doi:10.1016/j.ymthe.2006.05.018

ARTICLE

Hematopoietic Dysfunction in a Mouse Model for Fanconi Anemia Group D1

Susana Navarro,[1] Nestor W. Meza,[1] Oscar Quintana-Bustamante,[1] José A. Casado,[1] Ariana Jacome,[1] Kimberly McAllister,[2] Silvia Puerto,[3] Jordi Surrallés,[3] José C. Segovia,[1] and Juan A. Bueren[1,*]

[1]*Hematopoiesis and Gene Therapy Division, CIEMAT/Marcelino Botín Foundation, Avenida Complutense, No. 22, 28040 Madrid, Spain*
[2]*National Institute of Environmental Health Sciences, National Institutes of Health, Bethesda, MD 20892, USA*
[3]*Department of Genetics, Universitat Autonoma, 08193 Barcelona, Spain*

**To whom correspondence and reprint requests should be addressed. Fax: +34 91 346 6484. E-mail: juan.bueren@ciemat.es.*

Available online 20 July 2006

We have investigated the hematopoietic phenotype of mice with a hypomorphic mutation in the *Brca2/Fancd1* gene (*Brca2*$^{\Delta 27/\Delta 27}$ mutation). In contrast to observations made in other Fanconi anemia (FA) mouse models, low numbers of hematopoietic colony-forming cells (CFCs) were noted in *Brca2*$^{\Delta 27/\Delta 27}$ mice, either young or adult. Additionally, a high incidence of spontaneous chromosomal instability was observed in *Brca2*$^{\Delta 27/\Delta 27}$ bone marrow (BM) cells, but not in *Brca2*$^{+/\Delta 27}$ or *Fanca*$^{-/-}$ BM cells. Although *Brca2*$^{\Delta 27/\Delta 27}$ CFCs were not hypersensitive to ionizing radiation, a very severe hematopoietic syndrome was observed in irradiated *Brca2*$^{\Delta 27/\Delta 27}$ mice. Conventional BM competition experiments showed a marked repopulation defect in *Brca2*$^{\Delta 27/\Delta 27}$ hematopoietic stem cells (HSCs), compared to wild-type HSCs. Moreover, we have observed for the first time in a DNA repair disease model a very significant proliferation defect in *Brca2*$^{\Delta 27/\Delta 27}$ HSCs maintained in their natural physiological environment. The progressive repopulation of wild-type HSCs transplanted into unconditioned *Brca2*$^{\Delta 27/\Delta 27}$ recipients is reminiscent of the somatic mosaicism phenomenon observed in a number of genetic diseases, including FA. The hematopoietic phenotype associated with the *Brca2*$^{\Delta 27/\Delta 27}$ mutation suggests that this FA-D1 mouse model will constitute an important tool for the development of new therapies for FA, including gene therapy.

Key Words: Fanconi anemia, Brca2, Fancd1, genetic instability, DNA repair, mitomycin C, hematopoietic stem cells, self-renewal, competitive repopulation ability, hematopoiesis

INTRODUCTION

Recent studies in mouse models of genetic diseases are clarifying the relevance of DNA repair proteins in hematopoiesis and, more specifically, in the function of hematopoietic stem cells (HSCs). In this respect, mice with mutations in proteins involved in double-strand break (DSB) repair, like *Rad50*$^{s/s}$ [1] and *Atm*$^{-/-}$ mice[2], developed a progressive hematopoietic failure resulting from a defect in the HSC function. Similarly, hematopoiesis of mice deficient in Ercc1, an endonuclease involved in both nucleotide excision repair (NER) and interstrand crosslink repair, was severely reduced in a manner consistent with that found in normal aging [3]. In contrast to the phenotype of these animals, mice deficient in mismatch repair (*MSH2*$^{-/-}$ mice) [4] or in NER (*Xpa*$^{-/-}$ mice) [3] had a milder hematopoietic phenotype. A number of laboratories, including our own, have also found a hematopoietic phenotype in mouse models of Fanconi anemia (FA) [5–7], although as in *MSH2*$^{-/-}$ or in *Xpa*$^{-/-}$ mice, basal hematopoietic defects in FA mice were not severe.

Fanconi anemia is a highly heterogeneous disease, for which 12 complementation groups have already been identified: FA-A/B/C/D1/D2/E/F/G/I/J/L/M [8,9]. With the exception of the FA-I group, genes corresponding to all these complementation groups have now been identified [9–11]. In dividing cells or in cells exposed to DNA aggression, eight FA proteins (FANCA/B/C/E/F/G/L/M) form a FA protein core. Both the FA protein core and probably the FANCI are necessary for the monoubiquitination of FANCD2 [8,12,13]. Ub-FANCD2 then interacts with other DNA repair proteins, including BRCA1 [12] and BRCA2 [14,15], also known as FANCD1 [16]. Although FANCD1/BRCA2 is pivotal in homologous recombination [17,18], the role that the other FA proteins

1525-0016/$30.00

doi:10.1016/j.ymthe.2006.05.018

have in the repair of DSBs remains under extensive investigation [19–22].

Significantly, FA patients with biallelic mutations in the *FANCD1/BRCA2* gene show a more severe phenotype and cancer proneness, compared to other FA patients [23]. This observation together with the high incidence of cancer in mice with hypomorphic mutations in *Brca2* [24] (the null expression of *Brca2* is embryonic lethal) [25] prompted us to investigate the relevance of *Brca2* mutations in mouse hematopoiesis and, more specifically, in the functionality of the self-renewing HSCs. Our data show that the homozygous deletion of exon 27 in the *Brca2* gene, the $Brca2^{\Delta 27/\Delta 27}$ mutation [24], confers a marked proliferation defect in the hematopoietic progenitors and in the self-renewing HSCs of the mouse. This proliferation defect facilitated the progressive repopulation of unconditioned $Brca2^{\Delta 27/\Delta 27}$ mice by transplanted wild-type (WT) bone marrow (BM) cells, mimicking the proliferation advantage of genetically corrected cells observed in patients suffering from inherited diseases like FA.

RESULTS

Deficient Numbers and Impaired Proliferative Potential of Myeloid Colony-Forming Cells from $Brca2^{\Delta 27/\Delta 27}$ Mice

We first conducted hematological and flow cytometry analyses of peripheral blood (PB) cells from adult $Brca2^{\Delta 27/\Delta 27}$, age-matched heterozygous and WT mice. We observed no significant differences in the numbers of PB cells between the three groups of mice (data not shown). Flow cytometry analyses of PB, BM, spleen, and thymus also showed no significant differences between $Brca2^{\Delta 27/\Delta 27}$ mice and heterozygous or WT mice (see Table 1).

Data in Fig. 1 show the cellularity and the number of colony-forming cells (CFCs) in the BM and spleen of $Brca2^{\Delta 27/\Delta 27}$ mice and heterozygous or WT mice. We observed slight decreases in the cellularity of femoral BM and spleen between adult $Brca2^{\Delta 27/\Delta 27}$ and age-matched heterozygous or WT mice (Fig. 1A), although this may simply reflect the lower weight of $Brca2^{\Delta 27/\Delta 27}$ mice (15% lower than that of WT mice). In contrast to mild differences in cellularity, we observed a very significant reduction in the proportion of CFCs in both the BM and the spleen of $Brca2^{\Delta 27/\Delta 27}$ mice, compared to the control group (on average, values were only 25–50% of control values; Fig. 1A). To investigate whether our observations could be related to premature aging of the animals, we also determined CFC values in 1.5-week-old mice. As shown in Fig. 1B, we obtained observations almost identical to those obtained in adult mice, suggesting a proliferation defect in $Brca2^{\Delta 27/\Delta 27}$ progenitor cells. This possibility was further investigated by comparing the sizes of colonies generated by the different mouse groups. While 45% of the colonies from WT and heterozygous mice were large colonies, only a minor proportion of the

TABLE 1: Comparative phenotypic analysis of peripheral blood, bone marrow, spleen, and thymus from WT, $Brca2^{+/\Delta 27}$ and $Brca2^{\Delta 27/\Delta 27}$ mice

