4-15-2005

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Akihiro Ohashi
Quillen-Dishner College of Medicine

Malgorzata Z. Zdzienicka
Leiden University Medical Center - LUMC

Junjie Chen
Quillen-Dishner College of Medicine

Fergus J. Couch
Quillen-Dishner College of Medicine

Citation Information

Ohashi, Akihiro; Zdzienicka, Malgorzata Z.; Chen, Junjie; and Couch, Fergus J.. 2005. Fanconi Anemia Complementation Group D2 (FANCD2) Functions Independently of BRCA2- and RAD51-Associated Homologous Recombination in Response to DNA Damage. *Journal of Biological Chemistry*. Vol.280(15). 14877-14883. https://doi.org/10.1074/jbc.M414669200 PMID: 15671039 ISSN: 0021-9258

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 15, Issue of April 15, pp. 14877–14883, 2005
Printed in U.S.A.

Fanconi Anemia Complementation Group D2 (FANCD2) Functions Independently of BRCA2- and RAD51-associated Homologous Recombination in Response to DNA Damage*

Received for publication, December 30, 2004, and in revised form, January 25, 2005
Published, JBC Papers in Press, January 25, 2005, DOI 10.1074/jbc.M414669200

Akihiro Ohashi‡, Malgorzata Z. Zdzienicka§, Junjie Chen¶‖, and Fergus J. Couch‡

From the ‡Department of Laboratory Medicine and Pathology and ¶Department of Oncology, College of Medicine, Rochester, Minnesota 55905 and §Department of Radiation Genetics and Chemical Mutagenesis-Human and Clinical Genetics, Leiden University Medical Center, Wassenaarseweg 72, 2333AL Leiden, The Netherlands

The BRCA2 breast cancer tumor suppressor is involved in the repair of double strand breaks and broken replication forks by homologous recombination through its interaction with DNA repair protein Rad51. Cells defective in BRCA2·FANCD1 are extremely sensitive to mitomycin C (MMC) similarly to cells deficient in any of the Fanconi anemia (FA) complementation group proteins (FANC). These observations suggest that the FA pathway and the BRCA2 and Rad51 repair pathway may be linked, although a functional connection between these pathways in DNA damage signaling remains to be determined. Here, we systematically investigated the interaction between these pathways. We show that in response to DNA damage, BRCA2-dependent Rad51 nuclear focus formation was normal in the absence of FANCD2 and that FANCD2 nuclear focus formation and mono-ubiquitination appeared normal in BRCA2-deficient cells. We report that the absence of BRCA2 substantially reduced homologous recombination repair of DNA breaks, whereas the absence of FANCD2 had little effect. Furthermore, we established that depletion of BRCA2 or Rad51 had a greater effect on cell survival in response to MMC than depletion of FANCD2 and that depletion of BRCA2 in FANCD2 mutant cells further sensitized these cells to MMC. Our results suggest that FANCD2 mediates double strand DNA break repair independently of Rad51-associated homologous recombination.

Breast cancer is one of the most common cancers affecting women. About half of all familial cases of breast cancer are caused by mutation in the breast cancer susceptibility genes *BRCA1*and *BRCA2*. The *BRCA2* gene encodes a 3,418-amino acid protein (1) containing eight conserved BRC motifs (2, 3) through which BRCA2 binds to Rad51, a protein with a crucial role in DNA recombination and repair (4–6). Because BRCA2 forms a complex with Rad51, a protein directly involved in DNA repair, BRCA2 may link DNA damage signaling pathways with the DNA damage repair machinery.

Recent studies indicate that Brca2-defective V-C8 lung fibroblasts are extremely sensitive to DNA cross-linking agents (7). This phenotype is similar to that of Fanconi anemia (FA)[1] cells (8, 9), suggesting that the BRCA2 and FA proteins may function together (10). Fanconi anemia is an autosomal recessive chromosomal disorder (8, 9). There are 11 complementation groups (A, B, C, D1, D2, E, F, G, I, J, and L) (11–13), and 9 of the *FA* genes have been cloned (*A, B, C, D1/BRCA2, D2, E, F, G*, and *L/PHF9*) (8, 9, 13–16). Seven of the proteins (A, B, C, E, F, G, and L) form a nuclear complex and play a role in the mono-ubiquitination of FANCD2 protein in response to DNA damage (16–21). This modification is required for the repair of DNA cross-links and the accumulation of FANCD2 at the sites of DNA damage (19, 22). Recently FANCL/PHF9 was shown to have ubiquitin-protein isopeptide ligase (E3) activity *in vitro* and to be essential for FANCD2 ubiquitination (14).

