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Fanconi anemia group A and C double-mutant mice: Functional evidence for a multi-protein Fanconi anemia complex

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Objective. **Fanconi anemia (FA) is a genetically heterogeneous disorder associated with defects in at least eight genes. The biochemical function(s) of the FA proteins are unknown, but together they define the FA pathway, which is involved in cellular responses to DNA damage and in other cellular processes. It is currently unknown whether all FA proteins are involved in controlling a single function or whether some of the FA proteins have additional roles. The aim of this study was 1) to determine whether the FA group A and group C genes have identical or partially distinct functions, and 2) to have a better model for human FA.**

Materials and Methods. **We generated mice with a targeted mutation in *fanca* and crossed them with *fancc* disrupted animals. Several phenotypes including sensitivity to DNA cross linkers and ionizing radiation, hematopoietic colony growth, and germ cell loss were analyzed in *fanca*−/−, *fancc*−/−, *fanca/fancc* double −/−, and controls.**

Results. **Fibroblast cells and hematopoietic precursors from *fanca/fancc* double-mutant mice were not more sensitive to MMC than those of either single mutant. *fanca/fancc* double mutants had no evidence for an additive phenotype at the cellular or organismal level.**

Conclusions. **These results support a model where both FANCA and FANCC are part of a multi-protein nuclear FA complex with identical function in cellular responses to DNA damage and germ cell survival.**

Fanconi anemia (FA) is a human autosomal-recessive disorder characterized by diverse clinical symptoms, including aplastic anemia due to progressive bone marrow failure, developmental abnormalities, and predisposition to cancer [1,2]. FA is also a genomic instability syndrome. FA cells exhibit spontaneous chromosome breakage [3] and are uniquely hypersensitive to DNA cross-linking agents such as mitomycin C (MMC) and diepoxybutane (DEB) [4–7].

FA is genetically heterogeneous. At least eight complementation groups (FANCA, B, C, D1, D2, E, F, and G) have been identified by cell fusion techniques [8–11]. Clinically, patients from different complementation groups are indistinguishable and therefore groups can only be assigned by molecular techniques. This finding suggests the hypothesis that the FA genes define a multi-component pathway involved in a single cellular response to DNA cross links [12]. Six FA genes (*FANCA*, *FANCC*, *FANCE*, *FANCF*, *FANCG*, and *FANCD2* have been cloned [10,13–17] but the precise biochemical role(s) of the FA pathway remains unknown (reviewed in [18,19]). The recently cloned FANCD2 is mono-ubiquitinated in response to DNA damage and then targeted to foci containing the BRCA1 protein [10,20]. We have shown that this mono-ubquitination of FANCD2 is defective in FA complementation groups A, B, C, E, F, and G, thus preventing the assembly of FANCD2/BRCA1 foci [20]. Studies on the interactions of FA proteins indicate that FANCA, FANCC, FANCE, FANCF, and FANCG form a multi-protein nuclear complex [21–24]. This complex acts upstream of FANCD2 and regulates its posttranslational modification in response to DNA damage consistent with a DNA damage sensing function.

Other work, however, has pointed toward other functions of the FA proteins, particularly FANCC. FANCC has been

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found to be located cytoplasmically as well as in the nucleus [25–28]. In addition, yeast-2-hybrid studies have identified several interacting proteins, which are cytoplasmic [29,30] and play a role in oxygen metabolism [31–34]. This has resulted in the hypothesis that at least FANCC and possibly all FA proteins are involved in the prevention of oxidative damage and damage-induced apoptosis in some cell types.

Several mouse models of FA have been generated to model human FA and for genetic studies. Two different knockouts of *fancc* have been generated [35,36] and recently mice defective in *fanca* [37] and *fancg* [38,39] have been reported. The phenotype of these mice is virtually identical and consists of hypoplasia of the gonads and cellular sensitivity to DNA cross linkers. Unlike in human FA, clinical anemia or tumor development has not been reported in any of these FA mice. This raised the possibility that there could be some functional redundancy of the FANCA, FANCC, and FANCG proteins and that a double knockout may be a better model for human FA. Therefore, in order to generate *fanca/fancc* double mutants, we generated a novel strain of mice with a targeted deletion of exon 37 of the *fanca* gene and crossed it with our previously reported fancc$^{\Delta exon8}$ mice. Here we report phenotypic and functional studies in this strain of mice.

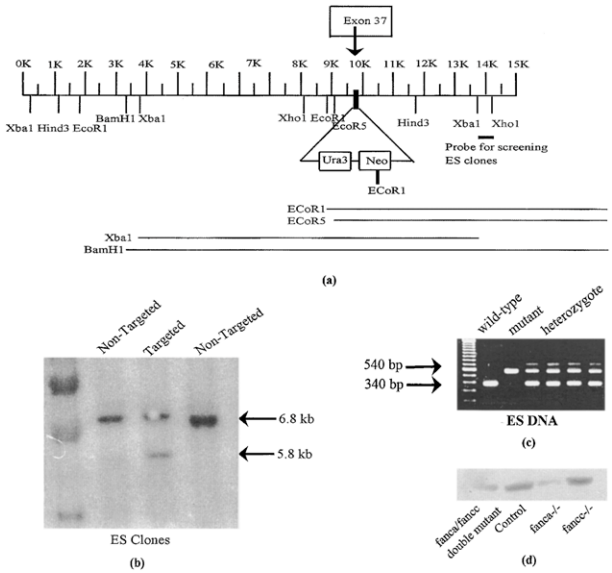

Figure 1. Targeted disruption of the murine *fanca* gene. **(a)** *fanca* targeting construct showing the deletion of exon 37. **(b)** Southern blot showing the targeted ES cell clones. Genomic DNA from the clones resistant to G418 selection were digested with *EcoRI* and analyzed with *Xba* I—*Xho* I probe. **(c)** PCR genotyping of *fanca* gene. **(d)** Western blot from the testis of *fanca*−/−, *fancc*−/−, *fanca*−/−/*c*−/−, and control mice showing the expression of fanca protein.

