Tempol Protects against Oxidative Damage and Delays Epithelial Tumor Onset in Fanconi Anemia Mice

Qing-Shuo Zhang,[1] Laura Eaton,[1] Eric R. Snyder,[2] Scott Houghtaling,[1] James B. Mitchell,[3] Milton Finegold,[4] Carter Van Waes,[2] and Markus Grompe[1]

[1]Oregon Stem Cell Center, Oregon Health and Science University, Portland, Oregon; [2]Tumor Biology Section, Head and Neck Surgery Branch, National Institute on Deafness and Other Communication Disorders and [3]Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, Maryland; and [4]Department of Pathology, Texas Children's Hospital, Houston, Texas

Abstract

Fanconi anemia (FA) is a genetic disorder characterized by congenital abnormalities, bone marrow failure, and marked cancer susceptibility. FA patients have an elevated risk of developing hematologic malignancies and solid tumors. Using *Fancd2*$^{-/-}$ knockout mice as a model of FA, we examined the potential of tempol, a nitroxide antioxidant and a superoxide dismutase mimetic, as a tumor-delaying agent for solid tumors. Dietary tempol increased the mean tumor-free survival time of *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice by 27% ($P < 0.01$), from 308 to 390 days, without changing the overall tumor spectrum. More strikingly, tempol delayed the onset of epithelial tumors and increased the mean epithelial tumor-free survival time by 38% ($P < 0.0001$), from 312 to 432 days, in *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice. These results show that tempol can significantly delay tumor formation in *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice. Furthermore, tempol treatment did not adversely affect the repopulating ability of FA hematopoietic stem cells. The reduction in oxidative DNA damage in tempol-treated FA fibroblasts and mice suggests that its tumor-delaying function may be attributed to its antioxidant activity. [Cancer Res 2008;68(5):1601–8]

Introduction

Fanconi anemia (FA) is an autosomal recessive or X-linked genetic disorder characterized by birth defects, progressive bone marrow failure, and cancer susceptibility. FA falls into at least 13 complementation groups and 13 causative genes (*FANCA, B, C, D1/BRCA2, D2, E, F, G/XRCC9, I, L/PHF9/Pog, J/BRIP1/BACH1, M/Hef, N/PALB2*) have been identified. All the FA proteins are believed to function in a common cellular signaling pathway that responds to DNA damage and maintains genome stability, although the precise function(s) of the FA pathway remains unknown (1, 2).

Patients with FA have multiple dysmorphic features including short stature, infertility, pigmentation defects, and microphthalmia. Most develop bone marrow failure usually during childhood. In older FA patients, the major complications are hematologic malignancies (typically acute myeloid leukemia) and solid tumors (most commonly aerodigestive and gynecologic carcinomas; ref. 3). Much progress has been made in treating the hematologic problems in FA due mainly to the significant advances in bone marrow transplantation. However, the treatment of solid tumors remains a big challenge, and given the increased risk of solid tumors with prolonged survival after transplantation (4), the development of chemoprevention has become an increasingly important issue for the physicians and scientists in the field.

Several murine models for FA, including *Fanca, Fancc, Fancg, Fancd2, Fanca-Fancc* double, and *Fancl* knockout mice, have been developed (2). All of these mice share some common phenotypes, such as defective germ cell development and cellular sensitivity to DNA cross-linking agents. However, unlike the other FA knockout mice, *Fancd2*$^{-/-}$ mutants show a strikingly increased risk for developing epithelial cancers (i.e., breast, ovarian, and liver; ref. 5). In addition, we showed that heterozygosity for p53 accelerates the onset of these tumors in *Fancd2*$^{-/-}$ mice, significantly decreasing the amount of time needed for the study of tumor formation (6). Therefore, *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice represent an improved model to test chemoprevention regimens in FA.

FA cells are well known to be hypersensitive to reactive oxygen species (ROS; ref. 7), which may be a significant source of DNA damage and therefore cause mutation and cancer (8, 9). Treatment with antioxidants can reduce oxidative DNA damage and thus has the potential to delay cancer in FA. Previous studies have shown antioxidant treatment can partially correct chromosomal abnormalities in lymphocytes or fibroblasts derived from FA patients (10, 11). Mice doubly mutant for *Fancc* and Cu/Zn superoxide dismutase (*Sod*) have a severe phenotype, suggesting that free oxygen radicals that can be metabolized by Sod may be particularly toxic in FA (12). The small molecule tempol (4-hydroxy-2,2,6,6-tetramethyl piperidine-*N*-oxyl) is a nitroxide antioxidant and a superoxide dismutase mimetic (13). Although other SOD mimetic antioxidants have been reported, tempol is stable and commercially available and has been shown to decrease tumorigenesis in C3H mice (14). More importantly, it was recently reported that tempol can delay the onset of lymphomas in mice with ataxia telangiectasia, another rare genetic chromosome instability syndrome associated with elevated oxidative stress (15). Considering the common finding of oxidative stress in FA and ataxia telangiectasia, we hypothesized that tempol might be able to function as a tumor-delaying agent in FA. In this study, we tested whether tempol could prevent or delay tumor formation using the *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mouse model.

Materials and Methods

Cell culture. PD352F, PD551F, and PD720F (of FA complementation groups G, C, and A, respectively) were human diploid FA fibroblasts maintained in the Oregon Health and Sciences University Fanconi Anemia Cell Repository (at passage numbers 10, 11, and 8, respectively). D551 was a normal human diploid fibroblast strain obtained from the American Type Culture Collection at passage number 3. All fibroblast cells were grown in

Note: Q-S. Zhang and L. Eaton contributed equally to this work.

Requests for reprints: Qing-Shuo Zhang, Oregon Stem Cell Center, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Portland, OR 97239. Phone: 503-494-6889; Fax: 503-494-5044; E-mail: zhangqi@ohsu.edu.