Recent studies have suggested genetic interactions between *FA* genes and the breast cancer susceptibility genes *BRCA1* and *BRCA2*. It has been reported that BRCA1 protein is required for mono-ubiquitination (19) and FANCD2 nuclear focus formation (19, 23). Furthermore, BRCA1 directly interacts with ubiquitinated FANCD2 and FANCA protein (19, 24). These reports strongly suggest that the BRCA1 and FA pathways are linked. In addition, it was recently reported that FA-D1 cells contain biallelic BRCA2 inactivating mutations and that expression of full-length BRCA2 in FA-D1 cells can partially reverse the MMC sensitivity of these cells (14). These findings indicate that *BRCA2* is the gene responsible for defects observed in FA-D1 cells. Supporting the involvement of BRCA2 and its associated protein Rad51 in the classical FA pathway, Digweed *et al.* (25) have reported that Rad51 focus formation in response to x-rays depends on the integrity of the FA pathway. In addition it has been suggested that mono-ubiquitination of FANCD2 promotes IR-induced BRCA2 focus formation (26). However, Godthelp *et al.* (27) reported normal Rad51 focus formation after MMC or IR treatment in all FA cells other than FA-D1 cells. Thus, the role of BRCA2 in the classical FA pathway in response to DNA damage remains to be resolved.

In this study, we further investigated the functional interaction between BRCA2 and FANCD2 following DNA damage. We used BRCA2- and FANCD2-deficient cells and cells treated with siRNAs specifically targeting BRCA2, Rad51, and FANCD2 and demonstrated that FANCD2 does not have a

* This work was supported by Grants CA87898 and CA102701 (to F. J. C.) and Grants CA89239 and CA92312 (to J. C.) from the National Institutes of Health and by grants from the Breast Cancer Research Foundation and the Prospect Creek Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Oncology, Mayo Clinic College of Medicine, 200 First St. S.W., Rochester, MN 55905. Tel.: 507-538-1545; Fax: 507-284-3906; E-mail: Chen.Junjie@mayo.edu.

[1] The abbreviations used are: FA, Fanconi anemia; FANC, FA protein; IR, ionizing radiation; MMC, mitomycin C; siRNA, small interfering RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HR, homologous recombination; GFP, green fluorescent protein; Gy, gray.

direct role in BRCA2- and Rad51-associated homologous recombination repair following DNA damage.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—V-C8 (Brca2-defective lung fibroblast cells) and BRCA2-reconstituted V-C8 cell lines were cultivated in Ham's F-10 (BioWhittaker Inc.) supplemented with 10% fetal bovine serum (7). The reconstituted V-C8 cell lines cell lines were established by transfecting with a full-length wild-type BRCA2 mammalian expression plasmid and selected for G418-resistant clones (5). BRCA2 expression in reconstituted clones was determined by Western blotting following immunoprecipitation using rabbit anti-BRCA2 antibody (5). Drs. Alan D. D'Andrea and Toshiyasu Taniguchi kindly provided FA-D1 cells (VU423/PMMP), FA-D1 cells stably expressing BRCA2 (A913 423/2-33), FA-D2 cells (PD20), and FA-D2-derivative cells stably expressing FANCD2 (PD20 + FANCD2). PD20 cells were cultured in RPMI 1640 medium supplemented with 15% fetal bovine serum. PD20 + FANCD2 cells also received 1 μg/ml puromycin. VU423/PMMP cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum containing 1 μg/ml puromycin. A913 423/2-33 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum containing hypoxanthine/aminopterin/thymidine and 200 μg/ml hygromycin.

Clonogenic Survival Assays—Cells were trypsinized, and 500–1,000 cells (2,500–5,000 cells for high doses) were seeded in a 100-mm dishes and left to attach for 4 h. For MMC treatment, cells were continuously exposed to 0, 0.01, 0.1, 1, or 10 ng/ml MMC for 8 days. For IR treatment, cells were exposed to γ radiation at the dose of 0, 4, 8, or 12 Gy. Eight days after those treatments, cells were stained and counted. Each survival curve represents the mean of at least two independent experiments. Error bars represent the standard deviations of the means.

