Differential p53 Engagement in Response to Oxidative and Oncogenic Stresses in Fanconi Anemia Mice

Reena Rani,1 Jie Li,1 and Qishen Pang1,2

1Division of Experimental Hematology, Cincinnati Children’s Hospital Medical Center; 2Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, Ohio

Abstract

Members of the Fanconi anemia (FA) protein family are involved in repair of genetic damage caused by DNA cross-linkers. It is not clear whether the FA proteins function in oxidative DNA damage and oncogenic stress response. Here, we report that deficiency in the Fancd2 gene in mice elicits a p53-dependent growth arrest and DNA damage response to oxidative DNA damage and oncogenic stress. Using a Fancd2−/− Trp53+/− double knockout model and a functionally switchable p53 retrovirus, we define the kinetics, dependence, and persistence of p53-mediated response to oxidative and oncogenic stresses in Fancd2−/− cells. Notably, oxidative stress induces persistent p53 response in Fancd2−/− cells, likely due to accumulation of unrepaired DNA damage. On the other hand, whereas wild-type cells exhibit prolonged response to oncogene activation, the p53-activating signals induced by oncogenic ras are short-lived in Fancd2−/− cells, suggesting that Fanca may be required for the cell to engage p53 during constitutive ras activation. We propose that the FA proteins protect cells from stress-induced proliferative arrest and tumor evolution by acting as a modulator of the signaling pathways that link FA to p53.

Introduction

Research on Fanconi anemia (FA) has recently generated great interest because the disease serves as an excellent model for hematopoietic failure and leukemic evolution. FA is a genetic disorder associated with bone marrow (BM) failure and cancers, particularly leukemia (1–3). FA is genetically heterogeneous, with 13 complementation groups identified thus far. The genes encoding the groups A (FANC A), B (FANC B), C (FANC C), D1 (FANC D1/BRCA2), D2 (FANC D2), E (FANC E), F (FANC F), G (FANC G), I (FANC I/KIAA1794), J (FANC J/BRIP1), L (FANC L), M (FANC M), and N (FANC N/PALB2) have been cloned (4–18). The biological function of FA proteins has been the subject of intense investigation in recent years.

Because cells deficient for FA genes are hypersensitive to DNA cross-linking agents, such as mitomycin C and diepoxybutane, it has been proposed that the FA proteins may be involved in the sensing and/or repair of DNA interstrand cross-links. This model is supported by evidence that shows that an intact FA nuclear complex is required for biochemical modification in the form of monoubiquitination of FANC D2 and FANCI as mutations in any one of the FA proteins that disrupt the complex prevent the activation of FANC D2 and FANCI, leading to cross-linker hypersensitivity and the characteristic broken and radial chromosome formation in FA cells (3, 18). The common damage to DNA in vivo is oxidative stress, and ample evidence has suggested that FA cells are in an in vivo prooxidant state (19) and that the FA proteins may play important roles in cellular responses to oxidative stress. For example, the FANCC protein has been found to interact with NADPH cytochrome P450 reductase and glutathione S-transferase P1-1 (20, 21), two enzymes involved in either triggering or detoxifying reactive intermediates, including reactive oxygen species. In addition, mice with combined deficiencies of the antioxidative enzyme, Cu/Zn superoxide dismutase, and the Fanc genes showed a defective hematopoiesis (22). Another FA protein, FANC G, interacts with cytochrome P450 2E1 (23) and mitochondrial peroxiredoxin-3 (24), suggesting a possible role of FANC G in protection against oxidative DNA damage. Recently, Saadatzadeh and colleagues (25) showed that oxidant hypersensitivity of Fancd2−/− cells was due to an altered redox regulation and hyperactivation of the serine-threonine kinase apoptosis signal-regulating kinase 1, an important kinase involved in oxidant-induced apoptosis. Moreover, oxidative stress induces complex formation by two major FA proteins, FANCA and FANCG (26). These observations corroborate a critical role for oxidative stress in FA phenotype and disease progression.

Cells from patients with FA gene mutations have high predisposition to leukemia and other cancers. However, little is known about whether these mutant cells have high susceptibility to oncogenic transformation. In response to oncogenic activation, normal cells induce genetically encoded programs that prevent deregulated proliferation and, thus, protect multicellular organisms from cancer progression. Two such programs induced by oncogenic activation are apoptosis and senescence, which are normally triggered by DNA damage or other stresses. Studies have shown that overexpression of antiapoptotic proteins, such as Bcl-2, or deletion of apoptosis-associated and senescence-associated proteins, such as p53, accelerates oncogene-induced tumorigenesis (27, 28). The theory that has risen from these findings is that oncogene-driven proliferation must be associated with inhibition of apoptosis and senescence to allow malignant outgrowth.