Tissue	Phenotype	WT	$Brca2^{+/\Delta 27}$	$Brca2^{\Delta 27/\Delta 27}$
Peripheral blood	Gr1	29.4 ± 2.6	37.6 ± 4.0	20.1 ± 3.6
	Mac	35.1 ± 1.6	40.4 ± 6.8	24.7 ± 4.8
	B220	43.0 ± 7.4	48.4 ± 9.1	47.6 ± 8.4
	CD3	9.7 ± 5.4	10.3 ± 7.8	14.4 ± 6.7
	CD4	7.2 ± 4.1	7.9 ± 6.4	11.6 ± 7.6
	CD8	1.9 ± 0.5	1.5 ± 0.4	1.9 ± 0.1
Bone marrow	Gr1	40.0 ± 19.9	37.7 ± 18.5	36.6 ± 18.6
	Mac	48.2 ± 1.9	43.8 ± 2.3	49.5 ± 4.7
	B220	24.4 ± 1.8	22.9 ± 6.4	24.7 ± 7.0
	CD3	1.3 ± 0.5	1.7 ± 0.2	1.9 ± 0.3
	Ter119	5.6 ± 1.2	5.7 ± 1.7	5.9 ± 0.9
	Lin^-	9.2 ± 1.3	10.0 ± 1.3	7.1 ± 0.1
	$Lin^-c\text{-}Kit^+$	6.5 ± 0.7	6.4 ± 0.9	4.9 ± 0.4
	Lin^-Sca1^+	0.24 ± 0.16	0.11 ± 0.02	0.22 ± 0.07
	$Lin^-Sca1^+c\text{-}Kit^+$	0.15 ± 0.02	0.08 ± 0.02	0.14 ± 0.04
Spleen	Gr1	7.6 ± 0.7	6.9 ± 0.8	6.5 ± 0.7
	Mac	5.9 ± 1.1	5.6 ± 0.3	4.9 ± 0.5
	B220	43.5 ± 4.5	44.5 ± 1.5	45.2 ± 1.2
	CD3	32.2 ± 2.7	30.9 ± 2.1	31.2 ± 1.6
Thymus	CD3	42.9 ± 20.1	37.5 ± 21.5	39.1 ± 19.5
	$CD4^+CD8^-$	9.6 ± 0.2	7.2 ± 1.0	6.8 ± 1.4
	$CD8^+CD4^-$	5.5 ± 0.6	5.9 ± 1.1	4.5 ± 0.9
	$CD4^+CD8^+$	86.1 ± 1.6	82.9 ± 1.0	93.0 ± 0.2
	$CD4^-CD8^-$	4.2 ± 0.8	8.4 ± 1.5	5.3 ± 2.3

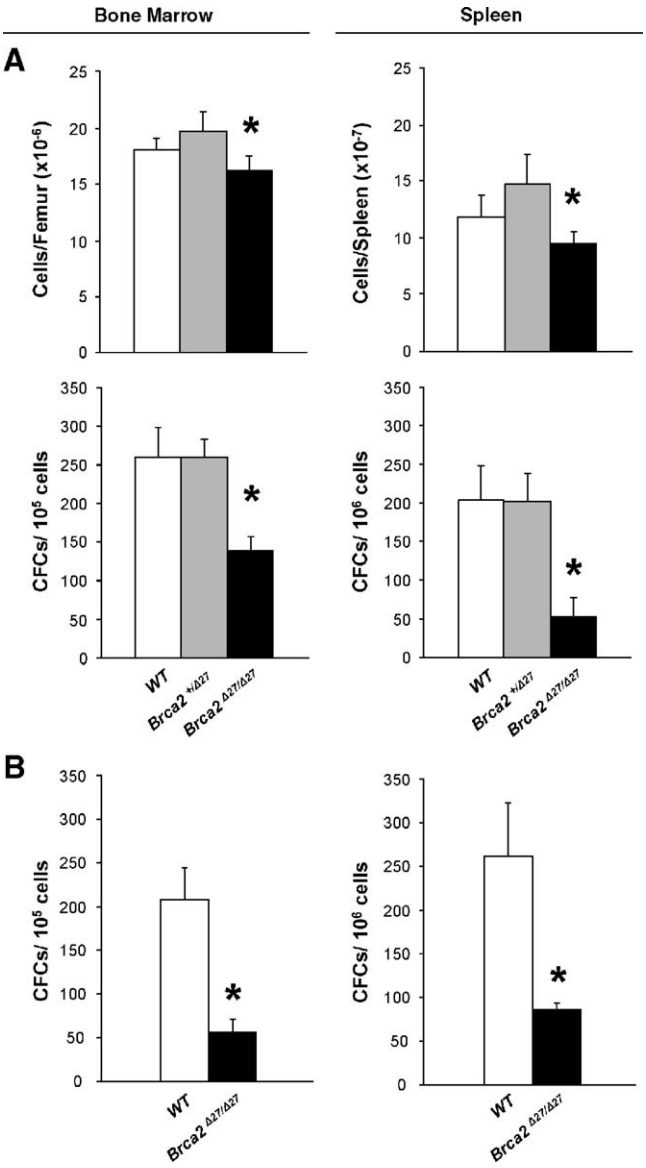

FIG. 1. Analysis of the cellularity and hematopoietic colony-forming cells in the bone marrow and spleen of *Brca2*$^{\Delta 27/\Delta 27}$ mice compared to age-matched *Brca2*$^{+/\Delta 27}$ and WT mice. Data represent the mean ± SEM values corresponding to three to five mice per group. (A) Data corresponding to 8- to 12-week-old mice are shown. (B) Data corresponding to 1.5-week-old mice. *Statistical significance between the *Brca2*$^{\Delta 27/\Delta 27}$ and WT groups ($P < 0.05$).

colonies (12%) from *Brca2*$^{\Delta 27/\Delta 27}$ mice were large (Fig. 2A). In a subsequent set of experiments, we subjected BM cells from *Brca2*$^{\Delta 27/\Delta 27}$ and from WT mice to *in vitro* incubation in liquid cultures. As shown in Fig. 2B, we also observed evident differences in the expansion of BM cells from *Brca2*$^{\Delta 27/\Delta 27}$ and WT mice. Growth defects were even more marked when we analyzed the content of CFCs along the incubation period (Fig. 2B). All these data are consistent with a proliferative defect in BM progenitors as a result of the *Brca2*$^{\Delta 27/\Delta 27}$ mutation.

Spontaneous and Mitomycin C-Induced Chromosomal Aberrations of *Brca2*$^{\Delta 27/\Delta 27}$ Bone Marrow Cells

To analyze the response of *Brca2*$^{\Delta 27/\Delta 27}$ hematopoietic progenitors to DNA crosslinking agents, we performed CFC assays with WT, *Brca2*$^{+/\Delta 27}$, and *Brca2*$^{\Delta 27/\Delta 27}$ BM cells in the presence of increasing concentrations of mitomycin C (MMC). As previously observed in other FA models [5,7], CFCs from *Brca2*$^{\Delta 27/\Delta 27}$ mice were

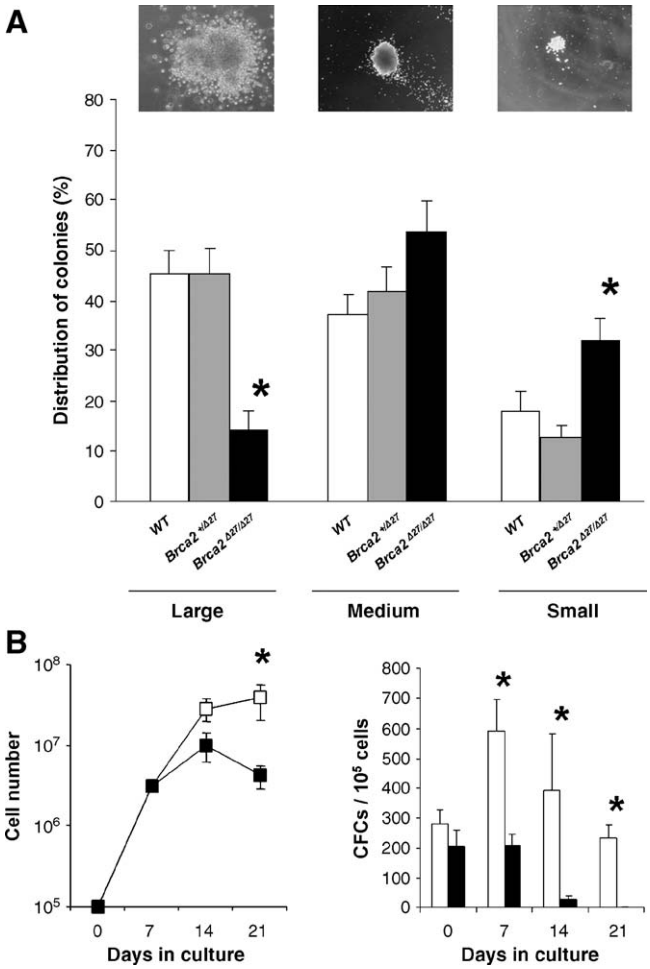

FIG. 2. Proliferation defect in hematopoietic colony-forming cells from *Brca2*$^{\Delta 27/\Delta 27}$ mice. (A) The distribution of large, medium, and small colonies generated by the bone marrow from WT, *Brca2*$^{+/\Delta 27}$, and *Brca2*$^{\Delta 27/\Delta 27}$ mice (three animals per group). Representative colonies from each category are shown. (B) The cumulative number of viable cells and proportion of CFCs in bone marrow cultures from WT (open symbols) and *Brca2*$^{\Delta 27/\Delta 27}$ (filled symbols) mice subjected to *ex vivo* expansion (see Materials and Methods). The means ± SEM of data corresponding to four different experiments, each with a different bone marrow sample, are shown. *Significant differences with respect to WT samples ($P < 0.05$).

