{+}, TER119^{+}) of WT and Prx II$^{-/-}$ mice. ATM is an essential regulator of stress induced DNA damage that is critical for hematopoietic stem cell self-renewal [19,20]. ATM transcript expression was significantly increased in the precursor cells of Prx II$^{-/-}$ mice, although the ATM expression in Prx II $^{-/-}$ total BMCs was slightly increased compared to that of WT (Fig. 4A). ATM activates p53 via multiple pathways in response to stress induced DNA damage that is also true to precursor cells of Prx II$^{-/-}$ mice with significantly increased expression of p53. In agreement with up-regulated p53, the p21 transcript level was also increased in this cell population, but not in total BMCs (Fig. 4A). As ATM, p53, and p21 transcription levels in the erythroid precursor of Prx II$^{-/-}$ mice were up-regulated, transcription levels of DNA damage–inducible protein 45 (GADD45) [21], cyclin D1, and proliferating cell nuclear antigen (PCNA) were significantly increased (Fig. 4B). Up-regulated PCNA and cyclin D1 could coordinate the cell cycle through DNA repair. Consistent with ROS increments in erythroid precursor cells of Prx II$^{-/-}$ mice, the transcript levels of oxidative stress associated genes such as nuclear factor E2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1) were significantly increased in erythroid precursor cells (Fig. 4C). These results suggest that Prx II deletion activated p53 signal pathways in response to DNA damage, induced by increased oxidative stress, and thus led to cell cycle arrested DNA repair in bone marrow precursor cells.