The tumor suppressor p53 is a key transcription factor that activates vital damage containment procedures to restrict aberrant cell growth in response to DNA damage, oncogene activation, and loss of normal cell contacts (29, 30). p53 restricts cellular growth by inducing senescence, cell cycle arrest, or apoptosis (31). Thus, p53 plays a major role in the prevention of cancer. Consistent with this, emerging evidence suggests that p53 deficiency may increase cancer development in patients with FA and FA mice. For example, studies have indicated a higher proportion of human papillomavirus-positive squamous cell carcinomas in patients with FA than in healthy controls, indicating that loss of functional p53 facilitates tumor development (32, 33). Mice deficient for Fancd1 or Fancd2...
have been shown to have accelerated tumor development in Trp53-deficient background (34, 35). In addition, Fanca deficiency accelerates the development of certain blood and solid tumors in mice heterozygous at Trp53 (36). Moreover, these studies found that the FA proteins and p53 cooperate in apoptosis and cell cycle checkpoint control after DNA damage (35, 37, 38). In this study, we show that p53 is engaged differentially in response to oxidative and oncogenic stresses in FA cells in vitro and in vivo.

Materials and Methods

Mice. Wild-type (WT), Fanca+/− mice and double knockout mice (p53+/−; Fanca−/−) were generated by interbreeding the heterozygous Fanca−/− and p53−/− mice. The genetic background of the mice was C57BL/6 mice, which were used at 6 to 10 wk of age. 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 lineage-depleted cells and differentiation assay. The femora and tibiae were harvested from the mice immediately after their sacrifice with CO2. BM cells were flushed from bones into Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) containing 10% FCS, using a 21-gauge needle and syringe. Low-density BM mononuclear cells (BMMNC) were separated by Ficoll Hypaque density gradient (Sigma) and washed with IMDM. BMMNC were depleted of lineage-committed cells using a lineage cell depletion kit (Miltenyi Biotec, Inc.) in accordance with the manufacturer’s instructions. For myeloid and lymphoid differentiation, BM Lin− cells were cultured with various cytokine combinations known to support myeloid [100 ng/mL stem cell factor (SCF), 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF)] or lymphoid [100 ng/mL SCF, 10 ng/mL each of interleukin-3 (IL-3) and IL-7] differentiation. After 5 d, cells were stained with antibodies against myeloid markers Gr-1 and Mac-1 or lymphoid markers B220 and CD3e, followed by flow cytometric analysis using a FACS Calibur (Becton Dickinson).

Mouse embryonic fibroblast isolation and culture. Mouse embryonic fibroblasts (MEF) were prepared from day 13.5 embryo derived from crosses of p53+/− with Fanca−/− mice. The head and the red organs were removed, and the torso was minced and dispersed with 0.25% trypsin-EDTA by using 20-gauge needle. Single-cell suspension obtained was cultured in DMEM containing 10% fetal bovine serum, glutamine, MEM nonessential amino acid, penicillin/streptomycin, and gentamicin under 5% CO2 for 3 d until the genotypes were confirmed. Cells were then trypsinized and expanded for experimental use. MEFS were further maintained on the basis of 3T3 protocol.

Construction of retroviral expression vectors. The full-length human Fanca cDNA was subcloned into the NotI site of retroviral vector MIEG3 to create MIEG3-FANCA. The WT mouse p53 (mp53wt), a temperature-sensitive mutant p53 (tp53V143A), and H-ras V12 cloned in MIEG3 were kindly provided by Dr. Yi Zheng (Cincinnati Children’s Medical Center). The plasmids (10 mg/μg) each were used to produce retroviral supernatant.