Materials and methods

Construction of the fanca gene-targeting vector and generation of fanca −/− mice

A Sv129 cDNA lambda library was screened with IMAGE clone 473236 (Genbank # AA038257). A 3′ phage clone (9B4) was characterized by restriction mapping and hybridization with exon-specific oligonucleotide probes. To generate a targeting vector, fanca exon 37 was removed by site-specific recombination in yeast as previously reported [40]. The following primer pairs were used to generate short homologous sequences flanking exon 37: GAAGATCTC CCAGTTACAGGCCC/CCATCGATCTGACACACAGA CTGTTGC and CCGCTCGAGGTGAGACAGTCTCCAG GG/CCCAAGCTTCTCATTGGAGAAGG. These sequences were cloned into plasmid pRay1 [40] to generate pRay1-37. The insert of lambda phage 9B4 was cloned into the *Not*I site in the yeast shuttle vector YCplac22. YCplac22-9B4 was then transformed into the recombination proficient yeast strain W303 [40]. pRay1-37 was transformed into the YCplac22-9B4 containing W303. Selection was carried out with TRP and URA to select for clones in which pRay1-37 had recombined with Ycplac22-9B4. An appropriate recombined plasmid was electroporated into 129/SvJ-derived embryonic stem cells (ES) and DNA from G418-resistant clones was analyzed with both the 5′ or 3′ probes on Southern blots (Fig. 1a and b). Subsequent hybridizations of the same blots with internal probes from the *Neo* cassette confirmed the homologous recombination in both clones and germ-line transmission was achieved with one of these clones.

Mouse strains and animal husbandry

Heterozygous F_1 mice were intercrossed to produce homozygous mutant mice in the syngeneic $129S_4$ background. *fanca*−/− male mice were also back-crossed with wild-type C57/BL females for five generations. *fanca*−/+ animals from the fifth generation were interbred to obtain −/− mice in a syngeneic C57/BL background. Both $129S_4$ and C57/BL colonies were then expanded for the experiments described.

fanca−/+ mice were crossed with our existing *fancc*−/+ mice [35] to produce double-mutant mice.

PCR genotyping

The *fanca* genotype was determined by PCR analysis on mouse tail DNA with a three-primer PCR—a common forward primer, TTCCTTCAAAGCTGCTGGGG; a reverse primer in the nontargeted exon 37, CAGTGACATCTTC CTTCCTAACTCC, to give a wild-type allele of 340 bp; and a reverse primer, GGTGAACGTTACAGAAAAG CAGGCT, in the recombinant neo allele to give a 540 bp mutant allele (Fig. 1c). The amplification conditions were 94°C × 3 minutes, followed by 37 cycles of (94°C × 30 seconds, 60°C × 30 seconds, and 72°C for 1 minute) and 72°C for 10 minutes using Boehringer Mannheim Taq polymerase and buffer. *fancc* genotyping was performed as explained previously [35].

fanca protein expression

fanca protein expression was analyzed both in testicular homogenate and splenocytes by Western blot using antiserum directed against the GST-FANCA fusion protein (amino acids 1–454) (kindly provided by Hans Joenje, Free University Medical Center, Amsterdam) [41].

Hematological assays

Peripheral blood was collected by retroorbital puncture using heparinized microcapillary tubes and transferred to EDTA-containing tubes. Measurements of blood cell counts, hemoglobin, hematocrit levels, MCV, PCV were performed on an ABC Vet automated analyzer (ANTECH Diagnostics, Portland, OR, USA).

Cell cultures, retroviral complementation, and chromosomal breakage

Ear fibroblast cultures were established [42] and were immortalized as previously reported [11]. Retroviral complementation of mutant fibroblasts was carried out using previously described vectors and procedures [43]. Briefly, fibroblast cell lines from *fanca*−/−, *fanca*−/−/*fancc*−/−, *fancc*−/−, and control were each resuspended in FANCA and FANCC viral supernatants and selected in puromycin (2 μg/mL) for one week. Chromosome breakage analysis was done as previously described [44]. The slides were stained with Wright's stain and chromosomes scored for breaks and radials per cell.

Growth inhibition assays in response to MMC or γ irradiation

Retrovirally transduced mouse ear fibroblast cell lines and controls from all four genotypes (described above) were seeded in triplicate in 96-well microtiter plates at a density of 500 cells/well in DMEM supplemented with 10% fetal bovine serum (FBS), l-glutamine, and penicillin/streptomycin. MMC was added at a concentration of 0–640 ng/mL or the cells were exposed to 0–11 Gy radiation. The cells were then incubated for 5 days at 37°C in a 5% CO_2 incubator. After five days, nucleic acid quantification was performed with a proprietary dye (CyQUANT; Molecular Probes, Eugene, OR, USA) and subsequently analyzed by an ELISA plate reader according to the manufacturer's directions (BMG Lab Technologies GmbH, Durham, NC, USA).