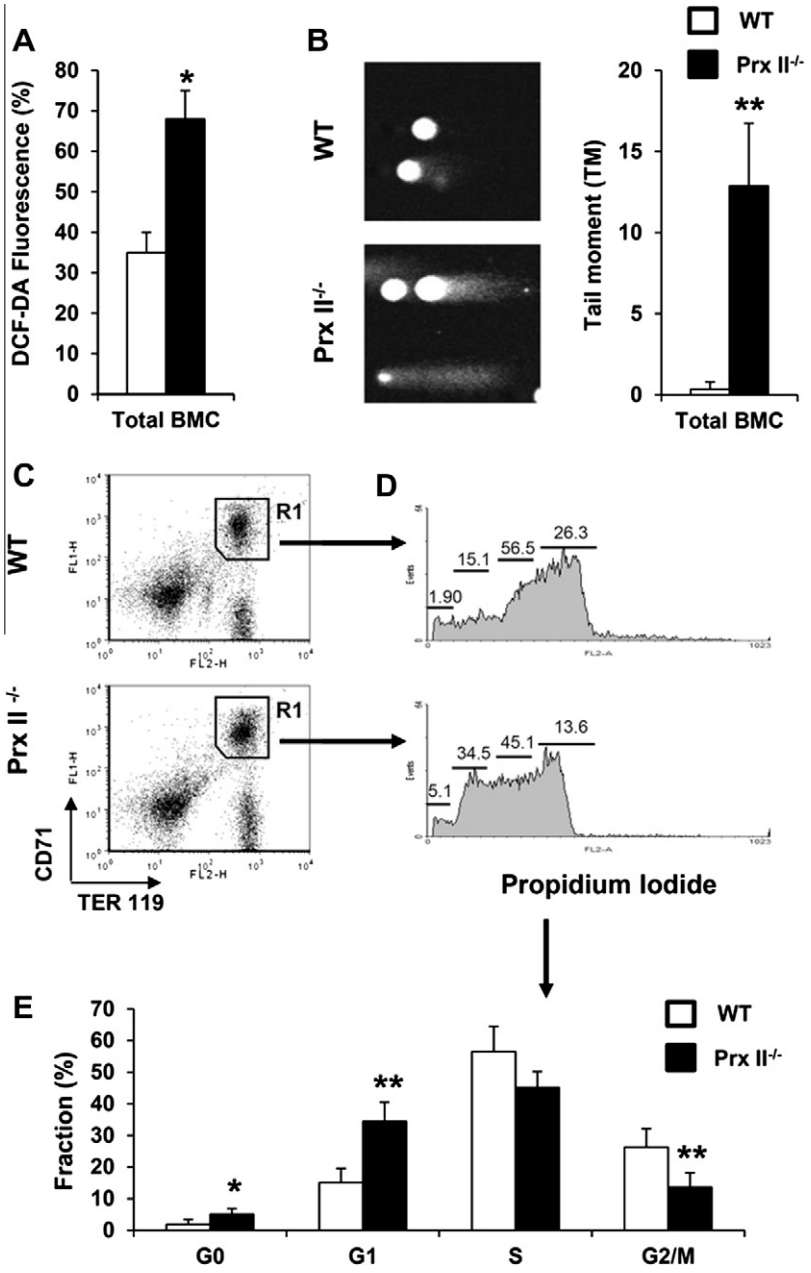

Fig. 3. DNA damage and cell cycle arrest in Prx II$^{-/-}$ BMCs. (A) Intracellular ROS level in WT and Prx II$^{-/-}$ BMCs. (B) DNA double strand breaks in WT and Prx II$^{-/-}$ BMCs by comet assay. (C) Flow cytometric analysis of WT and Prx II$^{-/-}$ bone marrow erythroid precursors according to the expression of CD71 and TER119. (D, E) Cell cycle distribution for CD71^{+}/TER119^{+} cells in range R1 by PI staining. Cell cycle was analyzed using propidium iodide (PI) in R1 sorted by FACS from WT and Prx II$^{-/-}$ bone marrow erythroid cells. Percentage of cells in G0, G1, S, and G2/M phages of cell is shown. Data are mean ± SEM ($n = 6$) $^{*}P < .05$, $^{**}P < .01$.

4. Discussion

Erythroid cells are highly prone to oxidative damage generated during erythropoiesis. Therefore antioxidant defense systems are well equipped to protect oxidative damage by maintaining redox homeostasis in the cells. Peroxiredoxins (Prxs) are antioxidant enzymes that scavenge hydrogen peroxide in mammalian cells. Among them, Prx II plays an important role in the metabolism of low level hydrogen peroxide in the erythrocyte and thus acts as a non-catalytic scavenger of hydrogen peroxide [22]. Our previous paper suggested that Prx II plays a key role in maintaining erythropoiesis by showing reduced life span of RBCs of Prx II$^{-/-}$ mice. In this study we examined the detailed role of Prx II in erythropoiesis using BMCs from WT and Prx II$^{-/-}$mice at the age of 3 weeks. Because it is important to understand the role of Prx II in erythropoiesis regardless of EPO level in blood. Here we found that loss of Prx II significantly increased apoptosis and cell cycle arrest leading to abnormal erythropoiesis, which was due to significantly increased DNA damage by ROS increased in Prx II$^{-/-}$BMCs compared to WT.

ROS may play a role in signal transduction at nontoxic concentrations [23], but higher levels of ROS can cause oxidative damage to macromolecules, resulting in lipid peroxidation, strand breaks, and oxidation of guanosine residues in DNA [24]. It has been suggested that oxidative stress might play a role in clinical and cellular manifestations. Several reports have implicated ATM, the ataxia-teleangiectasia gene product, as a serine protein kinase that senses and controls cellular reactions to this form of stress [25]. Based on the report, we investigated mRNA levels of ATM in WT and Prx II$^{-/-}$