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MEM with nonessential amino acids (Life Technologies/Bethesda Research Laboratories) supplemented with 10% heat-inactivated FCS (Life Technologies/Bethesda Research Laboratories) without antibiotics. Cells were maintained in a fully humidified 5% CO_2, containing atmosphere at 37°C.

Animals. *Fancd2* and *Fancc* mutant mice were generated previously in this laboratory and maintained on 129S4 background. *Fancd2*$^{+/-}$ *Trp53*$^{+/-}$ breeding pairs were crossed to generate *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice and littermate controls, as described previously (6). ROSA26 transgenic mice were developed originally by Dr. Philippe Soriano and maintained on 129S4 background (16). Animals were kept at Medical Research Building in Oregon Health and Science University and treated in accordance with the guidelines of the Institutional Animals Care and Use Committee.

Tempol treatment. A cohort of 58 *Trp53*$^{+/-}$ mice (39 *Fancd2*$^{-/-}$, 12 *Fancd2*$^{+/-}$, and 7 *Fancd2*$^{+/+}$) were generated. Upon weaning (3–4 weeks of age), the mice received bacon-flavored mouse chow either with or without tempol (Bioserv; powdered tempol mixed at 11 g/kg). All animals were weighed and examined weekly and sacrificed upon observation of a palpable mass, loss of >20% body weight, or other obvious indicators of poor health. All visible tumors, ovaries, and, in most cases, liver and spleen, as well as any other abnormal masses, were collected for histologic analysis. Survival curves were generated and statistically analyzed using Prism 4.0 software.

Histology. Tumor samples and selected tissues were fixed in 10% phosphate-buffered formalin and stained with H&E as reported previously (5).

***In vivo* competitive repopulation assay.** Recipient mice (6–10 weeks old) were lethally irradiated in a split dose of 1,200 rad (600 rad each, 4 h apart) 1 day before transplantation. Donor bone marrow cells were isolated from *Fancc*$^{-/-}$ and ROSA26 transgenic mice (129S4 strains; 8–10 weeks old). Unfractionated marrow cells were counted, and 2 million mixed *Fancc*$^{-/-}$ and *ROSA26*$^{+/-}$ donor cells were transplanted into each recipient mouse by retroorbital injection (0.1 mL of cell suspension per mouse). Blood samples were taken through the retroorbital plexus 6 months after transplantation. The animals were then euthanized, and bone marrow was taken out. DNA was isolated from blood and bone marrow using MasterPure DNA Purification kit (EPICENTRE Biotechnologies). Quantitative real-time PCR analysis was performed to analyze the contribution of *Fancc*$^{-/-}$ or *ROSA26* genotypes to mature blood cells and bone marrow in recipient mice.

Western blotting. A small group of mice (littermates) were treated for 6 months with special diet (two *Fancd2*$^{-/-}$ mice on tempol diet, two *Fancd2*$^{-/-}$ mice on placebo diet, two wild-type mice on tempol diet, and one wild-type mouse on placebo diet). Mice were euthanized, and thymi samples were taken for protein expression analysis by Western blot. The primary antibodies for p53, phosphorylated p53, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and p21 were monoclonal antibody (mAb) OP03L (Calbiochem), rabbit polyantibody (Cell Signaling), mAb (Novus Biologicals), and goat polyantibody (Santa Cruz Biotech), respectively. Secondary antibodies were from Santa Cruz Biotech.

Quantitative real-time PCR. DNA was isolated from peripheral blood or bone marrow samples. Quantitative real-time PCR was performed with Bio-Rad MyiQ single-color real-time PCR detection system using SYBR green dye. For the amplification of ROSA26 transgenic DNA, the following primers were used: MG1717, CATCAGCCGCTACAGTCAACAG; MG1718, CAGCCATGTGCCTTCTTCCGC. To amplify *Fancc* mutant DNA, the following primers were used: MG1711, GAGCTGCCTGATACGGATGCTG; MG1791, GGGCTGCTAAAGCGCATGCTC.

8-Hydroxy-2′-deoxyguanosine assay. For *in vitro* cultured fibroblasts, cells were trypsinized and resuspended in 200 mL PBS. After lysis with proteinase K, DNA was extracted and purified according to the method of Vogelstein and Gillespie (17). DNA was then digested with nuclease P1 and alkaline phosphatase (Roche), and 1 mg of DNA per sample was used for 8-hydroxy-2′-deoxyguanosine (8-OHdG) content analysis via the BIOXYTECH 8-OHdG-EIA kit (Oxis International). Calibration curves were generated with six standardized samples (0.5, 2.0, 8.0, 20.0, 80.0, and 200.0 ng/mL), and the data were fitted to a four-variable logistic function.

For the determination of 8-OHdG level in mice samples, a cohort of 14 *Fancd2*$^{-/-}$ mice and 12 wild-type controls were equally divided into two groups and treated with either tempol or control diet. After 6 months of treatment, urine samples were collected daily for three consecutive times and 15 μL from each fraction for the same mouse were pooled together and then diluted 5-fold immediately before the 8-OHdG analysis via the BIOXYTECH 8-OHdG-EIA kit.