Retroviral-mediated gene transfer. Helper virus-free Phoenix packaging cells (kindly provided by Dr. Gary Nolan, Stanford University) were plated at the density of 3 × 104 in 10-cm tissue culture dish and incubated for 24 h. Cells were then transfected by calcium phosphate precipitation with 10 μg of retroviral plasmids (15 h at 37°C). Retroviral supernatant was collected at different time points (24, 36, and 48 h), respectively. After transfection, the virus-containing medium was filtered (0.45 μm filter, Millipore) and aliquoted at −80°C. WT, Fanca−/−, p53−/− Fanca−/−, and p53−/− Fanca−/− MEFs were plated at the density of 8 × 103 cells per 10-cm dish and incubated overnight at 37°C. For infections, culture medium was replaced by appropriate retroviral supernatant in the presence of 4 μg/mL polybrene (Sigma) for 12 h. Infection process was repeated twice. At 24 h after infection, EGFP-positive cells were used for different assays, as indicated in Results.

Colony and cell proliferation assay. Low-density BMMNCs were plated in a 35-mm tissue culture dish in 4 mL of semisolid medium containing 3 mL of MethoCult M 3134 (Stem Cell Technologies) and the following growth factors: 100 ng/mL SCF, 10 ng/mL IL-3, 100 ng/mL GM-CSF, and 4 units/mL erythropoietin (Peprotech). On day 10 after plating, erythroid and myeloid colonies were enumerated. Hematopoietic clonal growth results were expressed as means (of triplicate plates) ± SD of three experiments. For proliferation, cells were cultured for 24 h in normal growth medium supplemented with 10 μmol/L Bd/rd (Sigma), harvested, and fixed in 70% ethanol. Bd/rd-labeled cells fixed in 70% ethanol were treated with 2 N HCl (20 min at room temperature), followed by addition of two volumes of 0.1 mol/L sodium borate (pH 8.5). The cells were incubated with an anti-Bd/rd mouse monoclonal antibody, washed, and incubated with FITC-conjugated antimouse antibody. Cells were counterstained overnight with 5 μg/mL of propidium iodide containing 40 μg/mL of RNase. The stained cells were analyzed by flow cytometry.

Apoptosis assay and cell cycle analysis. Cells were stained with Annexin V and 7AAD 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 fixed with 0.25% formaldehyde in PBS and permeabilized with 0.3% Nonidet P-40. Cells were then stained with propidium iodide containing 1 mg/mL RNase A, followed by fluorescence-activated cell sorting (FACS) analysis for G0/G1, S, and G2/M populations using a FACS caliber.

Immunocytochemistry. Cells were plated on 18-mm diameter glass coverslips and allowed to recover for 24 h before treatment with H2O2. Treated cells were fixed with 4% formaldehyde in PBS for 20 min at room temperature. Cells were then washed with PBS and permeablized with 0.2% Triton X-100 in 3% bovine serum albumin (BSA)/PBS for 10 min at room temperature. The cells were then washed with PBS and blocked with 10% BSA/PBS solution for 1 h at room temperature. Cells were then incubated with primary antibodies against p53, p53Ser20, or p21WAF1 (Cell Signaling Technology) in 10% BSA/PBS solution at room temperature for 2 h. After extensive washes, cells were incubated with Rhodamine Red X–conjugated goat anti-rabbit IgG or Rhodamine Red X–conjugated goat anti-mouse IgG (Jackson Immuno Research). DNA was then labeled with 4′,6-diamidino-2-phenylindole(Sigma). Slides were finally mounted in a mounting medium (Vector) and were visualized under Carl Zeiss invert Axiovert 200M microscope with OpenLab 4.0.3 software (Improvision).

Temperature shifts and cell proliferation analysis. For the temperature shift experiment, 1 × 105 tsp53 transduced p53−/−: ‘Fanca+/− and p53−/− ‘Fanca−/− MEF cells were cultured in triplicate in six-well tissue culture plates. Plates were incubated at 37°C or 32°C. Every third day, cells were trypsinized and counted with a hemacytometer, and the same number of cells was replated in six-well tissue culture plates.

Senescence-associated β-galactosidase. Senescence-associated β-galactosidase (SA-β-gal) activity was determined using a SA-β-gal staining kit (Cell Signaling Technology) according to the manufacturer’s instructions. Briefly, cells were washed once with PBS and fixed with 0.5% glutaraldehyde. Cells were further washed with PBS and stained with X-gal solution (1 mg/mL X-gal) overnight at 37°C. The percentage of positive-stained cells was quantified under a phase-contrast microscope.