doi:10.1016/j.ymthe.2006.05.018

highly sensitive to MMC (Fig. 3A). Next we investigated the presence of cytogenetic abnormalities in *Brca2*$^{\Delta 27/\Delta 27}$ BM cells subjected to *in vitro* stimulation for 2 days, both in the presence and in the absence of MMC. As an additional control, we included cells from *Fanca*$^{-/-}$ mice in these experiments. As expected, we observed a low number of chromatid breaks in cells from WT and *Brca2*$^{+/\Delta 27}$ mice, regardless of the presence of MMC. Compared to non-MMC-treated samples, cells from *Fanca*$^{-/-}$ and *Brca2*$^{\Delta 27/\Delta 27}$ mice

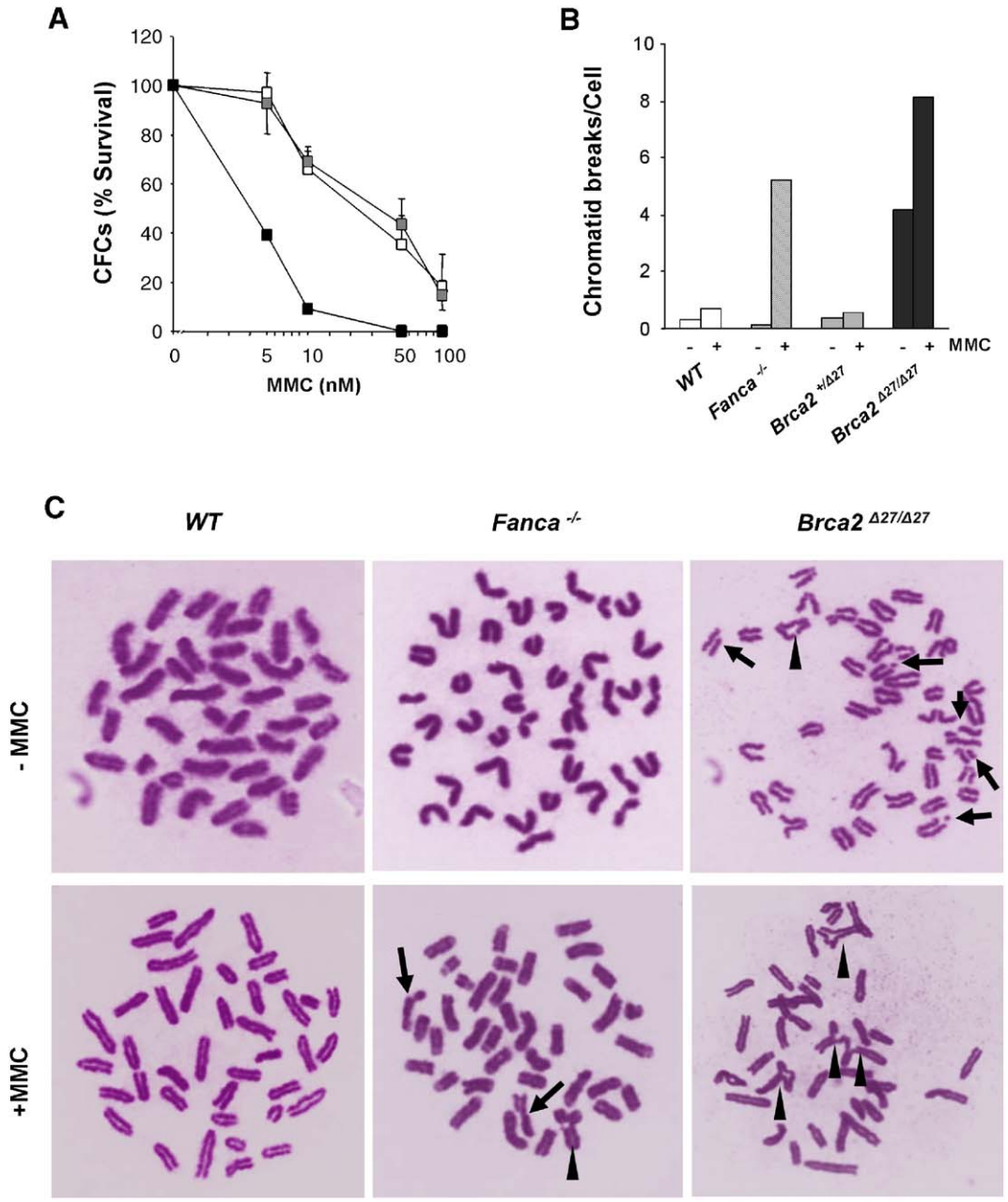

FIG. 3. Mitomycin C sensitivity and chromosomal instability of bone marrow progenitors from *Brca2*$^{\Delta 27/\Delta 27}$ mice. (A) Analysis of the sensitivity of bone marrow progenitors from WT (open symbols), *Brca2*$^{+/\Delta 27}$ (gray symbols), and *Brca2*$^{\Delta 27/\Delta 27}$ (black symbols) mice to mitomycin C (MMC). Each point represents the mean ± SEM corresponding to three experiments. (B) Analysis of the number of chromatid breaks in bone marrow cells from WT, *Fanca*$^{-/-}$, *Brca2*$^{+/\Delta 27}$, and *Brca2*$^{\Delta 27/\Delta 27}$ mice in the absence (−) or the presence (+) of 30 nM MMC. Data from one representative experiment are shown. A minimum of 50 metaphases were scored in each case. (C) Representative metaphase spreads showing spontaneous and MMC-induced chromatid breaks (arrows) and interchromatid exchanges, such as radial and complex figures (arrowheads) in WT, *Fanca*$^{-/-}$, and *Brca2*$^{\Delta 27/\Delta 27}$ bone marrow cells.

showed a marked increase in the number of chromatid breaks after a 30 nM exposure. Moreover, a high number of spontaneous chromosomal aberrations was characteristic of *Brca2*$^{\Delta 27/\Delta 27}$ cells (Fig. 3B). The presence of MMC-induced and spontaneous chromosome aberrations in these cells is shown in Fig. 3C. All these data are consistent with results previously obtained in FA-D1 patients, who show a high rate of chromosomal aberrations, even in the absence of a genotoxic insult [26].

Impaired Hematopoietic Response of *Brca2*$^{\Delta 27/\Delta 27}$ Mice to Ionizing Radiation

Based on previous observations showing that FA-D1 is the only FA complementation group incapable of generating Rad51 foci after DNA damage [27], we investigated whether fibroblasts from *Brca2*$^{\Delta 27/\Delta 27}$ mice were capable of generating Rad51 foci after an exposure of 12 Gy. Consistent with results obtained in FA-D1 patients, fibroblasts from *Brca2*$^{\Delta 27/\Delta 27}$ mice did not generate Rad51 foci after irradiation (Fig. 4A).