Results

Tempol reduced oxidative DNA damage in FA fibroblasts and in FA mice. We first tested whether tempol was able to reduce endogenous ROS-mediated DNA damage in FA cells. To do so, we used a competitive ELISA for quantitative measurement of 8-OHdG, a commonly used marker for oxidative DNA damage. Human FA primary fibroblasts PD720F, PD551F, and PD352F belong to common FA complementation groups A, C, and G and bear disrupted FA genes for *FANCA, FANCC*, and *FANCG*, respectively. 8-OHdG contents in these three FA fibroblasts were analyzed, along with normal human primary fibroblast strain D551, under a range of acute peroxidation levels in the absence and presence of tempol. For cells preincubated with tempol, the medium was replaced with fresh medium to minimize any effects attributable to free tempol. All FA fibroblasts exhibited a baseline increase in 8-OHdG levels relative to the normal fibroblasts (Fig. 1*A*). Treatment with 100 μmol/L tempol decreased 8-OHdG levels in all cell types and at all peroxide concentrations tested. Tempol treatment resulted in an average of 18% reduction in baseline 8-OHdG levels, reducing levels to within 1 SD of the 8-OHdG level in the normal control. FA fibroblasts of complementation group A (PD720F) exhibited the most significant decrease. With increasing peroxidation, FA cells of complementation group C (PD551F) maintained a greater difference between tempol-treated and untreated cultures. In summary, these data indicate that tempol treatment could reduce the elevation in baseline oxidative DNA damage observed in these cultured FA fibroblast cells and alleviate further oxidative DNA damage generated by peroxidation.

Next, the antioxidative properties of tempol were further evaluated in *Fancd2*$^{-/-}$ mice *in vivo*. A cohort of *Fancd2* mutant or wild-type mice were treated with either tempol or placebo for over 6 months and used for the determination of urinary 8-OHdG levels. Urine samples were collected daily on 3 consecutive days, and 15 μL of urine from each collection for the same mouse was pooled together to minimize possible fluctuations. In the cases of placebo treatment, we observed slightly higher average 8-OHdG level in urine samples of mutant mice compared with wild-type mice, although two of the seven placebo-treated mutant mice showed much higher levels of 8-OHdG (20% and 30%, respectively; above average). In the cases of tempol treatment, both *Fancd2* mutant and wild-type mice produced a substantial decrease in average 8-OHdG levels compared with their placebo-treated counterparts (Fig. 1*B*). Based on a statistical analysis, tempol-treated *Fancd2* mutant mice had an 18% lower average urinary 8-OHdG than placebo-treated mutants ($P = 0.04$), whereas tempol-treated wild-type mice had 10% lower average urinary 8-OHdG than placebo-treated wild-type mice ($P = 0.08$). Overall, these data indicated that tempol might reduce the level of oxidative damage in both *Fancd2* mutant and wild-type mice *in vivo*, consistent with the results in cultured human cells above.

Tempol treatment delayed the onset of epithelial tumors in FA mice. To test the chemoprevention possibility of tempol in FA

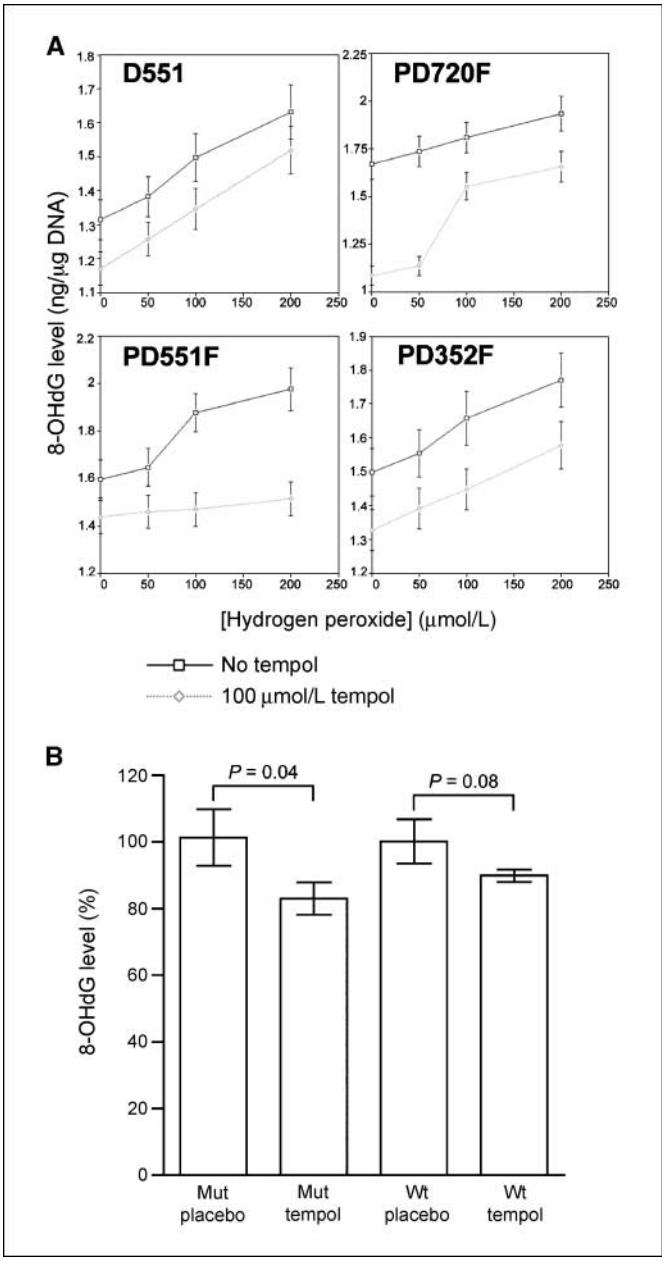

Figure 1. Oxidative damage in human FA fibroblasts and *Fancd2* mutant mice. *A,* 8-OHdG levels in human FA fibroblasts under increasing concentration of H_2O_2. *B,* 8-OHdG levels in *Fancd2* mutant mice treated with tempol or placebo. The 8-OHdG level in placebo-treated wild-type mice was set as 100%. *Columns,* mean; *bars,* SE.