Immunoprecipitation, Immunoblotting, and Immunostaining—Immunoprecipitation was carried out as described previously (5). Briefly, immunoprecipitation of BRCA2 was carried out using 1 μg of anti-BRCA2 polyclonal antibody (BRCA2C) (5). Proteins bound to protein G beads were eluted by boiling in SDS gel sample buffer and separated by SDS-PAGE. Immunoblotting was performed using anti-BRCA2 antibodies (BRCA2C) (5), anti-Rad51 antibodies (5), and anti-FANCD2 antibodies (FI17, Santa Cruz Biotechnology, Inc.) at a concentration of 5 μg/ml. Immunoblotted proteins were visualized by chemiluminescence. Immunofluorescence was performed (5) using affinity-purified anti-Rad51 antibodies and anti-FANCD2 antibodies at a concentration of 1–2 μg/ml. Treatment with MMC or IR was carried out as described previously (14, 19, 22, 25). Images were captured with a 100× oil lens on an Axioplan fluorescence microscope. At least 150 cells in 5–10 different fields were counted for each experiment.

Homologous Recombination (HR) Repair Assay—An HR repair assay was carried out as described previously (28). Briefly, the efficiency of HR was assessed using an I-SceI expression plasmid (pCBASce) and an I-SceI repair reporter plasmid (DR-GFP) composed of two differentially mutated GFP genes, one of which contained a unique I-SceI restriction site. The assay works through gene conversion repair of a double strand break caused by I-SceI digestion. DR-GFP plasmids repaired by homologous recombination express GFP (29). The relevant cells were transfected with either 2 μg of DR-GFP plus 8 μg of pCBASce or 2 μg of DR-GFP plus 8 μg of control plasmids. 48 h after transfection, the cells were harvested, and the number of GFP-expressing cells was assessed by flow cytometry.

RNA Interference—RNA interference was performed using the Smart Pool siRNA (Dharmacon) for BRCA2, Rad51, and FANCD2. A pSSHUP vector with BRCA2 short hairpin RNA was used for PD20 cells or PD20 + FANCD2 cells. A pSSHUP vector (courtesy of Daniel Billadeau, Mayo Clinic) that produced a short hairpin RNA against BRCA2 was generated with the following oligonucleotides: forward, 5′-gatccccGTTTAGAAAGCCAAGCTACttcaagagaGTAGCTTGGCTTTCTAAACtttttggaaa-3′; reverse, 5′-gggCAAATCTTTCGGTTCGATGaagttctctCATCGAACCGAAAGATTTGaaaaacctttt-3′. Transfection of siRNA oligonucleotides was carried out with LipofectAMINE 2000 (Invitrogen) in 6-well plates according to the manufacturer's specifications. Cells were incubated for 48 h posttransfection prior to MMC treatment.

MTT Assay—The sensitivity of siRNA-transfected cells to MMC was evaluated using an MTT assay as described previously (30). 48 h after transfection, 5,000 cells were seeded into 96-well plates with 100 μl of culture medium, incubated for 16 h, and exposed to various concentrations of MMC for 72 h. MTT solution (CellTiter 96 AQueous One Solution Reagent, Promega) (20 μl) was added to each well and incubated for 1.5 h. The absorbance of each well was measured in a microplate reader at 490 nm, and the absorbance in treated cells relative to untreated cells was displayed.