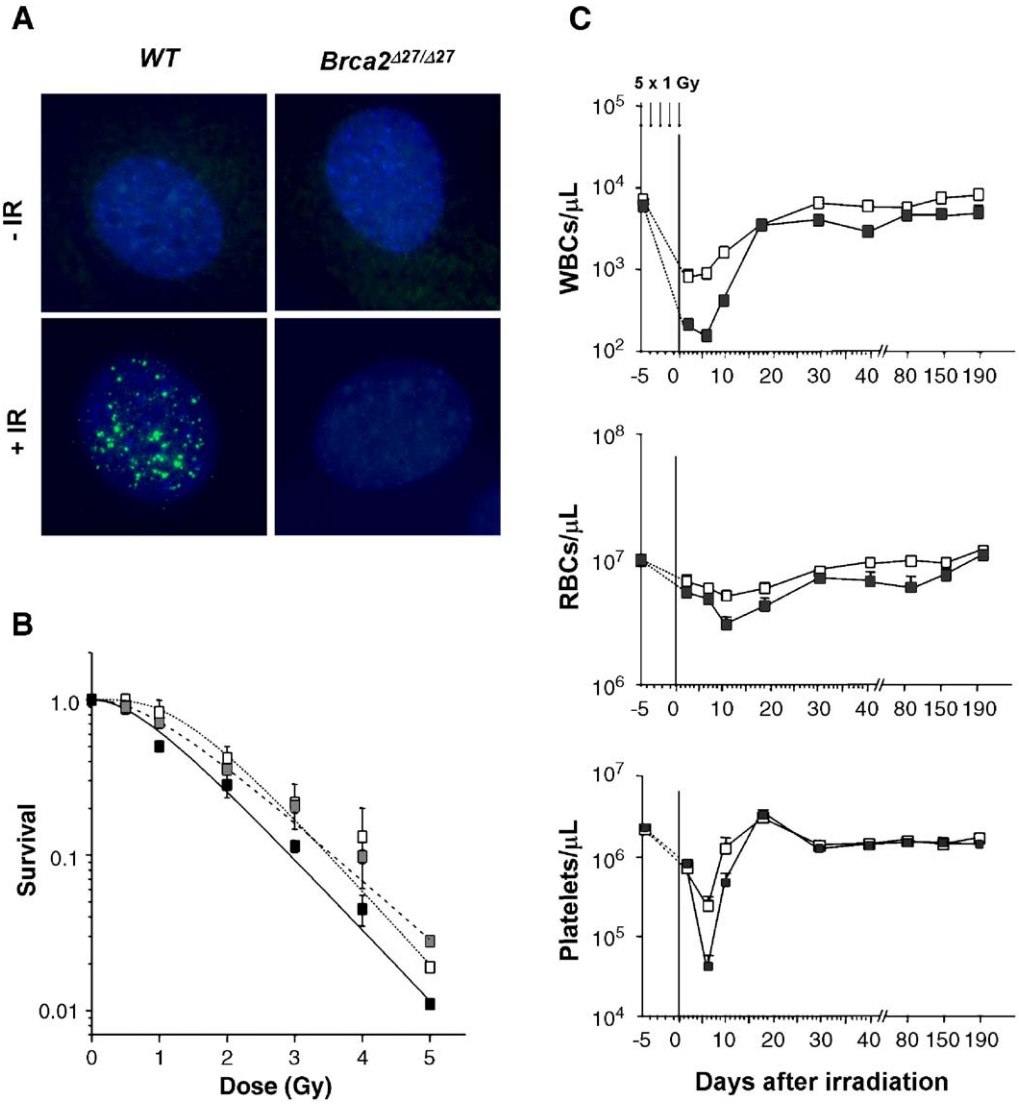

FIG. 4. Impaired response of *Brca2*$^{\Delta 27/\Delta 27}$ mice to ionizing radiation. (A) Pictures show representative analyses of Rad51 foci in fibroblasts from WT and *Brca2*$^{\Delta 27/\Delta 27}$ mice. (−/+ IR, cells were sham-irradiated or irradiated with 12 Gy.) (B) Survival curves of bone marrow CFCs from WT (open symbols), *Brca2*$^{+/\Delta 27}$ (gray symbols), and *Brca2*$^{\Delta 27/\Delta 27}$ (black symbols) mice after *in vitro* irradiation. Survival data were adjusted to the multitarget model, as previously described [23]. The parameters obtained from the mathematical model were the following: WT, Do = 0.91 Gy, n = 4.9; *Brca2*$^{+/\Delta 27}$, Do = 1.13 Gy, n = 2.0; *Brca2*$^{\Delta 27/\Delta 27}$, Do = 0.94 Gy, n = 2.3. (C) Hematopoietic syndrome of WT (open symbols) and *Brca2*$^{\Delta 27/\Delta 27}$ (filled symbols) mice exposed to total body irradiation (5 doses of 1 Gy delivered at 24-h intervals). Seven days after the irradiation protocol was started, numbers of leukocytes (WBCs), erythrocytes (RBCs), and platelets were periodically analyzed in peripheral blood.

doi:10.1016/j.ymthe.2006.05.018

To investigate potential differences in the radiosensitivity of the hematopoietic progenitors from WT, *Brca2*$^{+/\Delta 27}$, and *Brca2*$^{\Delta 27/\Delta 27}$ mice, we exposed BM cells from these animals to increasing doses of X-rays and then cultured them in semisolid medium to determine the survival rate of the CFCs. Although *Brca2*$^{\Delta 27/\Delta 27}$ CFC survivals were generally lower compared to *Brca2*$^{+/\Delta 27}$ and WT CFCs, Do values corresponding to the different curves were not significantly different between each other (ANOVA, $P>0.05$; see Fig. 4B). This indicates that the *Brca2*$^{\Delta 27/\Delta 27}$ mutation does not significantly modify the radiosensitivity of these CFCs.

To investigate more deeply the hematopoietic response of *Brca2*$^{\Delta 27/\Delta 27}$ mice to irradiation, we subjected mice to total body irradiation with a fractionated dose of 5 Gy (five doses of 1 Gy spaced 24 h apart, as previously described [22]). We then followed the hematological recovery of the animals periodically. As shown in Fig. 4C, the hematopoietic syndrome produced in the animals was markedly more severe in *Brca2*$^{\Delta 27/\Delta 27}$ mice compared to WT animals. As shown in the figure, both the period and the severity of the leukopenia, anemia, and thrombopenia were much more significant in *Brca2*$^{\Delta 27/\Delta 27}$ compared to WT animals. To confirm the high radiosensitivity of these animals, we gave an acute dose of 7 Gy to the animals. While 90% of WT mice survived an acute dose of 7 Gy, this dose was supralethal in *Brca2*$^{\Delta 27/\Delta 27}$ mice, who died within a period of 5–9 days postirradiation (data not shown).

Defective Competitive Repopulation Ability of *Brca2*$^{\Delta 27/\Delta 27}$ Hematopoietic Stem Cells

Aiming to investigate the HSC functionality of *Brca2*$^{\Delta 27/\Delta 27}$ mice, we investigated the competitive repopulating ability (CRA) of BM from WT and *Brca2*$^{\Delta 27/\Delta 27}$ mice. As illustrated in Fig. 5A, we mixed BM samples from WT and *Brca2*$^{\Delta 27/\Delta 27}$ mice (males in all instances) together with the same number of BM cells from WT females and transplanted them into myeloablated recipients. To verify that endogenous hematopoiesis was not significant, we used female mice with a heterozygous point mutation in the ferrochelatase (Fech) gene [28] as recipients (see Fig. 5A). Starting 1 month after transplantation, we periodically determined the proportion of Y-chromosome-positive cells in the PB of recipient mice by means of real-time quantitative PCR (RT-qPCR; see Materials and Methods). Data obtained at 30 days posttransplantation showed that the CRA of *Brca2*$^{\Delta 27/\Delta 27}$ BM cells was markedly below that corresponding to WT BM cells (Fig. 5B). Furthermore, CRA values from *Brca2*$^{\Delta 27/\Delta 27}$ BM progressively dropped as the posttransplantation time was increased (Fig. 5B), indicating that, at least under the proliferative stress mediated by a BM transplantation, the repopulating function of *Brca2*$^{\Delta 27/\Delta 27}$ HSCs is markedly impaired.

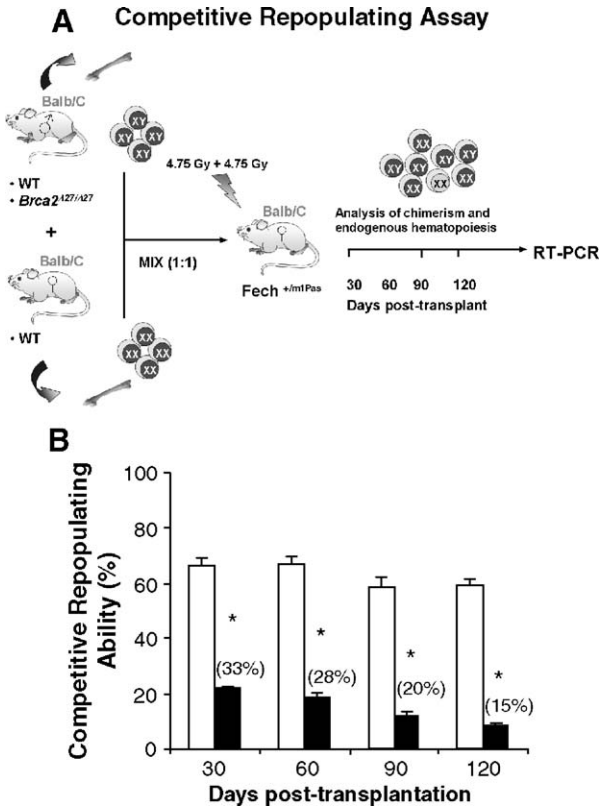

FIG. 5. Defective competitive repopulation ability of hematopoietic stem cells from *Brca2*$^{\Delta 27/\Delta 27}$ mice. (A) Experimental approach used to determine the competitive repopulation ability (CRA) of hematopoietic stem cells from WT and *Brca2*$^{\Delta 27/\Delta 27}$ mice (see Materials and Methods). (B) Competitive repopulation ability of *Brca2*$^{\Delta 27/\Delta 27}$ stem cells (black bars) compared to that observed in control WT mice (white bars). Relative values of CRA from *Brca2*$^{\Delta 27/\Delta 27}$ with respect to WT stem cells are shown in parentheses. In all instances the level of endogenous reconstitution of the irradiated recipients was below 10%. *Statistical significance between the WT and the *Brca2*$^{\Delta 27/\Delta 27}$ groups ($P<0.05$).