mice, a cohort of *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice along with *Fancd2*$^{+/-}$ or *Fancd2*$^{+/+}$ controls were divided into two groups and treated with either tempol or control diet. Because a previous study with *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice in this laboratory found that tumor progression and incidence were more pronounced in females than males (6), we chose to use only females in this work. As shown in Fig. 2*A*, *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice on tempol diet showed a significantly longer ($P < 0.01$) mean tumor-free survival (mean survival of 390 days) than the mice on placebo diet (mean survival of 308 days). Four early deaths were seen in the *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice that received tempol, all of which were lymphomas; the first four deaths in the *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice fed with placebo diet were also due to lymphoma. The first tumor of epithelial origin was seen at 250 days in the mice on control diet, whereas the earliest epithelial tumor in the *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice on tempol appeared at 378 days of age. The oldest mouse to succumb to an epithelial tumor in the *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice on placebo diet was 386 days old, and the last tumor of epithelial origin in the *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice on tempol diet was seen at 509 days of age. This profound difference between the two groups of *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice is perhaps most apparent when comparing the mean epithelial tumor-free survival at 432 days for mice on tempol and 312 days for those on control diet (Fig. 2*B*). Statistical analysis revealed that tempol treatment significantly increased the mean epithelial tumor-free survival time by 38% in *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice ($P < 0.0001$). There was no significant difference in mean nonepithelial tumor-free survival time between tempol treatment and placebo treatment ($P = 0.15$; see Fig. 2*C*).

The non-FA (*Fancd2*$^{+/-}$ or *Fancd2*$^{+/+}$) *Trp53*$^{+/-}$ controls on placebo diet had a mean survival of 491 days, and those on tempol diet survived an average of 566 days. The majority of the tumors (91%) seen in non-FA/*Trp53*$^{+/-}$ mice were nonepithelial in origin. In both groups, only one *Fancd2*$^{+/-}$ *Trp53*$^{+/-}$ mouse developed an epithelial tumor (Fig. 2*B*) at ~18 months of age, and in both cases, a nonepithelial tumor was also found in the same animal.

Tempol treatment did not significantly alter the tumor spectrum seen in *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice. Thirty-nine *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice developed 44 tumors in total, as a few of them had more than one tumor (summarized in Table 1). Twelve of 22 tumors (55%) in tempol-treated group were of epithelial origin, whereas 10 of 22 tumors (45%) in placebo-treated group were epithelial in origin. These percentages are very similar to those seen previously in this laboratory (6). Eight of the 12 (75%) epithelial tumors seen in tempol-treated mice were ovarian or mammary carcinomas (see representative tumor histologic pictures in Fig. 3) compared with 7 of 10 (70%) seen in placebo-treated mice. In both groups, nonepithelial tumors were mainly lymphoma or sarcoma. Whereas there was a greater variety in the kinds of nonepithelial tumors seen in tempol-treated mice compared with those receiving the placebo diet, the overall percentages of nonepithelial type tumors are virtually identical across the two groups, as well as when compared with results previously described by this laboratory (6). These data indicated that tempol treatment did not change the overall tumor spectrum seen in *Fancd2*$^{-/-}$*Trp53*$^{+/-}$ mice.

Tempol treatment did not affect the repopulation capability of FA hematopoietic stem cells. Because hematopoietic defects are a major complication in FA, we next tested whether tempol treatment would affect FA hematopoietic stem cells (HSC). To do this, an *in vivo* competitive repopulation approach, as depicted in Fig. 4*A*, was used. After being transplanted with a mixture of bone marrow cells from *Fancc*$^{-/-}$ and *ROSA26*$^{+/-}$, experimental *Fancd2*$^{-/-}$ mice were divided into two groups for either tempol or placebo treatment. The treatment started >24 h after the last dose of irradiation to minimize the radioprotective effect of tempol (18) and continued for 6 months. The relative contribution of *Fancc*$^{-/-}$ and *ROSA26*$^{+/-}$ genotypes to peripheral blood DNA and bone marrow DNA, which reflected the relative capacity of donor HSC to repopulate the blood system, was determined by quantitative real-time PCR analysis (19). Consistent with the previous report that wild-type bone marrow cells had a selective advantage over FA cells during repopulation at the stem cell level (19), we observed that *ROSA26*$^{+/-}$ bone marrow cells, which have an