Immunohistochemistry. Spleen, thymus, and bone were fixed with formalin and embedded in paraffin for sectioning. After deparaffinization and hydration, sections were blocked with 10% serum in PBS for 30 min and then incubated with 1:50 diluted anti-p53 or 1:50 diluted anti-p21WAF1 (all from Santa Cruz Technologies) primary antibodies overnight at 4°C, followed by three washing with PBS at 5-min interval. Detection of p53 and p21WAF1 staining was performed using the biotin peroxidase complex method (Vectorstain ABC kits, Vector Laboratories). Color was developed with diaminobenzidine tetrahydrochloride, and nuclei were stained with hematoxylin.

Preparation of cell extracts and immunoblotting. To prepare cell protein, single-cell suspension was prepared from BM MNCs, spleenocytes, or trypsinized MEFs, washed with ice-cold PBS, and resuspended in ice-cold lysis buffer containing 50 mmol/L Tris-HCL (pH 7.4), 0.1% NP40, and 1 mol/L NaCl supplemented with protease and phosphatase inhibitors [10 μg/mL aprotinin, 25 μg/mL leupeptin, 10 μg/mL pepstatin A, 2 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L NaPO4, 25 mmol/L Na2 and
2 mmol/L sodium orthovanadate] for 30 min on ice. Cell debris was removed from the lysates by centrifuging them at 14,000 rpm for 30 min. Protein concentration was quantified by using Bio-Rad reagent. Cell lysates (100 μg) were resolved on (12%) SDS-PAGE and transferred onto nitrocellulose membranes. Immunoblots were then incubated with primary antibodies specific for p53, p53Ser20, and p21WAF1 (Santa Cruz Biotechnologies), H2AX (Upstate Biotechnology), and phosphorylated pRB, phosphorylated p38, phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2), and the pan kinases p38 and ERK1/2 (all from Cell Signaling) or β-actin (Sigma) for 12 to 16 h at 4°C. Signals were revealed after incubation with antimouse or antirabbit secondary antibodies.

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

**Figure 1.** Fanca−/− cells are hypersensitive to oxidants. A, left, 2 × 10^5 BMMNCs from Fanca−/− mice and their WT littermates were treated with increasing concentration of H2O2 and cultured in triplicate wells at 37°C for 45 min. Cells were then washed and cultured for 24 h in normal growth medium supplemented with 10 μmol/L BrdUrd, and the level of BrdUrd incorporation was determined by flow cytometry. Columns, mean of three experiments; bars, SD. Right, 1 × 10^5 BMMNCs from Fanca−/− mice and their WT littermates were cultured in semisolid medium containing increasing concentration of H2O2, and progenitor proliferation was analyzed by clonogenic assay. Columns, mean of three experiments; bars, SD. B, equal density (1 × 10^5) of MEFs isolated from Fanca−/− mice or their WT littermates were treated with or without H2O2 (50 μmol/L) and cultured in triplicate wells at 37°C for 4 d, wherein viable cells were counted each day. Experiments were performed on three MEF cultures derived from individually isolated embryos for each genotype. C, WT, Fanca−/− MEFs at passage 4 were incubated with or without H2O2 (10 μmol/L) for 5 d, with medium and H2O2 changed each day. Cells were then stained for SA-β-gal. D, WT, Fanca−/− MEFs at passage 4 were incubated with or without H2O2 (10 μmol/L) for 5 d, with medium and H2O2 changed each day. Whole cell extracts or total RNAs were subjected to Western blot (left and right) or RT-PCR (middle) analysis, respectively.
Results

Hematopoietic cells and MEFs from Fanca−/− mice are hypersensitive to oxidants. Because FA patients have severe defects in hematopoietic function and because it is not clear whether the FA proteins function in oxidative stress response, we used mice deficient for the FA complementation group A (Fanca) gene to examine the sensitivity of primary FA cells to oxidative stress. We first treated BMMNCs from Fanca−/− or their WT littermates with increasing concentrations of the oxidant hydrogen peroxide (H2O2) and analyzed cell proliferation using the BrdUrd incorporation assay. We observed that treated Fanca−/− cells proliferated much slower than their WT counterparts (Fig. 1A, left). Because FA hematopoietic failure is a direct consequence of the defect in the hematopoietic stem cell/progenitor (HSC/P) compartment, we tested whether the FA HSC/P cells were hypersensitive to oxidative damage. Indeed, the number of colonies formed by Fanca−/− HSC/P cells was markedly decreased with 100 μmol/L of H2O2, whereas WT HSC/P cells showed little effect with H2O2 treatment (Fig. 1A, right).