Defective Repopulation Ability of *Brca2*$^{\Delta 27/\Delta 27}$ Hematopoietic Stem Cells Maintained under Physiological Steady-State Conditions

In the last set of experiments, we aimed to investigate the proliferating properties of *Brca2*$^{\Delta 27/\Delta 27}$ HSCs maintained under their physiological conditions. With this aim, we transplanted unconditioned *Brca2*$^{\Delta 27/\Delta 27}$ and WT mice with either 2×10^6 or 2×10^7 BM cells from WT male donors (see Fig. 6A). Under these experimental conditions, the repopulation of *Brca2*$^{\Delta 27/\Delta 27}$ mice with donor WT BM cells would indicate a regeneration defect in *Brca2*$^{\Delta 27/\Delta 27}$ HSCs maintained in their natural microenvironment.

Transplantation of 2×10^6 WT BM cells into unconditioned animals was incapable of engrafting any recipient, regardless of their genotype ($n=6$; not shown). Similarly, when we transplanted 2×10^7 WT BM cells into WT recipients, no significant engraftment was in any animal ($n=5$). In contrast to these observations, three of

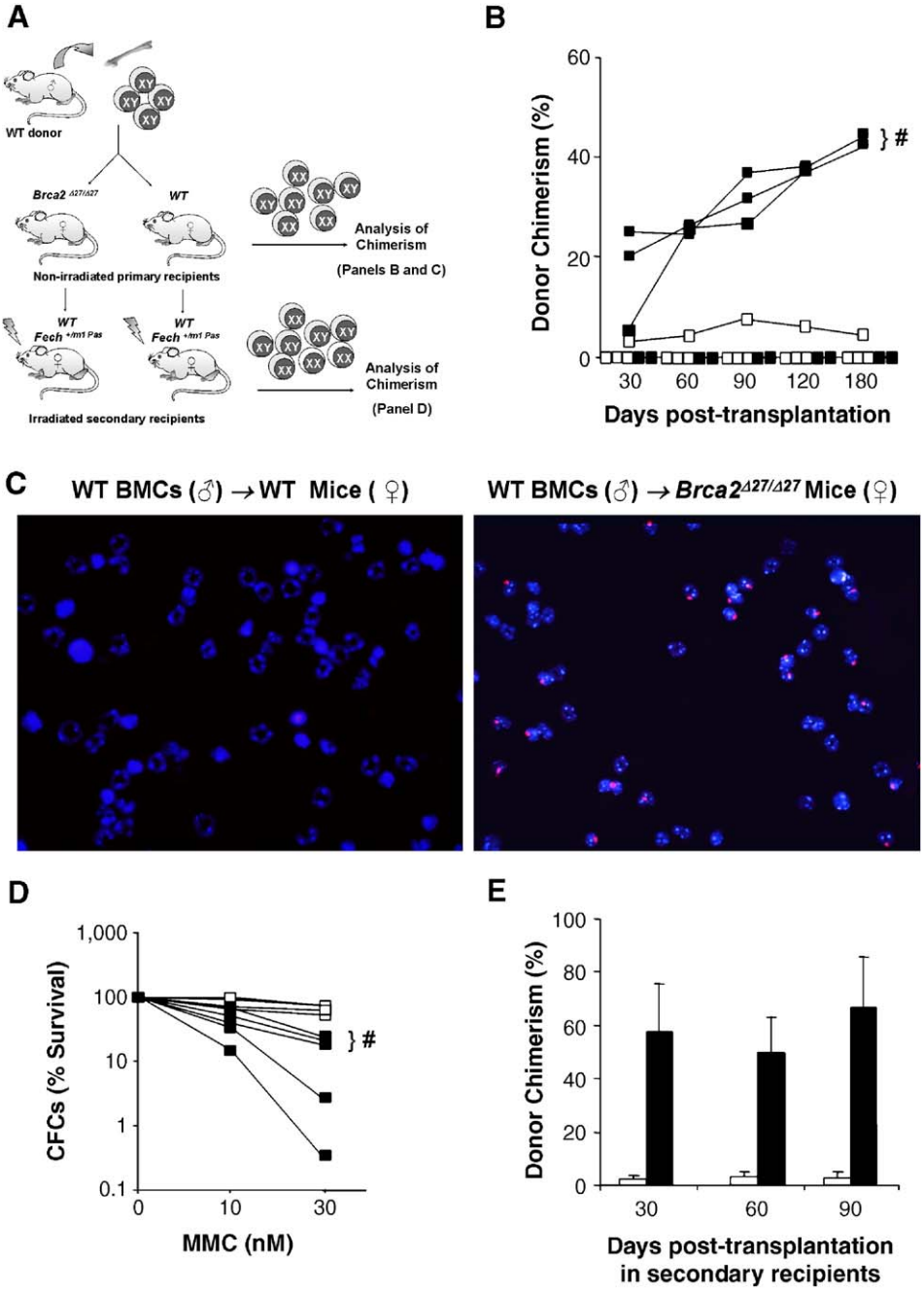

FIG. 6. Proliferation advantage of wild-type hematopoietic stem cells transplanted into unconditioned *Brca2*$^{\Delta 27/\Delta 27}$ mice. (A) Experimental protocol used for evaluating the regeneration capacity of *Brca2*$^{\Delta 27/\Delta 27}$ HSCs under steady-state physiological conditions. In these experiments, bone marrow cells (either 2×10^6 or 2×10^7 cells) from male WT mice were transplanted into unconditioned female recipients (either WT or *Brca2*$^{\Delta 27/\Delta 27}$; see details under Materials and Methods). (B) Analysis of engraftment of donor WT cells in WT (open symbols) and *Brca2*$^{\Delta 27/\Delta 27}$ (filled symbols) recipients determined by RT-qPCR in peripheral blood cells. Individual data corresponding to recipients transplanted with 2×10^7 BM cells are shown. (#) Engrafted mice. (C) Representative *in situ* hybridization analyses of the Y chromosome in bone marrow samples from WT and *Brca2*$^{\Delta 27/\Delta 27}$ unconditioned recipients transplanted with WT bone marrow cells, as indicated in (A). Bone marrow samples were analyzed 6 months after transplantation. (D) Partial reversion of the MMC sensitivity of bone marrow progenitors from *Brca2*$^{\Delta 27/\Delta 27}$ mice produced as a consequence of the transplantation of 2×10^7 WT BM cells. (#) Data from the three engrafted mice in (B). (E) Sustained repopulation of WT primary bone marrow cells in myeloablated secondary recipients. Bone marrow from unconditioned primary recipients, WT (open symbols) and *Brca2*$^{\Delta 27/\Delta 27}$ (filled symbols) mice, was harvested 6 months after transplantation and then infused into irradiated secondary recipients. The level of endogenous reconstitution of secondary recipients was always below 10%.

doi:10.1016/j.ymthe.2006.05.018

five *Brca2*$^{\Delta 27/\Delta 27}$ recipients showed a progressive reconstitution by donor cells, which reached levels close to 45% at 6 months posttransplantation (Fig. 6B). Since the presence of donor T lymphocytes in recipient PB might generate false positive results of long-term engraftment, animals were killed at 6 months posttransplantation, and we performed *in situ* hybridization analyses of BM—in which the proportion of T cells is lower than 3%—with a Y-chromosome-specific probe. Significantly, the three engrafted *Brca2*$^{\Delta 27/\Delta 27}$ recipients shown in Fig. 6B showed a high proportion (around 30%) of BM cells labeled with the Y-chromosome probe (see a representative analysis in Fig. 6C; engraftments in these BM samples were also confirmed by RT-qPCR; data not shown). When we conducted similar transplantation protocols in *Fanca*$^{-/-}$ mice ($n=3$) and *Fancc*$^{-/-}$ mice ($n=3$), no evidence of engraftment was apparent even 6 months posttransplantation (data not shown).

To evaluate whether the engraftment observed in unconditioned *Brca2*$^{\Delta 27/\Delta 27}$ recipients corrected the hematopoietic phenotype of these animals, we assessed the MMC sensitivity of progenitors from WT and *Brca2*$^{\Delta 27/\Delta 27}$ mice transplanted with 2×10^7 BMCs. As shown in Fig. 6D, the MMC sensitivity of CFCs from engrafted *Brca2*$^{\Delta 27/\Delta 27}$ mice was markedly reversed compared to samples from nonengrafted *Brca2*$^{\Delta 27/\Delta 27}$ mice.