MMC-induced colony formation assay

Both the retrovirally corrected and noncorrected transformed mouse ear fibroblasts from all the four genotypes were seeded in triplicate in 100-mm dishes at a density of 500 cells/well in DMEM supplemented with 10% FBS, l-glutamine, and penicillin/streptomycin. MMC was added at a concentration of 0–640 ng/mL. The cells were then incubated for 5 days at 37°C in a 5% CO_2 incubator. After 5 days, the plates were washed with 1× phosphate-buffered saline (PBS), stained with methylene blue, and the colonies were counted.

Hematopoietic colony growth assays

Four- to six-week-old sex-matched *fanca*−/−, *fanca*−/−/*fancc*−/−, *fancc*−/−, and control mice (n = 6) were analyzed in a blinded fashion. Genotype codes were not broken until after progenitor growth studies had been completed. Femoral marrow samples were obtained from the mice after cervical dislocation and total viable cell counts were performed. Unfractionated bone marrow (BM) cells (1×10^5) were cultured under standard conditions [35]. Burst forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte macrophage (CFU-GM) were counted after 7 and 14 days of culture at 37°C at 5% CO_2, using an inverted microscope. In each experiment, mitotic inhibitory factors were added to methylcellulose cultures containing growth factors. Multiple doses of recombinant murine interferon-γ (IFN-γ) (0.05, 0.1, 0.5, 1.0, 5.0, and 10 ng/mL) (Genzyme, Cambridge, MA, USA), tumor necrosis factor-α (TNF-α) (0.05, 0.5, and 5.0 ng/mL; R & D Systems), and tumor growth factor-β (TGF-β) (0.05, 5.0, and 50 ng/mL; Sigma) were tested.

BFU-E and CFU-GM formation was also tested in response to different doses of mitomycin C (0–30 nM).

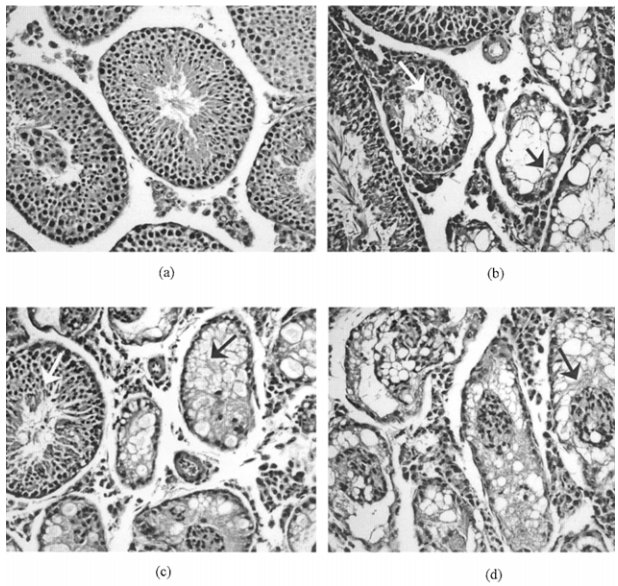

Figure 2. Gonadal abnormalities of *fanca*−/−, *fancc*−/−, *fanca*−/−/*c*−/− $129S_4$ mice. Histological sections (200× magnificatioin) of testis from 4-month-old mice. **(a)** Control testis. The control testis show all stages of normal spermatogenesis. **(b)** *fanca*−/− testis. **(c)** *fanca*−/−/*c*−/− double-mutant testis. **(d)** *fancc*−/− testis. In all the three genotypes, a mosaic pattern of seminiferous tubules devoid of germ cells (black arrow) and normal tubules (white arrow) can be seen. There was a severe degeneration of germinal epithelium and an increase in Leydig cells in *fanca*−/−, *fancc*−/−, *fanca*−/−/*c*−/−, showing that the double mutants do not have a more severe defect than either single mutant alone.

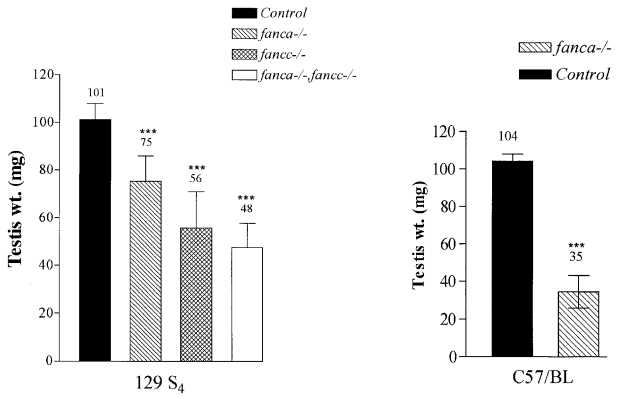

Figure 3. Testis weights of *fanca*−/− and control mice both in **(a)** 129 S_4 background and **(b)** C57/BL. *fanca*−/− testis were smaller in C57/BL in comparison to in 129 S_4 background. There was no significant difference between the *fancc*−/− and double-mutant testis. Values are mean ± SD of 10 independent samples (***$p < 0.0001$ for testicular weights of *fanca*−/−, *fancc*−/−, *fanca/fancc* double mutants in comparison to controls, as analyzed by one-way ANOVA).