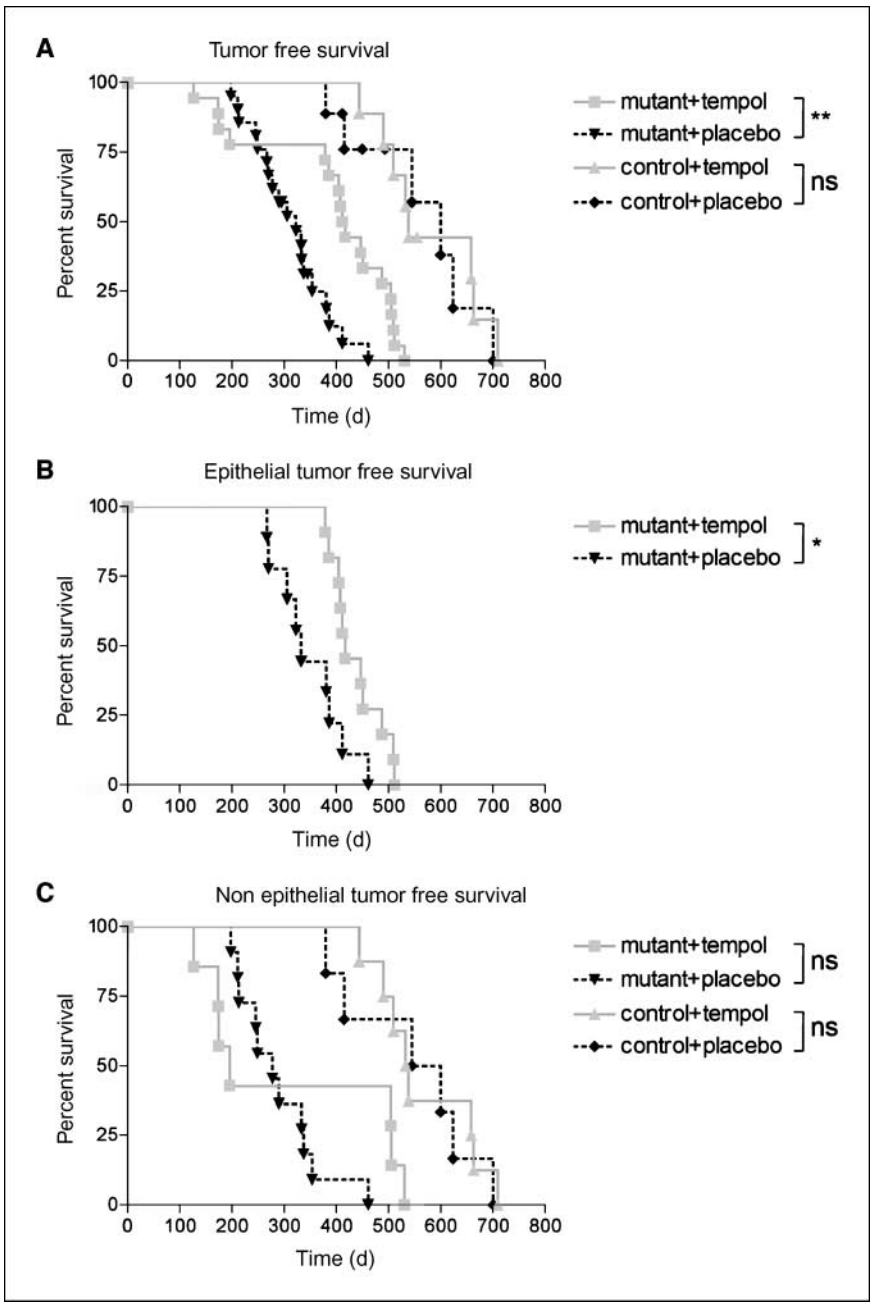

Figure 2. Tempol treatment delayed the onset of epithelial tumors in FA mice. *A,* tumor-free survival curves of all tumor types show tempol treatment is chemopreventive for *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice. *B,* epithelial tumor-free survival curves show a significant delay of epithelial tumor formation in tempol-treated mutant mice. In both tempol-treated and placebo-treated non-FA (*Fancd2*$^{+/-}$ or *Fancd2*$^{+/+}$) *Trp53*$^{+/-}$ control mice, only one mouse developed epithelial tumor for each group. *C,* nonepithelial tumor-free survival curves suggest tempol does not significantly delay the onset of nonepithelial tumors in mutant mice. Statistical analysis was performed with Prism 4.0 software using log-rank test. ** and *, $P < 0.01$ and $P < 0.0001$, respectively; *ns,* not significant ($P > 0.05$).

intact FA pathway, had a higher repopulation capability than the *Fancc*$^{-/-}$ marrow. Six months after the initial transplantation, the ratio of *Fancc*$^{-/-}$ versus *ROSA26*$^{+/-}$ genotypes in recipient bone marrow dropped from 1:1 to ~1:5, with no difference between tempol treatment and placebo treatment ($P = 0.85$), as shown in Fig. 4*B*. We also checked the ratio of *Fancc*$^{-/-}$ versus *ROSA26*$^{+/-}$ genotypes in recipient peripheral blood and did not find a significant difference between tempol and placebo treatment either ($P = 0.06$), demonstrating that tempol treatment did not have any negative effect on the repopulation capability of the donor FA hematopoietic stem cells. Unfortunately, however, it did not enhance survival of *Fancc*$^{-/-}$ HSC, either.

Tumor-delaying function of tempol in FA mice may be attributable to its antioxidant activity and not to the activation of the p53 damage response pathway. Besides the reduction of oxidative DNA damage, the activation of p53-dependent redox-mediated damage response pathway has also been proposed to underlie the tumor-delaying effect of tempol (14, 20). One crucial piece of evidence for the possible involvement of p53 in redox-mediated signaling after tempol treatment came from Western blot analysis of the isolated thymocytes treated *in vitro* with 1 mmol/L tempol and IR, which revealed enhancement of p53 phosphorylation in tempol-treated cells versus controls (20). Considering that serum concentration of tempol in treated mice is at the level of 90 to 100 μmol/L (15), much lower than the *in vitro* conditions used in the reference cited above, we sought to measure changes in the endogenous level of p53 phosphorylation after tempol treatment. For this purpose, we

treated a cohort of mice (*Fancd2*$^{-/-}$ or wild type) with either tempol or placebo diet for 6 months. Tissue samples were made from thymuses and used for immunoblotting assay with antibodies against p53 or phosphorylated p53. There was no significant difference in the protein levels of either p53 or phosphorylated p53 between tempol-treated or placebo-treated mice (Fig. 5). Similarly, we also found no difference after tempol treatment in the level of p21, one of the p53 targets involved in DNA repair and cell cycle control (data not shown). These results suggested that the tumor-delaying function of tempol observed in FA mice was unlikely to be mediated through the activation p53 phosphorylation.