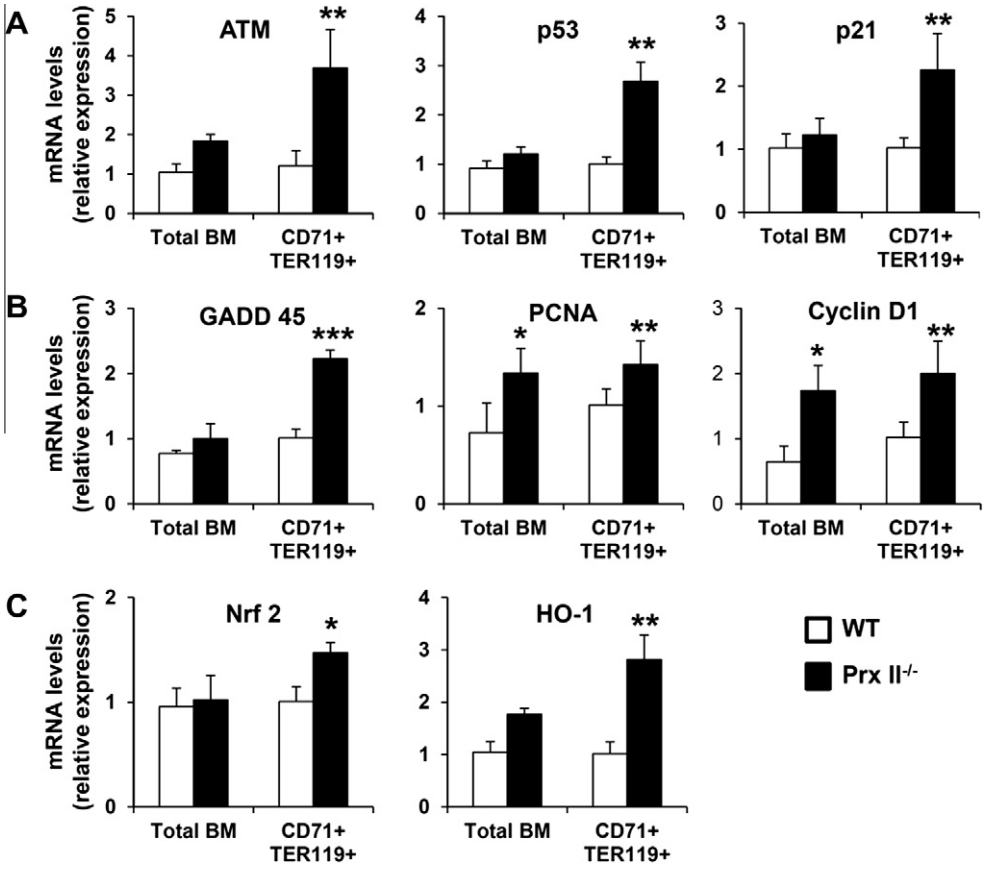

Fig. 4. QRT-PCR analysis in FACS sorted erythroblasts ($CD71^+/TER119^+$: R1) and BMCs from WT and Prx $II^{-/-}$ mice. (A) QRT-PCR analysis of ATM pathway genes (B) DNA repair and cell cycle associated genes (C) antioxidant genes in WT and Prx $II^{-/-}$ mice. Data means ± SEM ($n = 6$) $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

BMCs. ATM was significantly increased in Prx $II^{-/-}$ BMCs, indicating that Prx II is required for regulation of erythroid cell maturation to inhibit DNA damage against oxidative stress during erythropoiesis. Oxidative stress can also induce p53 activation, followed by cell cycle arrest, presumably to allow DNA repair to occur before replication or mitosis [26,27]. Activation of p53 binding to target DNA is also regulated without increased protein levels of p53. Phosphorylation of p53 by ATM, or DNA-dependent protein kinase is important for sequence specific DNA binding of p53 in response to DNA damage [28]. One of the targets of p53 is p21, a G1 cyclin-dependent kinase inhibitor, through which p53 arrests the cell cycle [29]. Interestingly, p21 deficient cancer cells increase susceptibility to p53-dependent apoptosis. Furthermore, p21 inhibits the activation of apoptosis, suggesting that p21 switches p53-dependent signaling from apoptosis to cell cycle arrest [30]. Prx II deletion increased expression of p53 and p21 transcription levels (Fig. 4A). That may be due to the increased ROS in bone marrow cells by the deletion of Prx II. Our results indicate that Prx II prevents DNA damage from ROS increased during erythropoiesis, which is associated with cell cycle arrest and increased apoptosis through activation of p53 signaling pathways.

Prx I and Prx III have been implicated to have their roles in erythropoiesis. Prx I knockout mice showed hemolytic anemia with hemoglobin instability and Heinz body formation along with an increased incidence of various malignancies [31]. The data show that the anemia in old Prx $I^{-/-}$ mice is due to shortened erythrocyte survival owing to an intrinsic defect of Prx I in red cells [31]. In addition, Prx III, originally isolated from murine erythroleukemia (MEL) cells and induced as a gene that is involved in erythroid cell differentiation [10], may take part in terminal erythroid differentiation. But, the function of Prx III in erythropoiesis has not been investigated. To know whether Prx I or Prx III are involved in regulating erythropoiesis, we compared expression levels of Prx I and Prx III in erythroid precursor cells of Prx $II^{-/-}$ and WT mice. Each gene expression level of Prx I and Prx III in total BMCs were similar compared to WT, but both gene expression levels were significantly decreased in $CD71^+$, $TER119^+$ erythroid cells of Prx $II^{-/-}$ mice (Supplementary Fig. 2). Like as the role of Prx II in erythropoiesis, Prx I and III may also play roles during erythropoiesis. Further studies will be performed using Prx $I^{-/-}$ and $III^{-/-}$ mice to understand detailed roles of Prx I and III in erythropoiesis.

In conclusion, our data, including that *N*-Acetyl-L-Cysteine (NAC) treatment significantly decreased immature erythroblasts and apoptotic cells increased in Prx $II^{-/-}$ BMCs (Supplementary Fig. 3), clearly demonstrate that Prx II plays an essential role in maintaining normal erythropoiesis by protecting DNA damage (Supplementary Fig. 4). Thus therapeutic approaches aimed at reducing ROS might be highly beneficial for the treatment of erythropoietic disorders.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.06.113.

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