Table 1. Evaluation of the cross-linker phenotype of primary ear fibroblasts derived from all 4 genotypes

Genotypes	Clastogen (ng/mL)	Percent cells with no breaks	Percent cells with radials
Control	None	54	4
	MMC (8 ng/mL)	40	4
	MMC (20 ng/mL)	44	26
	DEB (100 ng/mL)	50	2
fanca−/−	None	46	4
	MMC (8 ng/mL)	15	48
	MMC (20 ng/mL)	4	70
	DEB (100 ng/mL)	12	58
fancc−/−	None	62	0
	MMC (8 ng/mL)	22	42
	MMC (20 ng/mL)	12	72
	DEB (100 ng/mL)	16	62
fanca−/−/c−/−	None	59	4
	MMC (8 ng/mL)	6	34
	MMC (20 ng/mL)	8	68
	DEB (100 ng/mL)	0	48

Results

Generation of mice with the fanca$^{\Delta 37}$mutation and fanca −/− fancc −/− double-mutant mice

We generated fanca/fancc double-knockout mice for two reasons. First, we sought to determine whether FANCA and FANCC have identical or partially distinct functions. Second, we hoped that double mutants may be a better model for human FA, if double knockout showed an additive phenotype. We first created a fanca−/− mouse strain by deleting exon 37 (Fig. 1a), since it has been shown to contain disruptive mutations in human FA patients. Interbreeding of heterozygous mice produced approximately 25% homozygous mutant pups, which indicated that there was no embryonic lethality associated with the deletion of exon 37. fanca−/− mice appeared morphologically normal and weighed the same as littermate controls without obvious congenital malformations or growth retardation (data not shown).

fanca−/+ mice were then crossed with *fancc*−/+ mice to obtain *fanca/fancc* double-mutant mice. Double heterozygous breeders produced the expected number of double-mutant pups (70/1120 ≈ 1/16). These weighed the same as littermate controls and had no macroscopic developmental abnormalities (data not shown).

Deletion of exon 37 was selected based on the human gene structure, in which it contains 139 bp, a number indivisible by 3. In contrast, the recent submission of the *Mus musculus fanca* gene (AF247181) shows 135 bp in exon 37, and therefore removal of exon 37 created an in-frame deletion of the protein, rather than a truncation. Western blotting carried on the testis of *fanca*−/−, *fancc*−/−, *fanca/fancc double* −/−, and control mice (Fig. 1D) showed that fanca protein was detectable in all strains, but at a lower level in the *fanca*−/− and *fanca/fancc double* −/− mice in comparison to the age-matched *fancc*−/− and control littermates.

Although the *fanca*$^{\Delta exon37}$ mice generated by us are not null mutants at the protein level, all other functional studies (see below) indicated that the internally deleted protein was nonfunctional.

Gonadal abnormalities of both fanca −/− and fanca fancc double −/− mice

fancc mutant mice have previously been reported to have reduced numbers of both male and female germ cells. The germ cell defect is more severe in females and is also influenced by the strain background [35]. Both *fanca*−/− and *fanca*−/−/*fancc*−/− mice also had striking abnormalities in the gonads. There was a severe degeneration of the germinal epithelium ranging from 40 to 90% (Fig. 2), and the testis from C57/BL strain showed fewer spermatocytes. The partial defect typical for FA mice with a mosaic pattern of seminiferous tubules with normal sperm production mixed with empty, Sertoli cell only tubules was observed [35–37]. As shown in Figure 3, the testicular weights of *fanca*−/− (34 mg ± 3) were significantly reduced in comparison to the control (104 mg ± 2) mice in C57/BL background. Interestingly, in the 129S_4 background, the *fanca*−/− testis were also smaller (75 mg ± 10) than control littermate animals (101 mg ± 7) but larger than in the C57/BL strain. The double-mutant testis (47 mg ± 10) was the smallest in comparison to the control mice but there was no significant difference between the *fancc*−/− (56 mg ± 4) and the double-mutant testis.

In the ovaries, there was marked variability in the expression of the phenotype in both *fanca* (16 animals analyzed) and *fanca/fancc* double mutants (14 animals analyzed) on the 129S_4 background. In some animals no germ