Discussion

Tempol has been shown to decrease tumorigenesis in wild-type C3H mice (14) and increase latency to tumorigenesis in *Atm*$^{-/-}$ mice and *Trp53*$^{-/-}$ mice (15, 20). In this work, we found that tempol could decrease oxidative DNA damage in human FA fibroblast cells and *Fancd2*$^{-/-}$ mice, as well as their wild-type counterparts. We tested the tumor-delaying potential of tempol in the *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mouse model, which exhibits an increased incidence of neoplasms. Tempol treatment significantly increased the mean tumor-free survival time of *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice without changing the overall tumor spectrum. Strikingly, tempol delayed the onset of epithelial tumors and increased the mean

Table 1. Tumor spectrum from *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ and control mice

	Control diet		Tempol diet	
	Onset (mo.)	Type of tumor(s)	Onset (mo.)	Type of tumor(s)
Fancd2$^{-/-}$ *Trp53*$^{+/-}$	6	*Lymphoma*	4	*Lymphoma, Leukemia*
	7	*Lymphoma*	6	*Lymphoma*
	7	*Lymphoma*	6	*Lymphoma*
	8	*Lymphoma*	6	*Lymphoma*
	8	Adenoma	12	Mammary adenocarcinoma
	9	Mammary adenocarcinoma	13	Ovarian granulosa cell tumor
	9	Uterine carcinoma	13	Ovarian carcinoma
	9	*Lymphoma*	14	Ovarian adenocarcinoma, *Stromal tumor*
	10	*Angiosarcoma*	14	Squamous cell carcinoma
	10*		14	Ovarian carcinoma, Mammary adenocarcinoma, Cystadenoma of the ovary
	10	Ovarian clear cell carcinoma		
	11	Mammary adenocarcinoma, *Stromal tumor of the ovary*	15	Ovarian adenocarcinoma
			15	Uterine carcinoma
	11	Ovarian adenocarcinoma, Mammary carcinoma	16	*Soft tissue angiosarcoma*
			16	*Hemangioma of the subcutis*
	11	*Osteosarcoma*	17	Mammary carcinoma, *Stromal tumor of the ovary*
	11	*Uterine sarcoma*		
	11*		17	*Acute myeloid lymphoma*
	12	*Lymphoma*	17	Mammary adenocarcinoma
	12	Ovarian adenocarcinoma	18	*Angiosarcoma*
	13	Uterine squamous cell carcinoma, Mammary adenocarcinoma, *Acute myeloid lymphoma*		
	14*			
	15	*Lymphoma, Leukemia*		
Fancd2$^{+/-}$ *Trp53*$^{+/-}$	13	*Osteosarcoma*	14	*Lymphoma*
	14	*Osteosarcoma*	16	*Plasmacytoma*
	18	Mammary sarcomatoid carcinoma, *Lymphoma*	17	*Hystiocytoma*
			18†	
	20	*Lymphoma, Angiosarcoma*	18	*Malignant fibrous hystiocytoma of the ovary,* Mammary carcinoma
	21	*Leiomyoma of the uterus, Pheochromocytoma*	22	*Malignant fibrous hystiocytoma of the ovary, Osteosarcoma*
	23	*Leukemia, Sarcoma*		
Fancd2$^{+/+}$ *Trp53*$^{+/-}$	10†		18	*Lymphoma, Malignant fibrous hystiocytoma of the ovary*
	14	*Lymphoma*		
	15†		22	*Rhabdomyosarcoma*
	16†		24	*Liposarcoma, Malignant fibrous hystiocytoma*

NOTE: Epithelial tumors are shown in regular font, whereas nonepithelial tumors are in italics.
*Mice were euthanized because of poor health from liver steatosis.
†Mice died from causes other than tumors.