We also established MEFs from Fanca−/− or WT mice and used this well-established cell type as a cellular model system for the evaluation of cellular and molecular alterations in oxidative response in FA mutant cells. Similar to BMMNCs, the growth of Fanca−/− MEFs was severely inhibited even at low concentrations of H2O2, which had little effect on the proliferation of WT cells (Fig. 1B). In addition, a sublethal dose (10 μmol/L) of H2O2 induced premature senescence, as tested by SA-β-gal staining in Fanca−/− MEFs (Fig. 1C). Together, these results indicate that loss of FA function results in cellular hypersensitivity to oxidants.

Oxidative stress induces premature senescence by activating several major signaling pathways, including the p16INK4a/pRB and ADP ribosylation factor/p53 pathways (39). Oncogene Ras has also been shown to induce cell growth arrest after oxidative injury by activating the mitogen-activated protein kinase (MAPK) pathway (40). We, therefore, examined the expression and activities of proteins and enzymes in the Ras, p53, and pRB pathways. We did not find significantly elevated Ha-Ras expression or the down-regulation or ADP ribosylation factor/p53 pathways (39). Oncogene Ras has also been shown to induce cell growth arrest after oxidative injury by activating the mitogen-activated protein kinase (MAPK) pathway (40). We, therefore, examined the expression and activities of proteins and enzymes in the Ras, p53, and pRB pathways. We did not find significantly elevated Ha-Ras expression or the down-regulation of H2O2, which had little effect on the proliferation of WT cells (Fig. 1B). In addition, a sublethal dose (10 μmol/L) of H2O2 induced premature senescence, as tested by SA-β-gal staining in Fanca−/− MEFs (Fig. 1C). Together, these results indicate that loss of FA function results in cellular hypersensitivity to oxidants.

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H2O2 induces G2-M cell cycle arrest and DNA double-strand breaks in Fanca−/− BM cells. One hallmark of FA cells in response to genotoxic stress is increased retardation of the cells at the G2-M phase of the cell cycle (1–3). We thus examined whether H2O2 induced abnormal G2-M cell cycle arrest in primary FA BM cells. Indeed, H2O2-treated Fanca−/− BM cells displayed a significant increase in the percentage of cells in G2-M compared with H2O2-treated WT cells (Fig. 2A). Because G2 cell cycle arrest is a major cellular response to DNA damage preceding the decision to repair or enter mitosis and because FA cells are defective in the repair of DNA double-strand breaks (DSB; refs. 1–3), we determined whether H2O2-treated Fanca−/− BM cells accumulated high levels of DNA damage by examining the well-established DSB marker, phosphorylated histone H2AX (γH2AX; ref. 42). As shown in Fig. 2B, there was no significant accumulation of DSB in WT BM cells, indicating that either the H2O2 treatments have little effect on WT BM cells or DNA damage induced by H2O2 had been mostly repaired in these WT BM cells. Strikingly, H2O2 induced significantly more DSB in Fanca−/− BM cells than in untreated Fanca−/− or H2O2-treated WT BM cells (Fig. 2B). Thus, like mitomycin C, H2O2 induces abnormal G2-M cell cycle arrest in primary FA BM cells, possibly in a DNA damage-dependent manner.

H2O2 overactivates DNA damage response in Fanca−/− cells in vitro and in vivo. Reasoning that the abnormal G2-M accumulation induced by H2O2 in Fanca−/− BM cells might have resulted from the prolonged activation of the oxidative DNA damage and DNA DSB checkpoints, we examined the expression of...
several oxidative damage response molecules p53, p53\textsuperscript{Ser20}, and p21\textsuperscript{WAF1}. We found increased expression of both the total and activated forms (p53\textsuperscript{Ser20}) of p53 in H\textsubscript{2}O\textsubscript{2}-treated Fanca\textsuperscript{−/−} cells compared with WT cells (Fig. 3A). Consistent with its activation, p21\textsuperscript{WAF1}, the target of p53, was up-regulated in H\textsubscript{2}O\textsubscript{2}-treated Fanca\textsuperscript{−/−} cells.

Next, we examined oxidative DNA damage response in vivo. For this purpose, we injected (i.v.) H\textsubscript{2}O\textsubscript{2} into WT and Fanca\textsuperscript{−/−} mice and examined the expression of p53, p53\textsuperscript{Ser20}, and p21\textsuperscript{WAF1}. Western blot analysis showed that all these DNA damage response molecules were elevated in response to H\textsubscript{2}O\textsubscript{2} treatment in BM and spleen harvested from Fanca\textsuperscript{−/−} mice (Fig. 3B). Similar results were noticed in the paraffin-embedded tissue sections of BM, spleen, and thymus prepared from H\textsubscript{2}O\textsubscript{2}-treated mice (Fig. 3C). These results suggested that loss of FA function causes strong oxidative DNA damage response, possibly through up-regulation of the p33 pathway.