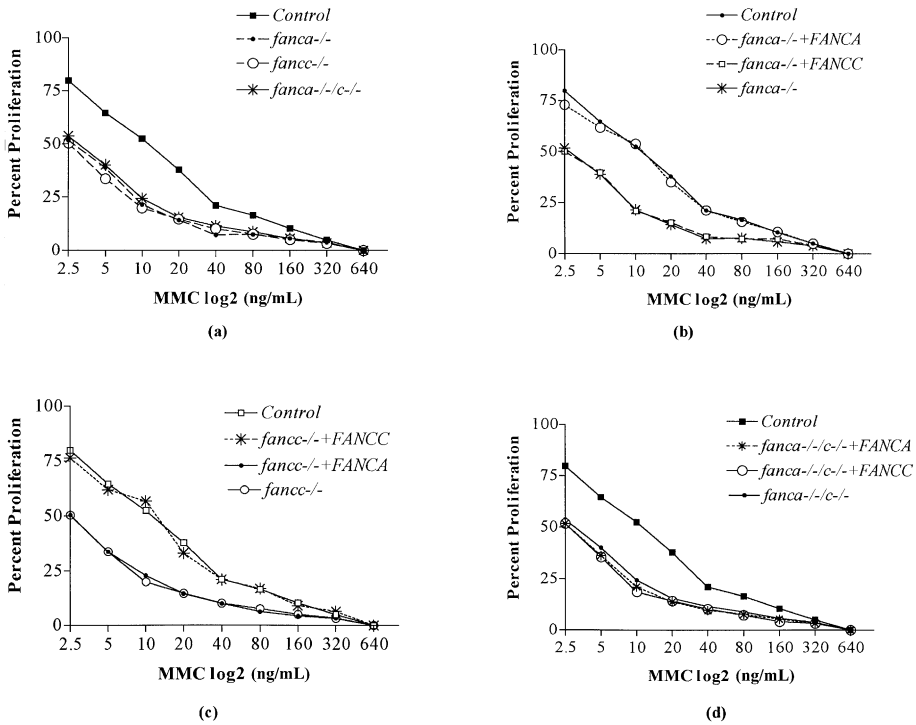

Figure 4. Murine *fanca*−/− and *fanca*−/−/*fancc*−/− cells show the classic Fanconi anemia DNA cross-linker sensitive phenotype. MMC growth inhibition assay was performed on both the corrected and noncorrected immortalized mouse ear fibroblasts derived from *fanca*−/−, *fancc*−/−, *fanca/fancc* double mutants, and control mice. The values are mean ± SD of six independent cultures (***$p < 0.0001$ for growth inhibition of *fanca*−/−, *fancc*−/−, and *fanca/fancc* double mutants in response to MMC, as analyzed by two-way ANOVA). The double mutants were not more sensitive to MMC in comparison to either mutant alone.

cells were found at all or if they were present, the gonad was primitive and undifferentiated. This pattern was consistent with a reduced number or absence of germ cells and identical to that previously reported in *fancc* mutants [35,36]. However, a fraction of mutant animals (both *fanca* and double mutants) displayed no histologically detectable ovarian abnormalities. The reason for the observed variability is unclear, but it could be due to environmental or stochastic factors. In contrast, *fancc*−/− ovaries were more consistently (15/15) underdeveloped, with no germ cells evident in comparison to their littermates. In these animals, almost complete replacement of the ovary by nests of thecal cells was observed. The important finding for the current study was, however, that 6/14 *fanca*−/−/*fancc*−/− mice studied had apparent normal ovarian histology despite being double mutant. Clearly, the ovarian defect was not more severe in double mutants than in either mutant alone.

Cross-linker sensitivity in fibroblasts

Primary ear fibroblasts were established from 4- to 6-week-old mice. After treatment with MMC and DEB, the mutant and double-mutant cells revealed chromosome breakage analogous to human FA patients (Table 1). This result provided evidence that the function of the fanca protein was significantly impaired even by the in-frame deletion of the protein and that the murine *fanca* gene has a similar function to the human gene.

The primary ear fibroblasts were then immortalized using SV40 plasmid [45] and each cell line was transduced with FANCA and FANCC retroviruses (kindly provided by Alan D'Andrea, Dana-Farber Cancer Center, Boston, MA, USA). This was followed by analysis for chromosomal breakage in response to MMC. As anticipated, the *fanca* and *fancc* cell lines were only corrected by the *FANCA* and *FANCC* cDNAs respectively. Interestingly, the double-mutant cell lines were not even partially corrected by either of the cDNAs alone (Table 2). The lack of partial correction of the inter-strand cross-links (ICL) sensitivity of double-mutant cells indicates that *fanca* and *fancc* function in the exact same process, responsible for maintaining genome stability.

To further corroborate the above findings and to obtain quantitative information, an MMC growth inhibition assay was performed on immortalized and retrovirally transduced mouse ear fibroblasts derived from *fanca*−/−, *fanca/fancc* double −/−, *fancc* −/−, and controls. As expected, both the single-mutant and double-mutant cells were more sensitive to MMC than the control cells. The appropriate retroviral vector was able to fully correct the MMC sensitivity of both

Table 2. Correction of cross-linker sensitivity of the mutant and double-mutant fibroblasts by corresponding human cDNA

Genotype	MMC (ng/mL)	Percent cells with 0 breaks	Percent cells with radials
Control	0	72	3
	15	58	6
fanca−/−	0	78	0
	15	8	76
fanca−/−+FANCA	0	38	0
	15	40	10
fanca−/−+FANCC	0	80	2
	15	14	80
fancc−/−	0	82	0
	15	18	66
fancc−/−+FANCC	0	63	0
	15	38	16
fancc−/−+FANCA	0	56	4
	15	18	66
fanca−/−c−/−	0	38	2
	15	8	78
fanca−/−/c−/−	0	56	2
+FANCA	15	6	68
fanca−/−/c−/−	0	44	2
+FANCC	15	8	60

fanca and *fancc* mutant cells. Whereas the IC_{50} for the control cells and the retrovirally corrected cells was ∼15 ng/mL, the IC_{50} of mutant cells and cells transduced with the nonmatching retrovirus was ∼2.5 ng/mL (Fig. 4). Importantly, the IC_{50} of the double-mutant cells did not differ from the single mutants. Furthermore, neither the *FANCA* nor the *FANCC* retrovirus had any effect on the MMC sensitivity of the double-mutant cells.