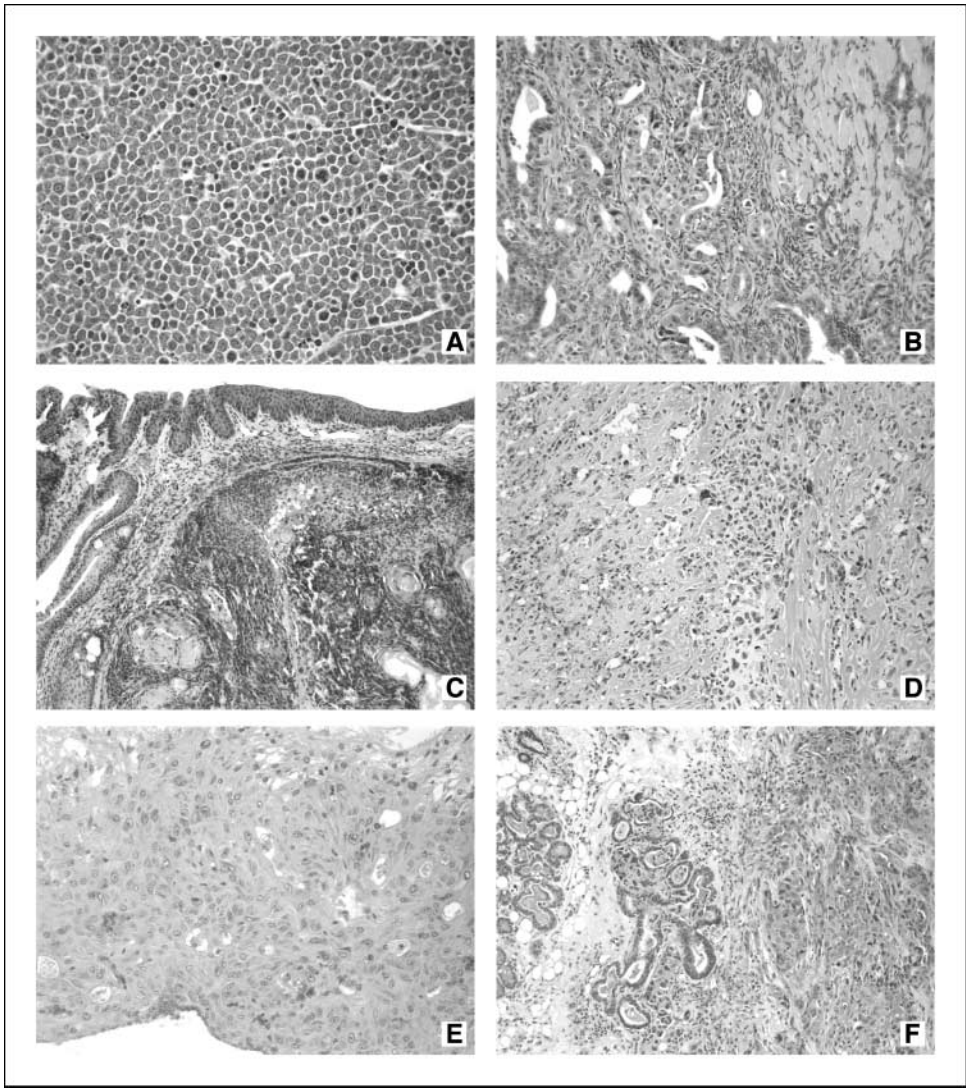

Figure 3. Representative tumor histology from *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice. *A,* thymic lymphoma occurred in a 12-mo-old mouse. The thymus is completely replaced and enlarged by a diffuse infiltrate of neoplastic thymocytes showing abundant mitoses. Original magnification, 250×. *B,* uterine adenocarcinoma arising from the endometrium in a 15-mo-old mouse. Adenocarcinoma is invading the myometrium and surrounding pelvic tissues. Original magnification, 200×. *C,* invasive squamous cell carcinoma in a mass above the right eye of a 13-mo-old mouse. Original magnification, 125×. *D,* osteosarcoma. H&E staining revealed a lytic lesion of the scapula with erosion of the cortex and soft tissue invasion by osteosarcoma in this 11-mo-old mouse. Rapidly dividing undifferentiated pleomorphic cells are embedded in a matrix of osteoid. Original magnification, 200×. *E,* ovarian carcinoma. The ovary is partially replaced and expanded by an adenocarcinoma in this 13-mo-old mouse. Original magnification, 250×. *F,* mammary carcinoma. An expansile and infiltrating poorly differentiated adenocarcinoma in the mammary gland of this 11-mo-old mouse. Original magnification, 160×.

epithelial tumor-free survival time by 38% in *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice. These results clearly show that tempol can significantly delay the formation of tumors in *Fancd2*$^{-/-}$ *Trp53*$^{+/-}$ mice, especially for the epithelial tumors, which are predominantly associated with *Fancd2* deficiency in female mice (5, 6) and rarely seen in *Trp53*$^{+/-}$ mice (21).

Previous work in other tempol-treated mice suggested that the tumor-delaying effect of tempol might be due to the reduction of oxidative DNA damage, either alone (14, 15) or in combination with the activation of p53-dependent redox-mediated signaling pathway (20). However, there were no changes in the protein levels of either p53, phosphorylated p53, or p21 in our tempol-treated FA mice versus placebo-treated controls, arguing against tempol-mediated enhancement of p53 phosphorylation, a key event in the proposed p53-dependent redox-mediated signaling.

A causal link between oxidative stress, DNA damage, and cancer has been proposed for over 20 years (22). Elevated ROS levels were found in multiple human cancer cell lines (23) and tumor tissues (24). The carcinogenic effects of ROS have been reported to regulate gene expression (25), increase proliferation (26), and contribute to genomic instability (27). Accordingly, it is not surprising that treatment with antioxidants was shown to attenuate oncogene-induced chromosomal instability and transformation (26, 27). In cancer-prone *Atm*-deficient mice model, where a continuous state of oxidative stress has been reported in numerous studies, treatment with several antioxidants, including tempol, EUK-189, and *N*-acetyl-cysteine, all had some tumor-delaying effects (15, 28, 29).

Several observations showed higher 8-OHdG levels in leukocytes from human FA patients (30, 31) and H_2O_2-exposed FA lymphoblast cells (32), consistent with the hypothesis that excess oxidative stress might be associated with FA phenotypes (7). Antioxidant enzymes, hypoxia, or low molecular weight antioxidants were able to reduce certain FA phenotypic alterations, including chromosomal abnormalities (10, 11, 33, 34). We found that human FA cells exhibited an increase in baseline 8-OHdG, which could be reduced by tempol. *Fancd2*$^{-/-}$ mice showed only a slight and nonsignificant increase in urinary 8-OHdG contents versus wild-type controls, although individual mutant mice bore much higher urinary 8-OHdG levels than average. Tempol treatment reduced urinary 8-OHdG levels in both *Fancd2*$^{-/-}$ and wild-type mice at the doses used in our study, clearly indicating that tempol is able to reduce oxidative DNA damage in both *Fancd2*$^{-/-}$ and wild-type mice. The difference in basal 8-OHdG contents between cultured FA fibroblasts and our FA mice is probably due to considerably higher