**Kinetics of H\textsubscript{2}O\textsubscript{2}-induced DNA damage response in Fanca\textsuperscript{−/−} mice.** The observation that H\textsubscript{2}O\textsubscript{2} induced strong DNA damage response in Fanca\textsuperscript{−/−} cells and tissues prompted us to study the kinetics of H\textsubscript{2}O\textsubscript{2}-mediated p53 signaling in Fanca\textsuperscript{−/−} mice. We treated (through tail vein injection) WT and Fanca\textsuperscript{−/−} mice with a single dose (250 \textmu mol/kg) of H\textsubscript{2}O\textsubscript{2} and sacrificed the injected animals 2, 4, 6, 12, and 24 hours after H\textsubscript{2}O\textsubscript{2} injection. The kinetics of DNA damage response in the BM and spleen of the treated mice was analyzed for the expression of p53\textsuperscript{Ser20} and \gamma H\textsubscript{2}AX. As shown in Fig. 4, p53 activation (in the form of p53\textsuperscript{Ser20}) was short-lived in both marrow (Fig. 4A) and spleen (Fig. 4B) of WT mice, which peaked at 4 hours but was decayed rapidly to background level by 6 hours after H\textsubscript{2}O\textsubscript{2} treatment. In Fanca\textsuperscript{−/−} mice, in contrast, high level of p53 activation was evident at 2 hours and persisted for 24 hours postinjection. Although the expression kinetics of \gamma H\textsubscript{2}AX, a well-established DSB marker (42), varied somewhat among the mouse tissues, Fanca\textsuperscript{−/−} mice showed prolonged expression of the DNA damage marker compared with WT mice, in which \gamma H\textsubscript{2}AX expression was undetectable by 12 to 24 hours after injection. Therefore, DNA damage response initiated by oxidative stress is persistent in Fanca\textsuperscript{−/−} mouse hematopoietic tissues in vivo.

![Figure 3.](https://www.aacrjournals.org/) H\textsubscript{2}O\textsubscript{2} overactivates DNA damage response in Fanca\textsuperscript{−/−} cells in vitro and in vivo. A, WT or Fanca\textsuperscript{−/−} mice were injected i.v. with H\textsubscript{2}O\textsubscript{2} (250 \textmu mol/kg body weight) or with equal volume (100 \textmu L) of PBS. The mice were then sacrificed 4 h later, and BM cells were isolated and stained for the p53\textsuperscript{Ser20}, p53, or p21\textsuperscript{WAF1} proteins. B, BM cells and splenocytes of PBS-treated or H\textsubscript{2}O\textsubscript{2}-treated WT and Fanca\textsuperscript{−/−} mice, as described in A were analyzed for the expression of the p53\textsuperscript{Ser20}, p53, or p21\textsuperscript{WAF1} proteins by Western blotting. Total protein levels were normalized using \beta -actin as a control. C, paraffin-embedded BM, spleen, or thymus sections from untreated or H\textsubscript{2}O\textsubscript{2}-treated mice were stained with the antibody against p53 or p21\textsuperscript{WAF1}.
The observation that H$_2$O$_2$-induced expression of the DSB marker $\gamma$H2AX seemed to be more pronounced and sustained in the BM than in spleen raised the question as to whether there was a lineage-specific effect on oxidative DNA damage in Fanca$^{-/-}$ mice. To address this, we determined H$_2$O$_2$-induced DSBs in lymphoid and myeloid cells (Fig. 4C) from the BM of Fanca$^{-/-}$ mice. Whereas increased and prolonged expression of $\gamma$H2AX was seen in Fanca$^{-/-}$ cells compared with WT cells, no significant difference in H$_2$O$_2$-induced DSBs was evident between lymphoid and myeloid cells (Fig. 4D).