Finally, to confirm the proliferation advantage of exogenous WT HSCs over the endogenous *Brca2*$^{\Delta 27/\Delta 27}$ HSCs, we harvested BM cells from primary unconditioned recipients 6 months after transplantation and then retransplanted them into irradiated secondary recipients (see Fig. 6A). We then investigated the presence of the original donor cells in the PB of secondary recipients by RT-qPCR in PB. As shown in Fig. 6E, when BM cells from *Brca2*$^{\Delta 27/\Delta 27}$ recipients were retransplanted into secondary mice, up to 70% of the PB cells from these recipients (data obtained 90 days after transplantation) were derived from the original BM graft. In contrast, when WT mice were used as primary recipients, we observed no reconstitution by the original donor cells. These data demonstrate the proliferation defect of endogenous *Brca2*$^{\Delta 27/\Delta 27}$ HSCs maintained in an undisturbed physiological environment.

DISCUSSION

Although several mouse models of FA have been already developed, FA mice generated so far only partially reproduce the phenotype of the disease [29]. Under physiological conditions, normal values of hematopoietic CFCs were observed in young adult *Fancc*$^{-/-}$ or *Fanca*$^{-/-}$ mice [5,7,30]. However, in older FA mice, or in BM samples from FA mice subjected to proliferative stimulation, reduced values of CFCs have been reported [5,7,31]. As happened in these models of FA, PB cell counts and flow cytometry analyses in the hematopoietic organs from *Brca2*$^{\Delta 27/\Delta 2}$ mice were normal. However, in contrast to data obtained in other FA mouse models, *Brca2*$^{\Delta 27/\Delta 27}$ mice contained very low numbers of CFCs in BM and spleen, an observation that was significant not only in adult mice, but also in 1.5-week-old animals (Fig. 1). Apart from a reduced number of hematopoietic CFCs, our data in Fig. 2 show a proliferative defect in *Brca2*$^{\Delta 27/\Delta 27}$ progenitor cells, thus explaining the apparent contradiction between the reduced number of CFCs observed in these animals and the normal values of Lin$^-$Sca1$^+$ cells in their hematopoietic tissues.

In addition to the subnormal hematopoiesis observed in *Brca2*$^{\Delta 27/\Delta 27}$ mice, several other parameters distinguish this FA-D1 mouse model from other models of FA. In this respect, the spontaneous chromosomal instability of *Brca2*$^{\Delta 27/\Delta 27}$ BM cells—not observed in *Fanca*$^{-/-}$ BM cells (Fig. 3)—mimics the cellular behavior of FA-D1 patients [23,26].

The response of the different FA cells to radiation is also of interest. As has been previously observed, the FA-D1 complementation group is the only one in which Rad51 foci are not formed after DNA damage [27,32,33]. Since the COOH-terminal domain of Brca2 is highly conserved and interacts with Rad51 [25,34], we investigated the generation of Rad51 foci in *Brca2*$^{\Delta 27/\Delta 27}$ fibroblasts after irradiation. Consistent with data obtained from human FA-D1 patients, irradiated cells from *Brca2*$^{\Delta 27/\Delta 27}$ mice did not generate Rad51 foci (Fig. 4A). Taking into account that Rad51 foci formation represents an important step in the cellular response to ionizing radiation [33] and that DSBs generated by irradiation are repaired by a Brca2-dependent pathway [20], we hypothesized that *Brca2*$^{\Delta 27/\Delta 27}$ hematopoietic progenitors would have an impaired response to IR. Although hematopoietic progenitors from *Brca2*$^{\Delta 27/\Delta 27}$ mice were not particularly sensitive to irradiation delivered *in vitro* (Fig. 4B), the hematopoietic syndrome produced after *in vivo* irradiation of *Brca2*$^{\Delta 27/\Delta 27}$ mice was very severe (Fig. 4C). Significantly, this impaired response to radiation has not been observed in *Fanca*$^{-/-}$ mice [22]. The response of *Brca2*$^{\Delta 27/\Delta 27}$ mice to radiation also mimics the response observed in a high proportion of human FA patients, who were highly sensitive to radiation *in vivo* [35], even though the *in vitro* radiosensitivity of their cells was not markedly high [36].

Regarding the experiments in HSC functionality, the CRA assays shown in Fig. 5 indicate that *Brca2*$^{\Delta 27/\Delta 27}$ BM grafts have a marked repopulation defect, compared to BM samples from WT animals. Similar observations have also been reported in *Fancc*$^{-/-}$ mice [6,30]. It is significant, however, that in our experiments with *Brca2*$^{\Delta 27/\Delta 27}$ mice, a progressive decrease in CRA was observed as the posttransplantation time was increased; something which is consistent with a progressive exhaustion in the repopulating function of the HSCs.

In addition to a CRA defect after transplantation into irradiated recipients, here we demonstrate a repopulation defect in *Brca2*$^{\Delta 27/\Delta 27}$ HSCs not disturbed from their physiological environment. As shown in Fig. 6, when 2×10^7 WT BM cells were transplanted into nonirradiated *Brca2*$^{\Delta 27/\Delta 27}$ recipients, a progressive increase in the reconstitution of WT donor cells was observed in three of five transplanted *Brca2*$^{\Delta 27/\Delta 27}$ mice. Taking into account that 2×10^7 BM cells constitute about 7% of the total mouse BM [37], and considering that only 7–9% of transplanted HSCs can home to the hematopoietic tissues of a nonirradiated recipient [38], the number of exogenous HSCs that may have homed in our *Brca2*$^{\Delta 27/\Delta 27}$ mice must represent about 0.6% of the endogenous number of HSCs. Strikingly, at 6 months posttransplantation, more than 40% of *Brca2*$^{\Delta 27/\Delta 27}$ hematopoiesis was of WT donor origin (Fig. 6B). Moreover, when BM from these primary recipients was transplanted into lethally irradiated WT mice, up to 70% of the PB cells from these secondary recipients corresponded to the original WT donor (Fig. 6E), thus confirming the proliferative defect of endogenous *Brca2*$^{\Delta 27/\Delta 27}$ HSCs. To our knowledge, this is the only DNA repair disease model in which a proliferation disadvantage of HSCs maintained under physiological conditions has been reported. The proliferation advantage of WT HSCs that progressively repopulated the hematopoietic tissues of unconditioned *Brca2*$^{\Delta 27/\Delta 27}$ mice is reminiscent of the somatic mosaicism phenomenon observed in a number of genetic diseases, including FA [39–41].

In addition to our observations on HSC dysfunction, *Brca2*$^{\Delta 27/\Delta 27}$ mice were characterized by a high incidence of epithelial tumors [24], also consistent with the phenotype of FA patients. Significantly, a marked predisposition for developing epithelial cancers was also observed in *Fancd2*$^{-/-}$ mice [42]. This observation, together with other phenotypic similarities between *Brca2*$^{\Delta 27/\Delta 27}$ and *Fancd2*$^{-/-}$ mice, suggested that the C-terminal of Brca2 functions in the same pathway as Fancd2 [42]. Although the role of Fancd2 in the functionality of HSCs has not been described so far, marked differences in the *in vivo* radiosensitivity of *Brca2*$^{\Delta 27/\Delta 27}$ and *Fancd2*$^{-/-}$ mice are evident, suggesting at least partial divergences in their biochemical pathways.

Taken together, although *Brca2*$^{\Delta 27/\Delta 27}$ mice do not develop aplastic anemia, the phenotype of these animals reflects more closely the disease observed in FA patients, compared to other FA mouse models so far developed. We, therefore, consider that this model will constitute an invaluable tool for the investigation of FA and for the development of new therapeutic strategies of inherited hematopoietic diseases, particularly for those in which the transplantation of genetically corrected cells into unconditioned recipients is considered a realistic option.

Materials and Methods

Animals. Balb/C mice with a homozygous deletion in the COOH-terminal of the *Fancd1/Brca2* gene [24] and their WT corresponding littermates were used throughout. Where indicated, Balb/C mice carrying a heterozygous point mutation in the ferrochelatase gene [28] (*Fech*$^{+/m1Pas}$ mice) were also used. In some experiments *Fanca*$^{-/-}$ mice [43] (129/FVB strain; kindly provided by Fre Artwert, Free University Medical Center, Amsterdam, The Netherlands) and *Fancc*$^{-/-}$ mice [5] (C57Bl strain; kindly provided by Manuel Buchwald, Hospital for Sick Children, Toronto, ON, Canada) were used. All experimental procedures were carried according to Spanish and European regulations (Spanish RD 223/88 and OM 13-10-89 of the Ministry of Agriculture, Food, and Fisheries, European Convention ETS-123).