These data were further verified by clonogenic assays in response to MMC using all of the cell lines described above. These data also clearly showed that the mutant cell lines were significantly sensitive to MMC in comparison to the heterozygous cell lines (Fig. 5). Mutant cell lines corrected with the retrovirus showed the restoration of the normal behavior towards the clastogen. In Figure 5, it can be seen that the IC_{50} for the control and corrected cell lines in this assay was ∼12 ng/mL, and for the mutant, double-mutant, and noncorrected cell lines the IC_{50} was ∼4 ng/mL.

γ-irradiation sensitivity of fibroblasts

The hypersensitivity of cells from FA patients to DNA cross-linking agents is well established [46], but the hypersensitivity to ionizing radiation has been controversial [3,47–49]. Nonetheless, the lower tolerance of human FA patients to IR has been known and applied to preconditioning for bone marrow transplantation [50]. Our previous studies [51] confirm that there is a differential sensitivity toward IR in *fancc*−/− in vivo. Accordingly, we did a dose response curve to IR on the mutant and control cell lines. The mutant cell lines were modestly more sensitive to IR (IC_{50} ∼2.5 Gy) in comparison to the control cell lines (IC_{50}

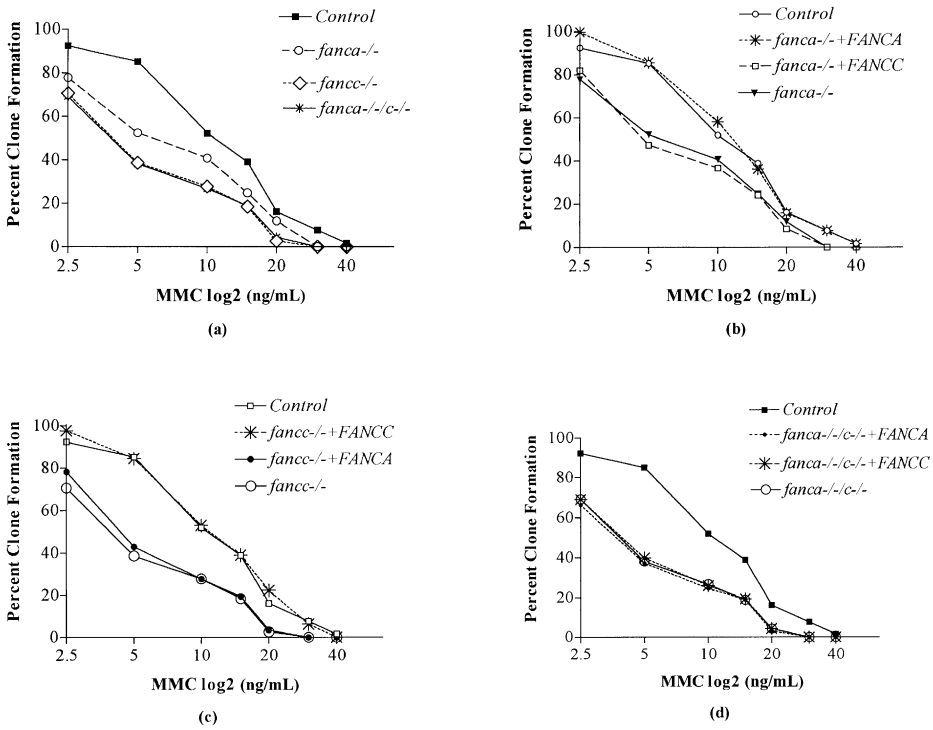

Figure 5. Clonogenic assay from mouse ear fibroblasts in response to MMC. The assay was performed on both the corrected and noncorrected immortalized mouse ear fibroblasts derived from *fanca*−/−, *fancc*−/−, *fanca/fancc* double mutants, and control mice. The values are mean ± SD of six independent cultures (***$p < 0.0001$ for growth inhibition of *fanca*−/−, *fancc*−/−, and *fanca/fancc* double mutants in response to MMC, as analyzed by two-way ANOVA). The double mutants were not more sensitive to MMC in comparison to either mutant alone.