The dependency and persistence of p53-mediated response to oxidative and oncogenic stresses in Fanca$^{-/-}$ cells. To explore the requirement for p53 activity and potential FA-p53 interaction in oxidative DNA damage response, we generated Fanca$^{-/-}$ mice with the deletion of the Trp53 gene, isolated primary MEFs from Fanca$^{+/+}$:Trp53$^{-/-}$ and Fanca$^{-/-}$:Trp53$^{-/-}$ mice, and expressed in these cells the temperature-sensitive p53 mutant tsp53V143A, which can be reverted to functional state at the permissive temperature of 32°C (43). The functional reversibility of the mutant tsp53V143A was verified by Western analysis,
demonstrating that H$_2$O$_2$ induced increased p21$^{\text{WAF1}}$ expression in all the tsp53$^{\text{V143A}}$-expressing cells cultured at 32°C (Fig. 5A). A prolonged time course analysis of cell growth indicated that, at nonpermissive temperature (37°C), loss of p53 function almost completely abolished growth response to H$_2$O$_2$ in cells with or without the Fanca protein (Fig. 5B), suggesting that H$_2$O$_2$-induced damage response requires a functional p53. Indeed, when the function of p53 was restored at 32°C, H$_2$O$_2$-induced growth inhibition was clearly evident in both Fanca$^{+/+}$ and Fanca$^{-/-}$ cells (Fig. 5C). However, we observed differential responses to H$_2$O$_2$-induced stress in cells with different Fanca status. In Fanca$^{+/+}$ cells, the p53 response to oxidative stress seemed to be relatively short (3–6 days) after the insult; whereas Fanca$^{-/-}$ cells underwent prolonged growth inhibition (Fig. 5C). Interestingly, TUNEL assay indicated that p53 deficiency increased H$_2$O$_2$-induced apoptosis in Fanca$^{-/-}$ cells, which could be inhibited by functional tsp53$^{\text{V143A}}$ (Fig. 5D). Thus, although p53 caused prolonged proliferative suppression in Fanca$^{-/-}$ cells, a functional p53 seemed to be required for the survival of the mutant cells under oxidative stress.

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growth arrest with few cells in the S-phase population (Fig. 6C), which was accompanied by strong induction of p21WAF1 (Fig. 6D). Whereas p53 restoration at day 12 could still trigger immediate growth arrest in Fanca+/+;Trp53−/− cells; Fanca−/−;Trp53−/− cells showed lack of activated ras-induced growth arrest and p21WAF1 expression (Fig. 6C and D). Therefore, loss of Fanca impairs p53 function during prolonged ras activation.

Discussion

Cellular stresses, most significantly oxidative DNA damage and oncogene insults, have been shown to activate p53 (45). Given that p53 activation can lead to cell growth arrest or apoptosis, cancer cells, in particular, must have developed strategies to regulate p53 activity. In addition to the p53-negative regulator Mdm2, several p53-interacting proteins have been shown to regulate p53 activity. The interaction of p53 with other cellular proteins is known to influence the activity of the tumor suppressor (46). Our finding that the Fanca protein coordinates with p53 to regulate oxidative and oncogenic stress responses supports the notion that a fine control of p53 activity is fundamentally important for cellular homeostasis and cancer prevention.

Patients with FA have an increased susceptibility to cancer, and cells from FA patients are deficient in repair of DNA damage induced by certain genotoxic agents. This suggests that the FA proteins may interplay with other tumor suppressor pathways that are involved in DNA damage repair and oncogenic signaling. Encouraged by recent reports that p53 deficiency increases cancer development in patients with FA and FA knockout mice (32–36), we formally tested the hypothesis that the FA proteins may functionally interact with the p53-activating signals in response to oxidative and oncogenic stresses. Our results suggest that the major FA protein, Fanca, may coordinate with p53 in regulation of oxidative and oncogenic stress responses. This notion is supported as follows: (a) the hypersensitive response to oxidative stress in tissues in vivo and cells in vitro derived from Fanca−/− mice is correlated with a persistent overactivation of the tumor suppressor p53; (ii) the loss of Fanca function causes prolonged oxidative DNA damage response through up-regulation of the p53 pathway; and (c) the functional status of p53 dictates the kinetics and persistence of response to oxidative and oncogenic stresses in these FA cells.