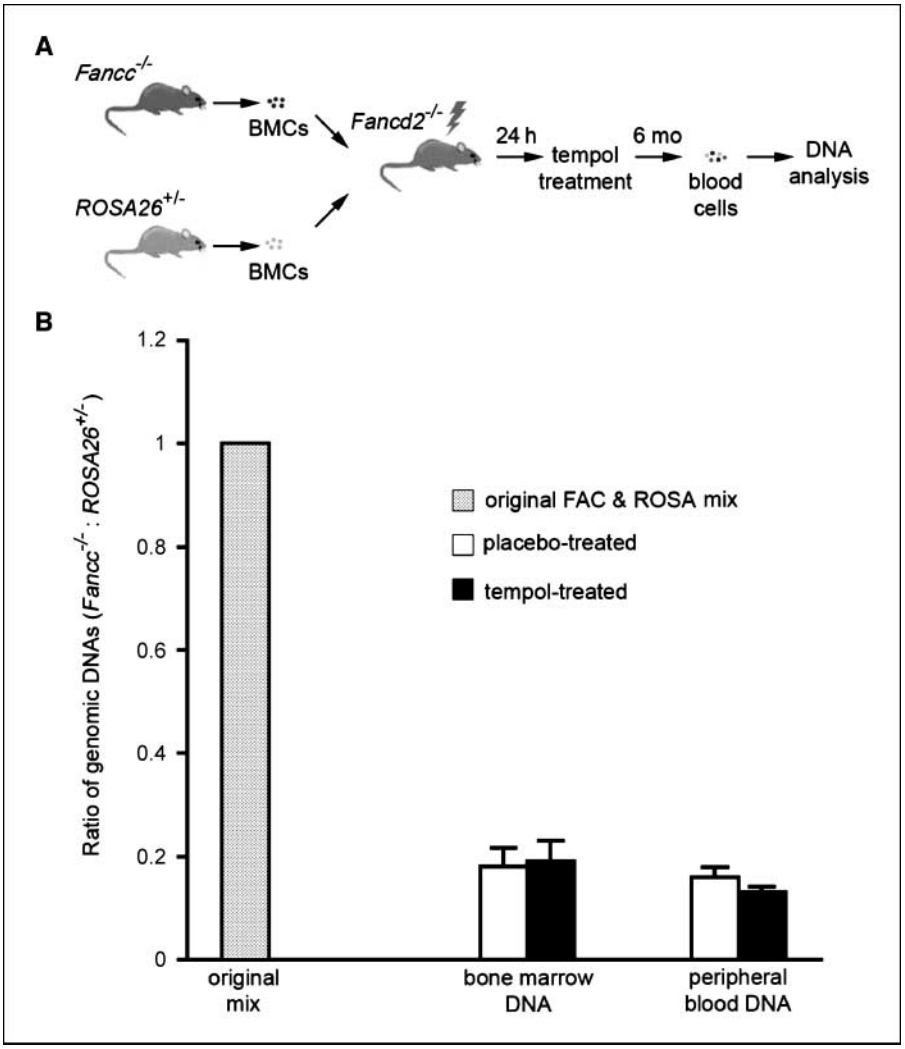

Figure 4. *In vivo* competitive repopulation of mixed *Fancc*$^{-/-}$ and *ROSA26*$^{+/-}$ bone marrow cells. *A,* strategy used in the competitive repopulation experiment. *B,* tempol treatment did not have any negative effect on the repopulation capability of FA hematopoietic stem cells. Three independent quantitative real-time PCR analyses were performed for each sample, and results from multiple animals were pooled together for each experimental group ($n = 7$ for bone marrow from placebo-treated mice, $n = 6$ for bone marrow from tempol-treated mice, $n = 12$ for peripheral blood from placebo-treated mice, and $n = 10$ for peripheral blood from tempol-treated mice). *Columns,* mean; *bars,* SE. $P > 0.05$ (Student's *t* test) for DNA from both bone marrow and peripheral blood.

pO_2 levels (20% O_2) in routine cell culture than living tissues (3–15%; ref. 35). Furthermore, consistent with the observation in FA mice, tempol treatment decreased 8-OHdG content in DNA in all the fibroblast cells tested, confirming the ability of tempol to reduce oxidative DNA damage in FA cells.

As a commonly used oxidative DNA damage marker, 8-OHdG is one of the major oxidatively modified DNA base products *in vivo* and is also the most mutagenic, resulting in GC-to-TA transversion unless repaired before DNA replication. A substantial body of evidence indicates that 8-OHdG is possibly an important factor in carcinogenesis (36, 37). Accumulation of 8-OHdG and other oxidative DNA damage could be more deleterious for FA than normal cells, given that DNA repair system in FA is already compromised. By reducing oxidative DNA damage, tempol might generate a beneficial effect on the defense against tumorigenesis in FA.

In summary, our data showed that the SOD mimetic tempol can delay the onset of epithelial tumors and extend tumor-free survival in FA with no adverse effect on hematopoietic system. Human FA patients are susceptible to many types of solid tumors, and most of them are epithelial in origin, including head and neck and

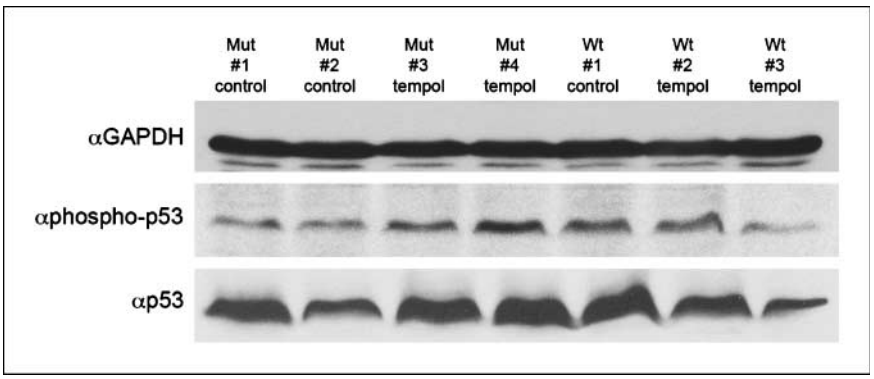

Figure 5. Western blot analysis of thymic proteins. Protein concentration was determined by Bio-Rad protein assay, and 100 μg of total proteins was loaded into each lane. Equal loading in each lane was confirmed by blotting with anti-GAPDH antibody.

gynecologic squamous cell carcinomas, esophageal carcinomas, and liver tumors (2). Tempol might be a good candidate to be tested in clinical trials for the prevention of solid tumors in human FA patients. It has been reported before that over 18% of primary ovarian epithelial cancers have a disrupted FA/BRCA pathway (38). Recent work also showed tissue-specific *FANCD2* gene suppression due to inherited mutations may be a cause of familial ovarian cancer (39). It remains to be determined whether tempol is beneficial for human patients who are at risk for ovarian cancers, such as members of ovarian cancer families.

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