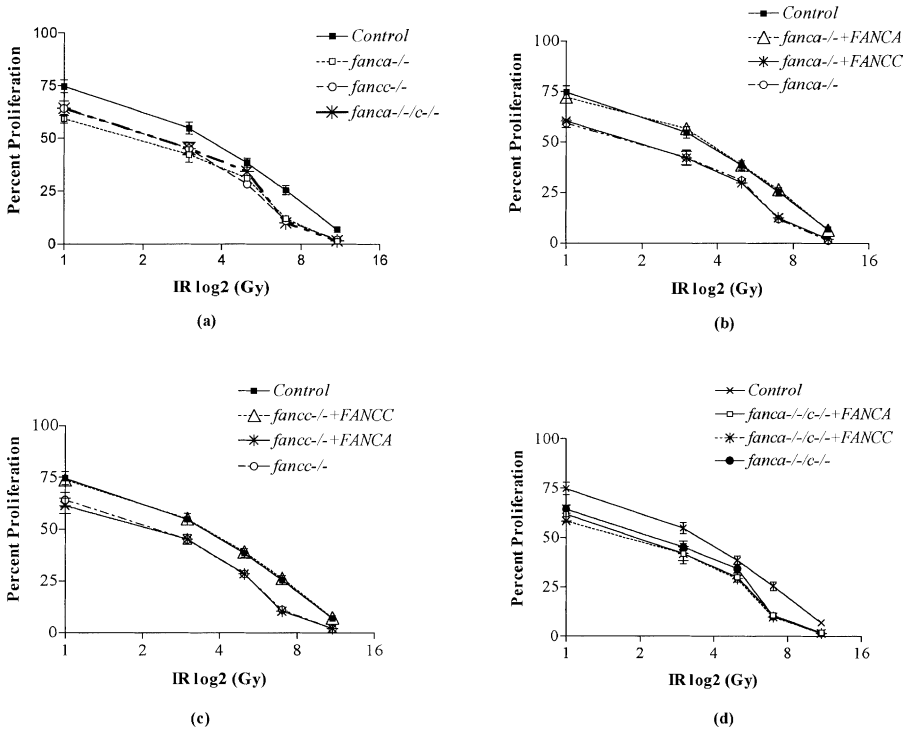

Figure 6. γ-irradiation inhibition assay. γ-irradiation growth inhibition assay was performed on both the corrected and noncorrected immortalized mouse ear fibroblasts derived from *fanca−/−*, *fancc−/−*, *fanca/fancc double mutants, and control mice. The values are mean* ± SD of six independent cultures (***$p < 0.0001$ for growth inhibition of *fanca−/−*, *fancc−/−*, and *fanca/fancc* double mutants in response to MMC, as analyzed by two-way ANOVA). The double mutants did not display more sensitivity to IR than either mutant alone.

∼3.8 Gy) (Fig. 6). Double-mutant cells displayed no greater sensitivity than either mutant alone.

Hematological abnormalities in fanca−/− and fanca/fancc double −/− mice

Periodically, complete blood counts and differential blood counts were performed on *fanca−/−*, *fanca/fancc* double *−/−*, and control animals. None of these mice in either genetic background showed signs of anemia or significant differences in the parameters analyzed up to one year (data not shown). In human FA patients, the anemia has an average age of onset of about 5 years [52,53]. However, abnormalities in cultured BM cells often precede the onset of clinical anemia [54]; consequently, we investigated cultured marrow from our knockout and double-knockout mice.

BM cells from $129S_4$ mice (4- to 6-week-old and also 6- to 8-month-old) were procured and cultured under conditions that establish erythroid and myeloid colonies. To ensure the reproducibility of the observations, congenic mice matched in sex and age were used and each experiment was repeated three times independently. In untreated cultures (no mitotic inhibitors added), the erythroid and myeloid colony growth of 4- to 6-week-old mice were similar (Fig. 7a and b). However, in 6- to 8-month-old mutant mice, the colony growth was somewhat reduced compared to controls (Fig. 7a and b). This decline of colony formation with age has been previously also reported for *fancc−/−* mice [35]. Importantly, the reduction in colony formation was similar in the double mutants as compared to either *fanca* or *fancc* single mutants. This is consistent with the hypothesis that the hematological defect in double-mutant mice is as mild as in single mutants. Hematopoietic colony assays were also performed in response to increasing doses of MMC. As expected, the mutant clonal progenitors were hypersensitive to MMC (Fig. 7c and d). However, similar to fibroblast cells, hematopoietic precursors from *fanca/fancc* double-mutant mice were not more sensitive to MMC than those of either single mutant.

Dose response curves to IFN-γ, TGF-β, and TNF-α were also generated. No differential hypersensitivity of any mutant hematopoietic colony-forming cells to either TGF-β or TNF-α was detected (data not shown), which is in accordance with our previously reported results [35]. However, our present results also did not show a differential sensitivity of any mutant cells to even IFN-γ, which is in contradiction to our earlier findings [35]. It should be noted that the previous studies were performed on animals from a mixed-strain C57Bl/129 background. In the current study, we were using congenic $129S_4$ mice. As mentioned earlier, the genetic background has a role in the phenotype of the FA animals, and perhaps the sensitivity to the mitotic inhibitors is also dependent on the contribution of the genetic back-