The mechanistic link between p53 signaling and FA has not been well defined. The involvement of p53 in FA pathophysiology has been highlighted by recent studies that show accelerated tumor development in Fanca1, Fanca2, or Fancc mice, also deficient for the Trp53 gene (34–36). Furthermore, developmental defects and increased apoptosis in Fanca2-deficient zebrafish could be corrected by p53 knockdown, suggesting that p53-dependent apoptosis may be an underlying mechanism for the developmental
defect in the Fancd2+/− fish (38). The current study establishes the requirement for p53 signaling in oxidative and oncogenic stress responses in Fanca-deficient cells. We have used primary MEFs from Fanca+/−;Trp53−/− and Fanca+/−;Trp53+/− mice combined with a functionally switchable p53 mutant to define the kinetics, dependence, and persistence of p53-mediated response to oxidative and oncogenic stresses in FA cells. One important finding of our study is that we observed different pattern of p53-dependent response to H2O2-induced and deregulated oncogene-induced stress in cells with different Fanca status. For instance, restoration of p53 function elicited a transient response to oxidative stress in Fanca+/− cells, whereas Fanca−/− cells underwent prolonged p53-dependent growth inhibition (Fig. 5). This persistent p53 engagement may be due to the fact that Fanca-deficient cells accumulated high levels of oxidative DNA damage as a consequence of impairment in DNA damage repair. We previously reported that BM progenitor cells from Fanca−/− mice contained high levels of oxidative DNA damage and exhibited persistent DNA damage response (47). Thus, the prolonged p53 response in Fanca−/− cells might have been induced by the unrepaired DNA damage, leading to persistent DNA damage response.

Another interesting finding in the current study is that, although activation of p53 by oxidative stress causes increased proliferative suppression in Fanca+/− cells compared with WT cells (Fig. 1), studies with the Fanca−/−;Trp53−/− double knockout cells indicated that p53 deficiency increased H2O2-induced apoptosis in Fanca−/− cells, which could be inhibited by functional tsp53V131A (Fig. 5). Thus, a functional p53 seems to be required for the survival of the mutant cells under oxidative stress. Circumstantial evidence indicates that the selectivity of p53 to regulate the prosurvival or proapoptotic signaling is affected by the growth environment, the type of stress used, the cellular context, and differential expression of the target genes (48, 49). In response to stress, p53 induces cell growth arrest, thus preventing the damaged cells from further replication (45, 50). More importantly, p53 can activate cell cycle checkpoint in cells with damaged DNA, which allows cells to repair the damage before reentering cell cycle. Indeed, it has been reported that p53-null cells are more sensitive to mitotic catastrophe induced by genotoxic drug (51, 52). Because H2O2-induced arrest in Fanca−/− cells with a functional p53 occurs at the G2–M cell cycle checkpoint, inactivation of p53 would lead to the impairment of the checkpoint. Consequently, failure of the damaged Fanca−/− cells to arrest at G2 would result in entry into mitosis and potential death through mitotic catastrophe and apoptosis.

Patients with FA have high predisposition to leukemia and other cancers. Whether FA cells have high susceptibility to oncogenic transformation is not known. We have used an in vitro system in which a functional p53 could be restored in Fanca−/−;Trp53−/− cells to investigate the dynamic interplay between Fanca and p53 in response to oncogenic stress. Whereas WT cells exhibit prolonged response to oncogene activation, the p53-activating signals induced by oncogenic ras were short-lived in Fanca−/− cells. Indeed, when p53 function was restored 12 days after ras activation, Fanca−/−;Trp53−/− cells showed lack of activated ras-induced growth arrest and p21WAF1 expression (Fig. 6). This suggests that a functional Fanca may be required for the cell to engage p53 during constitutive ras activation. In contrast to oxidative DNA damage, which triggers p53 activation only transiently, activated Ras elicits persistent p53-activating signals in WT cells. Furthermore, our results show that the Fanca function is required to maintain p53 response to Ras-induced growth arrest, as restoration of p53 function after 12 days of constitutive ras activation failed to suppress proliferation of cells deficient in Fanca.

In conclusion, our results derived from both in vivo and in vitro studies using the restorable p53 model shed new light on the potential interplay between p53 and the FA pathway during cellular response to oxidative and oncogenic stresses. Our studies also define the important kinetic difference between WT and FA cells during prolonged response to oxidative DNA damage and activated oncogene signals. Additional studies into functional interaction between the p53 and FA pathways in DNA damage and oncogenic stress response may aid us in better understanding on how cells can bypass the normal checkpoints and continue to proliferate in the presence of damaged DNA and oncogenic activation. In the context of FA, new insights on the role of the FA proteins in oxidative DNA damage response/repair and oncogene activation can suggest new pathways and proteins to target for therapeutic prevention of cancer progression of the disease.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Reena Rani, Jie Li and Qishen Pang


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