Hematopoietic cells and in vitro culture assays. Cells from the BM, spleen, and thymus were dispersed in Iscove's modified Dulbecco's medium (IMDM; Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS (Sigma Chemicals, St. Louis, MO, USA). Blood samples were obtained by tail vein excision and anticoagulated with EDTA (10–20 mM final concentration). Hematological counts were performed in a Technicom H1E (Bayer, Miles, Inc., Tarrytown, NY, USA) hematological analyzer. For *ex vivo* expansion, BM cells were seeded at a final concentration of 10^5 cells/ml in IMDM supplemented with 20% FBS, 2 mM L-glutamine (GIBCO Cell Culture Systems), 50 U/ml penicillin–50 U/ml streptomycin (GIBCO), hrIL-11 (100 ng/ml) and mrSCF (100 ng/ml) (both from Chemicon International, Inc.), hrTPO (100 ng/ml) (Kirin Brewery Co., Ltd., Tokyo, Japan), and ProGP (300 ng/ml) (progenipoietin; dual hFlt3 and hG-CSF receptor agonist kindly provided by Monsanto Co., St. Louis, MO, USA) and followed weekly for cell number and viability. At weekly intervals, cells were diluted at the initial cell concentration. To determine the number of CFCs, samples were plated in MethoCult GF M3534 culture medium (StemCell Technologies, Vancouver, BC, Canada) and cultured at 37°C in 5% CO_2 and fully humidified air. Colonies of at least 50 cells were scored after 7 days of culture.

Flow cytometry. Hematopoietic cells were lysed in ammonium chloride lysis solution to eliminate erythrocytes and stained with different cocktails of monoclonal antibodies raised against mouse differentiation markers, which include B220, Mac1, CD3, Gr1, Ter119, CD4, and CD8 (all of them from BD Pharmingen, Palo Alto, CA, USA). To identify primitive progenitors ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) in BM cells, samples were first stained with a cocktail of biotinylated differentiation markers (Lin) and then stained with Sca-1–FITC (Pharmingen), c-Kit–PE (Pharmingen), and streptavidin-tricolor (Caltag, Burlingame, CA, USA). Samples were analyzed in an EPICS XL flow cytometer (Coulter Electronics, Hialeah, FL, USA). Dead cells were excluded with propidium iodide (2 μg/ml).

Structural chromosomal abnormalities. Liquid cultures of BM cells were left untreated or were treated with 30 nM MMC for a 48-h period. To obtain metaphases, colcemid (0.1 μg/ml; Gibco, Paisley, UK) was added 2 h prior to harvesting, and the cells were processed following standard cytogenetic procedures. Briefly, cells were dropped onto clean slides and air-dried overnight. Slides were stained with 10% Giemsa in phosphate buffer, pH 6.8. At least 50 metaphases per sample were analyzed for chromosome aberrations including gaps, chromosome and chromatid breaks, acentric fragments, and chromosome- and chromatid-type exchanges.

Sensitivity to MMC and ionizing radiation. To assess the susceptibility of the BM progenitor cells to DNA crosslinking agents, BM cells were cultured in MethoCult medium containing increasing concentrations of MMC (Sigma). For assessing the radiation sensitivity of the progenitors, BM cell suspensions were irradiated with an X-ray machine (Phillips, Hamburg, Germany) at 300 kV, 10 mA, HVL 2.3 mm Cu, with a dose rate of 1.034 Gy/min.

Analysis of Rad51 foci. Ear fibroblasts from WT and *Brca2*$^{\Delta 27/\Delta 27}$ mice were grown in two-chamber slides treated with 0.2% gelatin. Eight hours after a 12-Gy irradiation, cells were washed with phosphate-buffered

doi:10.1016/j.ymthe.2006.05.018

saline (PBS) and fixed with 3.7% paraformaldehyde for 15 min. Cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min. After blocking with 10% bovine serum albumin, 0.1% NP-40 in PBS (blocking buffer) for 1 h, a mouse monoclonal antibody was used to detect Rad51 (1/200 dilution; Abcam, Cambridge, UK). After washing, the secondary antibody (Alexa Fluor 488, 1/2000 dilution; Molecular Probes, Leiden, The Netherlands) was added. Cells were counterstained with 4′,6-diamidino-2-phenylindole using Vectashield (Vector Laboratories) mounting medium and analyzed under a fluorescence microscope (Zeiss Axioplan2) using a 100× objective (Zeiss). At least 200 nuclei were scored.

Bone marrow competition experiments. Competitive repopulation assays were conducted essentially as previously described [44]. Briefly, BM cells from WT or *Brca2*$^{\Delta 27/\Delta 27}$ mice (always males) were mixed together with the same number of BM cells from WT females. Aliquots of the chimeric BM consisting of 10^7 cells were transplanted into Fech$^{+/m1Pas}$ female recipients previously irradiated with a myeloablative regimen of X-rays (two doses of 4.75 Gy spaced 4 h apart [45]). At various times after transplantation, DNA from PB was extracted, and the proportion of cells positive for the Y chromosome was determined by RT-qPCR. Endogenous reconstitution was determined by the presence of the Fech mutation, as described below.

Repopulation of unconditioned recipients with donor hematopoietic stem cells. To assess the possibility of engrafting unconditioned *Brca2*$^{\Delta 27/\Delta 27}$ mice with donor WT BM cells, WT or *Brca2*$^{\Delta 27/\Delta 27}$ female recipients were transplanted with 2×10^6 or 2×10^7 BM cells from WT male mice. Thereafter, the presence of donor cells was determined by RT-qPCR and in situ hybridization (see Fig. 6A). A subsequent transplantation of 10^6 BMCs from primary unconditioned recipients into irradiated secondary *Fech*$^{+/m1Pas}$ recipients was done, to confirm the proliferation advantage of exogenous WT HSCs over the endogenous *Brca2*$^{\Delta 27/\Delta 27}$ HSCs.

Analyses of chimerism. Quantification of chimerism was accomplished by using a RT-qPCR approach using a Rotor Gene RG-3000 (Corbett Research Products, Foxboro, MA, USA). Genomic DNA was extracted using a DNAeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. Primers for the male-specific sequence were SRY-F, 5′-TGTTCAGCCCTACAGCCACA-3′, and SRY-R, 5′-CCTCTCACCACGGGACCAC-3′, and detected with the TaqMan probe SRY-T, 5′-FAM–ACAATTGTCTAGAGAGCATGGAGGGCCA–BHQ1-3′. Amplification of murine genomic β-actin sequence was achieved using the primers β-actin-MF, 5′-ACGGCCAGGTCATCACTATTG-3′, and β-actin-MR, 5′-ACTATGGCCTCAAGGAGTTTTGTCA-3′, and detected with the TaqMan probe β-actin-T, 5′-TR–AACGAGCGGTTCCGATGCCCT–BHQ2-3′. Amplification was carried out in a multiplex reaction using TaqMan Universal Mastermix, NoAmpErase UNG (Applied Biosystems Roche, NJ, USA). The thermal profile was one hold of 10 min at 95°C and 55 cycles of 20 s at 95°C, 30 s at 58°C.

The contribution of endogenous hematopoiesis was similarly analyzed by RT-qPCR quantifying the mutated DNA sequence of the ferrochelatase gene from receptor mice. DNA samples from transplanted mice were digested overnight with *Pag*I (Fermentas) restriction endonuclease to avoid nonspecific amplification of the wild-type ferrochelatase gene. The primers for the ferrochelatase mutated specific sequence were as follows: FechM-F, 5′-CGCACCCCACACACGAC-3′, and FechM-R, 5′-CGCACCCCACACACGAC-3′. The TaqMan probe was Fech-T, 5′-Cy5–CTTCCCATTCAAAAGTAAGTTATGCTGTGTGGAG–BHQ2-3′. The amplification reactions were carried out under the conditions already described. The thermal profile was one hold of 10 min at 95°C and 45 cycles of 20 s at 95°C, 30 s at 60°C. Findings from RT-qPCR assays were supported using fluorescence *in situ* Y hybridization in cytospin preparations of bone marrow samples from transplanted mice. The samples were fixed in methanol:acetic acid (3:1) for 30 min at −20°C and dehydrated by serial ethanol washing. Hybridization and detection were carried out according to the STAR FISH hybridization manufacturer's protocol (Cambio Ltd., Cambridge, UK).

Statistical analysis. Data are represented as means ± standard error of the mean. The significance of differences was determined by the nonparametric Wilcoxon Mann–Whitney *W* test, and multiple comparisons were determined by the *F* test ANOVA using Statgraphics Plus 5.0 software package (Manugistics, Inc., Rockville, MD, USA). Fitting of the radiation sensitivity curves was performed with the Origin 7.0 (Northampton, MA, USA) software package.

ACKNOWLEDGMENTS

The authors acknowledge the excellent technical work of Mr. Sergio García, Israel Ormán, Jesús Martínez, and Elena López. This work was supported by grants from the European Program "Life Sciences, Genomics, and Biotechnology for Health" (CONSERT; Ref. 005242), Comisión Interministerial de Ciencia y Tecnología (SAF2005-00058), and Red de Investigación Cooperativa sobre Anemia de Fanconi (ISCIII; Ministerio de Sanidad y Consumo, Ref. G03/073).

RECEIVED FOR PUBLICATION JANUARY 27, 2006; REVISED MAY 23, 2006; ACCEPTED MAY 23, 2006.