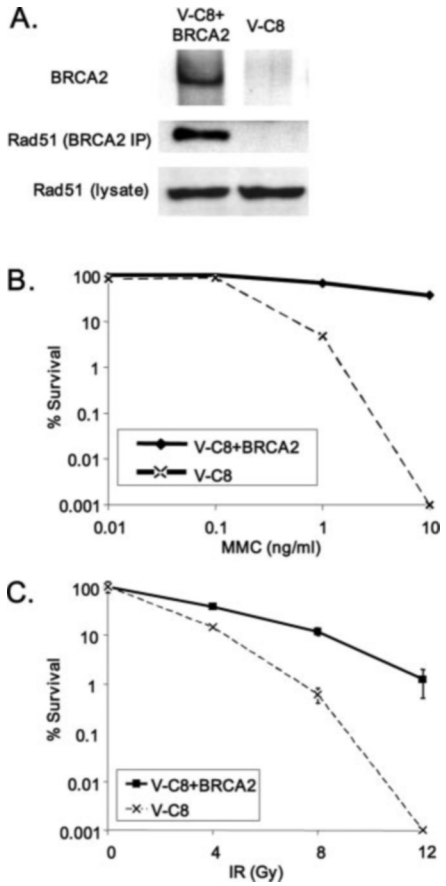

FIG. 1. **BRCA2 is required for cell survival following DNA damage.** *A*, reconstituted BRCA2 in V-C8 cells co-immunoprecipitates with Rad51. V-C8 and V-C8 + BRCA2 cell extracts were immunoprecipitated (*IP*) with anti-BRCA2 antibodies and immunoblotted with anti-BRCA2 (*top panels*) or anti-Rad51 antibodies (*middle panel*). Whole cell Rad51 was used as a loading control (*bottom panel*). *B* and *C*, reconstituted BRCA2 in V-C8 cells enhances survival in response to MMC (*B*) and IR (*C*). V-C8 and V-C8 + BRCA2 cells were treated with different amounts of MMC (*B*) and IR (*C*), and cell survival was measured by clonogenic survival. *Solid lines* indicate V-C8 + BRCA2 cells, and *dotted lines* indicate V-C8 cells. Data are presented as the mean ± S.E. (n = 2).

RESULTS

BRCA2 Is Required for Cell Survival and Rad51 Focus Formation following MMC and Ionizing Radiation—Recent studies indicate that BRCA2-defective cells are extremely sensitive to cross-linking agents (7). To confirm that this hypersensitivity in V-C8 cells deficient in Brca2 is due to the loss of Brca2, we generated V-C8-derivative cell lines stably expressing wild-type human BRCA2 (V-C8 + BRCA2). Expression of human BRCA2 in these V-C8 cells was confirmed by immunoblotting (Fig. 1*A*). We noted that the exogenously expressed human BRCA2 associated with endogenous Rad51 in these cells (Fig. 1*A*, *middle panel*) and that the expression level of Rad51 remained the same as that in V-C8 cells (Fig. 1*A*, *bottom panel*). The reconstitution of a BRCA2·Rad51 DNA repair complex in the V-C8 + BRCA2 cells using exogenous human BRCA2 suggested that BRCA2-dependent DNA repair was active in these cells. This was confirmed by demonstrating that the sensitivity of V-C8 cells to double strand breaks and cross-links induced by IR and MMC was largely rescued by the introduction of human BRCA2 into these cells (Fig. 1, *B* and *C*). In addition, although V-C8 cells showed defective Rad51 focus formation following MMC or IR treatment, we found that BRCA2-reconstituted