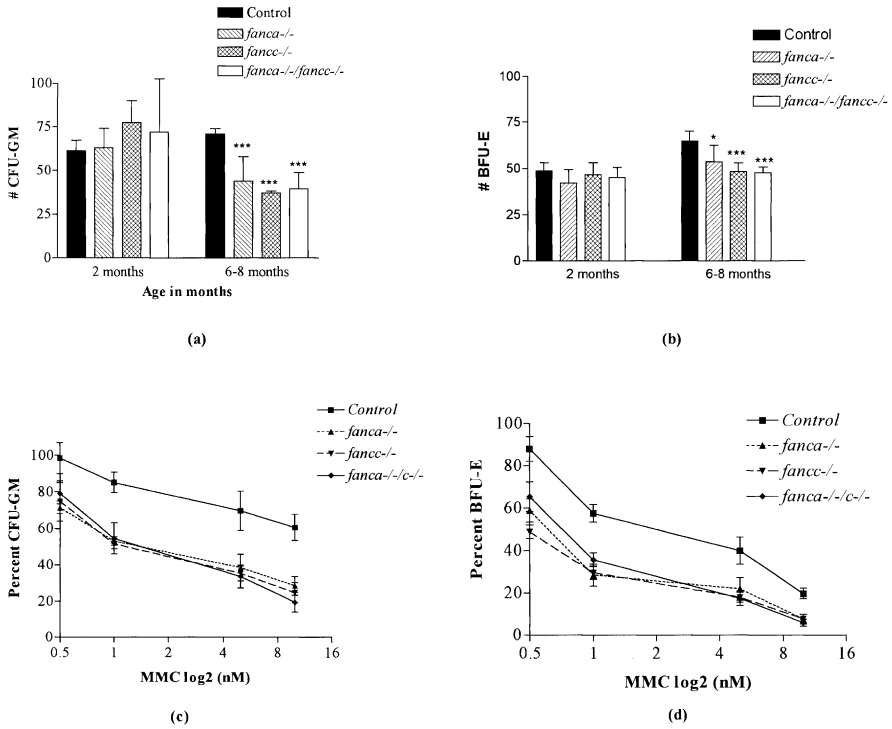

Figure 7. Hematopoietic colony growth assay. Comparison of **(a)** myeloid colony growth and **(b)** erythroid colony growth in response to the age of mice. In untreated cultures (no mitotic inhibitors added), the BFU-E and CFU-GM were similar in 4- to 6-week-old mice. However, in 6- to 8-month-old *fanca*−/−, *fancc*−/−, *fanca/fancc* double-mutant mice, the colony growth was reduced (***$p < 0.001$ as analyzed by one-way ANOVA) in comparison to the control mice. The reduction in colony formation was similar in the double mutants as compared to either *fanca* or *fancc* mutants alone. Each experiment (n = 3) was repeated independently in triplicate. **(c)** CFU-GM, and **(d)** BFU-E in response to different doses of MMC (0–30 nM). Both the BFU-E and CFU-GM (***$p <$ 0.0001 as analyzed by one-way ANOVA) from the mutant and double-mutant mice were hypersensitive to MMC. However, the hematopoietic precursors from *fanca/fancc* double-mutant mice were not more sensitive to MMC than either single mutant alone. The values are mean ± SD of 8 separate cultures.

ground. Importantly, double-mutant bone marrow cells of the 129 strain were also not sensitive to IFN-γ, once again confirming the lack of an additive phenotype

Discussion

The Fanconi anemia pathway is relatively new in evolution, and to date complete conservation of the 6 cloned FA genes can be found only in vertebrates. Therefore, the genetic analysis of the FA pathway has relied on the known mutant organisms, both human and mouse. The extensive overlap of the clinical and cellular phenotype of human FA patients has always suggested that all the FA genes/protein participate in the same biochemical function(s), essential to genomic integrity, but also in the function of hematopoietic and germ-line stem cells. Nonetheless, several reports have raised the question whether some of the FA proteins, in particular FANCC, may have additional roles. First, FANCC has been found to be cytoplasmic as well as nuclear [25–28], and one report indicated that forced nuclear localization of FANCC actually prevented its proper function in MMC resistance [55,26]. In contrast, the other FA proteins are predominantly located in the nucleus. Second, yeast 2-hybrid screens have shown FANCC to interact with a variety of cytoplasmic proteins, including NAD-cytochrome p450 reductase [56,57], glutathione transferase [31], and heat shock protein [30]. These observations have suggested a role of FANCC in oxidative damage responses in the cytoplasm. Third, abnormalities of stat1 signaling have been reported in FANCC mutant cells [58], but not FANCA or other FA complementation groups. Fourth, one study showed expression of FANCC and FANCA mRNA in different tissues and cell types in the developing mouse [59]. Together, these observations have raised the possibility that FANCC has additional functions beyond participation in the FA pathway. It is conceivable, for example, that multiple pathways for the repair of interstrand DNA cross links exist and that a defect in any of them would cause a similar phenotype. If FANCA and FANCC participated in distinct, parallel DNA damage response pathways, mutations in both genes may cause an additive phenotype.

Our present study used mouse genetics to address the question of whether FANCA and FANCC are epistatic or not. FA mutant mice have several well-described phenotypes [35–38], but their defect is less severe than in human patients. The phenotypes include: 1) severe cellular hypersensitivity to DNA cross-linking agents; 2) mild cellular hypersensitivity to ionizing irradiation; 3) markedly reduced numbers of germ cells, worse in females and worse in the C57/BL strain com-

pared to $129S_4$; and 4) reduced formation of hematopoietic progenitor colonies with age, and in response to mitotic inhibitors. We examined all of these phenotypes in *fanca* and *fancc* single as well as in *fanca/fancc* double-mutant mice and cells. In none of the assays double-mutant animals or cells displayed an additive phenotype, which went beyond the findings of the single mutants. In cellular assays where retroviral complementation was possible, addition of either FANCA or FANCC to the double-mutant cells resulted in no improvement of cellular function.

In summary, our data indicate that the murine *fanca* and *fancc* genes are epistatic in those functions that were analyzed in this paper, including cross-linker sensitivity, hematopoietic colony growth, and the germ cell deficiency. Disruption of either gene results in complete deficiency of the entire FA pathway and indistinguishable cellular and organismal phenotypes. It is, however, possible that the FANCA or FANCC proteins could have additional, yet-to-be-identified cellular roles that are unique. Should novel functions be uncovered, it again will be important to perform an epistasis analysis as described here.

The findings reported here are consistent with the model first put forward by A. D'Andrea and coworkers, in which most of the FA proteins interact in a multi-subunit FA nuclear complex with one function [60]. This view has most recently found strong support by the observation that FANC-A, B, C, E, F, and G cells all have a common defect in their inability to mono-ubiquitinate FANCD2 protein in response to DNA damage [20].

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