REFERENCES

1. Bender, C. F., *et al.* (2002). Cancer predisposition and hematopoietic failure in Rad50(S/S) mice. *Genes Dev.* **16:** 2237–2251.
2. Ito, K., *et al.* (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* **431:** 997–1002.
3. Prasher, J. M., *et al.* (2005). Reduced hematopoietic reserves in DNA interstrand crosslink repair-deficient Ercc1$^{-/-}$ mice. *EMBO J.* **24:** 861–871.
4. Reese, J. S., Liu, L., and Gerson, S. L. (2003). Repopulating defect of mismatch repair-deficient hematopoietic stem cells. *Blood* **102:** 1626–1633.
5. Whitney, M. A., *et al.* (1996). Germ cell defects and hematopoietic hypersensitivity to gamma-interferon in mice with a targeted disruption of the Fanconi anemia C gene. *Blood* **88:** 49–58.
6. Haneline, L. S., *et al.* (1999). Loss of FancC function results in decreased hematopoietic stem cell repopulating ability. *Blood* **94:** 1–8.
7. Rio, P., *et al.* (2002). In vitro phenotypic correction of hematopoietic progenitors from Fanconi anemia group A knockout mice. *Blood* **100:** 2032–2039.
8. Levitus, M., *et al.* (2004). Heterogeneity in Fanconi anemia: evidence for 2 new genetic subtypes. *Blood* **103:** 2498–2503.
9. Meetei, A. R., *et al.* (2005). A human ortholog of archaeal DNA repair protein Hef is defective in Fanconi anemia complementation group M. *Nat. Genet.* **37:** 958–963.
10. Levran, O., *et al.* (2005). The BRCA1-interacting helicase BRIP1 is deficient in Fanconi anemia. *Nat. Genet.* **37:** 931–933.
11. Levitus, M., *et al.* (2005). The DNA helicase BRIP1 is defective in Fanconi anemia complementation group J. *Nat. Genet.* **37:** 934–935.
12. Garcia-Higuera, I., *et al.* (2001). Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell* **7:** 249–262.
13. Meetei, A. R., *et al.* (2004). X-linked inheritance of Fanconi anemia complementation group B. *Nat. Genet.* **36:** 1219–1224.
14. Wang, X., Andreassen, P. R., and D'Andrea, A. D. (2004). Functional interaction of monoubiquitinated FANCD2 and BRCA2/FANCD1 in chromatin. *Mol. Cell. Biol.* **24:** 5850–5862.
15. Hussain, S., *et al.* (2004). Direct interaction of FANCD2 with BRCA2 in DNA damage response pathways. *Hum. Mol. Genet.* **13:** 1241–1248.
16. Howlett, N. G., *et al.* (2002). Biallelic inactivation of BRCA2 in Fanconi anemia. *Science* **13:** 13.
17. Moynahan, M. E., Pierce, A. J., and Jasin, M. (2001). BRCA2 is required for homology-directed repair of chromosomal breaks. *Mol. Cell* **7:** 263–272.
18. Xia, F., *et al.* (2001). Deficiency of human BRCA2 leads to impaired homologous recombination but maintains normal nonhomologous end joining. *Proc. Natl. Acad. Sci. USA* **98:** 8644–8649.
19. West, S. C. (2003). Molecular views of recombination proteins and their control. *Nat. Rev. Mol. Cell. Biol.* **4:** 435–445.
20. Venkitaraman, A. R. (2004). Tracing the network connecting BRCA and Fanconi anaemia proteins. *Nat. Rev. Cancer* **4:** 266–276.
21. Surralles, J., *et al.* (2004). Molecular cross-talk among chromosome fragility syndromes. *Genes Dev.* **18:** 1359–1370.
22. Casado, J. A., Nuñez, M. I., Segovia, J. C., Ruiz de Almodovar, M., and Bueren, J. A. (2005). Non-homologous end joining defect in Fanconi anemia hematopoietic cells exposed to ionizing radiation. *Radiat. Res.* **164:** 635–641.
23. Wagner, J. E., *et al.* (2004). Germline mutations in BRCA2: shared genetic susceptibility to breast cancer, early onset leukemia, and Fanconi anemia. *Blood* **103:** 3226–3229.
24. McAllister, K. A., *et al.* (2002). Cancer susceptibility of mice with a homozygous deletion in the COOH-terminal domain of the Brca2 gene. *Cancer Res.* **62:** 990–994.
25. Sharan, S. K., *et al.* (1997). Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature* **386:** 804–810.

26. Hirsch, B., *et al.* (2004). Association of biallelic BRCA2/FANCD1 mutations with spontaneous chromosomal instability and solid tumors of childhood. *Blood* **103:** 2554–2559.

27. Godthelp, B. C., *et al.* (2006). Inducibility of nuclear Rad51 foci after DNA damage distinguishes all Fanconi anemia complementation groups from D1/BRCA2. *Mutat. Res.* **22:** 39–49.

28. Tutois, S., *et al.* (1991). Erythropoietic protoporphyria in the house mouse: a recessive inherited ferrochelatase deficiency with anemia, photosensitivity, and liver disease. *J. Clin. Invest.* **88:** 1730–1736.

29. Wong, J. C., and Buchwald, M. (2002). Disease model: Fanconi anemia. *Trends. Mol. Med.* **8:** 139–142.

30. Carreau, M., *et al.* (1999). Hematopoietic compartment of Fanconi anemia group C null mice contains fewer lineage-negative $CD34^+$ primitive hematopoietic cells and shows reduced reconstruction ability. *Exp. Hematol.* **27:** 1667–1674.

31. Li, X., *et al.* (2005). Ex vivo culture of $Fancc^{-/-}$ stem/progenitor cells predisposes cells to undergo apoptosis, and surviving stem/progenitor cells display cytogenetic abnormalities and an increased risk of malignancy. *Blood* **105:** 3465–3471.

32. Godthelp, B. C., Artwert, F., Joenje, H., and Zdzienicka, M. Z. (2002). Impaired DNA damage-induced nuclear Rad51 foci formation uniquely characterizes Fanconi anemia group D1. *Oncogene* **21:** 5002–5005.

33. Tarsounas, M., Davies, D., and West, S. C. (2003). BRCA2-dependent and independent formation of RAD51 nuclear foci. *Oncogene* **22:** 1115–1123.

34. Wong, A. K., Pero, R., Ormonde, P. A., Tavtigian, S. V., and Bartel, P. L. (1997). RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J. Biol. Chem.* **272:** 31941–31944.

35. Alter, B. P. (2002). Radiosensitivity in Fanconi's anemia patients. *Radiother. Oncol.* **62:** 345–347.

36. Marcou, Y., D'Andrea, A., Jeggo, P. A., and Plowman, P. N. (2001). Normal cellular radiosensitivity in an adult Fanconi anaemia patient with marked clinical radiosensitivity. *Radiother. Oncol.* **60:** 75–79.

37. Schofield, R., and Cole, L. J. (1968). An erythrocyte defect in splenectomized X-irradiated mice restored with spleen colony cells. *Br. J. Haematol.* **14:** 131–140.

38. Cerny, J., *et al.* (2002). Homing of purified murine lymphohematopoietic stem cells: a cytokine-induced defect. *J. Hematother. Stem Cell Res.* **11:** 913–922.

39. Waisfisz, Q., *et al.* (1999). Spontaneous functional correction of homozygous Fanconi anaemia alleles reveals novel mechanistic basis for reverse mosaicism. *Nat. Genet.* **22:** 379–383.

40. Gregory, J. J., Jr., *et al.* (2001). Somatic mosaicism in Fanconi anemia: evidence of genotypic reversion in lymphohematopoietic stem cells. *Proc. Natl. Acad. Sci. USA* **98:** 2532–2537.

41. Gross, M., *et al.* (2002). Reverse mosaicism in Fanconi anemia: natural gene therapy via molecular self-correction. *Cytogenet. Genome Res.* **98:** 126–135.

42. Houghtaling, S., *et al.* (2003). Epithelial cancer in Fanconi anemia complementation group D2 (Fancd2) knockout mice. *Genes Dev.* **17:** 2021–2035.

43. Cheng, N. C., *et al.* (2000). Mice with a targeted disruption of the Fanconi anemia homolog Fanca. *Hum. Mol. Genet.* **9:** 1805–1811.

44. Albella, B., Segovia, J. C., Guenechea, G., Pragnell, I. B., and Bueren, J. A. (1999). Preserved long-term repopulation and differentiation properties of hematopoietic grafts subjected to ex vivo expansion with stem cell factor and interleukin 11. *Transplantation* **67:** 1348–1357.

45. Varas, F., Bernad, A., Almendral, J. M., and Bueren, J. A. (1996). Relevance of myeloablative conditioning in the engraftment of limiting numbers of normal and genetically marked lympho-hematopoietic stem cells. *Bone Marrow Transplant.* **18:** 981–989.