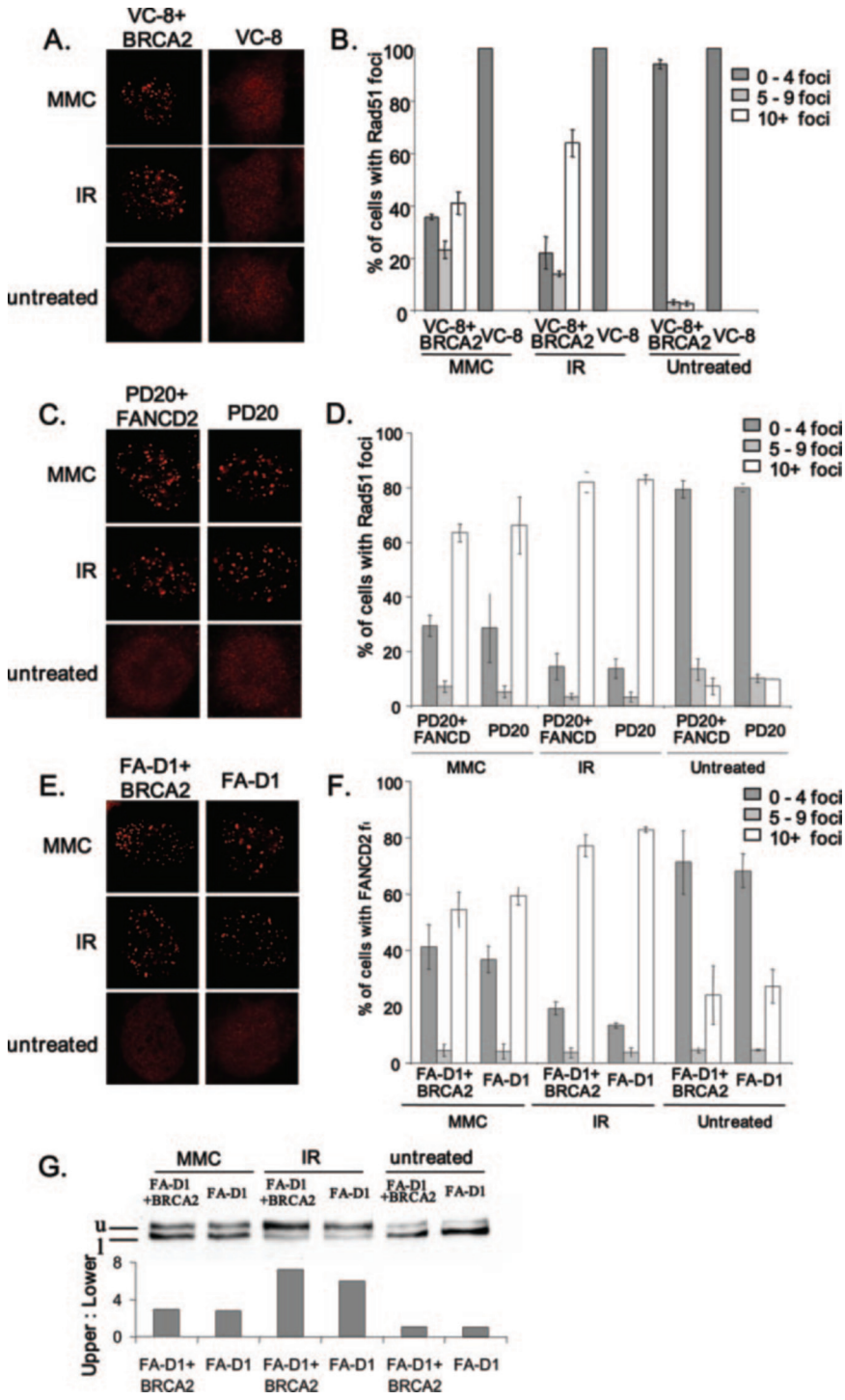

FIG. 2. **FANCD2 functions independently of Rad51 and BRCA2 in response to DNA damage.** *A*, V-C8 reconstituted with BRCA2 form Rad51 foci. V-C8 and V-C8 + BRCA2 cells were treated with 10 ng/ml MMC or 8 Gy of IR and immunostained with anti-Rad51 antibodies. *B*, quantification of Rad51 focus formation in V-C8 and reconstituted cells. Nuclei were grouped by the number of Rad51 foci per nucleus. Data are presented as the mean ± S.E. (n = 3). *C*, disruption of FANCD2 does not alter Rad51 focus formation. PD20 cells and PD20 FANCD2-reconstituted cells were treated with 40 ng/ml MMC or 12 Gy of IR and immunostained with anti-Rad51 antibodies. *D*, quantification of Rad51 focus formation in FA-D2 and reconstituted cells. Nuclei were grouped as in *B*. *E*, BRCA2 is not required for FANCD2 focus formation after DNA damage. FA-D1 and FA-D1 + BRCA2 cells were treated with 25 ng/ml MMC or 12 Gy of IR and immunostained with anti-FANCD2 antibodies. *F*, quantification of FANCD2 focus formation in FA-D1 and reconstituted cells. Nuclei were grouped by number of foci. Data are presented as the mean ± S.E. (n = 3). *G*, BRCA2 does not influence FANCD2 mono-ubiquitination. FA-D1 and FA-D1 BRCA2-reconstituted cells were treated with 40 ng/ml MMC or 12 Gy of IR and immunoblotted with anti-FANCD2 antibody. The letters *u* and *l* indicate the mono-ubiquitinated (FANCD2-L) and nonubiquitinated (FANCD2-S) forms of FANCD2, respectively. Band intensity of FANCD2 was measured by densitometry. The *lower panel* shows band intensity of FANCD2-L relative to FANCD2-S.

V-C8 cells displayed a 64.3% increase in the number of cells containing ≥5 foci following MMC treatment and a 78.0% increase after IR treatment (Fig. 2, *A* and *B*). Taken together, these data confirm that BRCA2 is required for Rad51 focus formation following DNA damage and suggest that the absence of BRCA2 confers hypersensitivity to DNA cross-linking agents through an inability to recruit Rad51 homologous recombination complexes to sites of DNA damage.

FANCD2 Is Not Required for Damage-induced Rad51 Focus Formation—Fanconi anemia cells, like V-C8 cells, are extremely sensitive to MMC. To explore the connection between BRCA2 and FANCD2, we examined whether Rad51 damage-induced nuclear focus formation depends on FANCD2. For this study, we used FA-D2 cells (PD20) and an FA-D2-derivative cell line stably expressing human FANCD2 (PD20 + FANCD2). As shown in Fig. 2*C*, Rad51 localized to nuclear foci following MMC or IR treatment in both PD20 cells and PD20 + FANCD2 cells. The percentage of Rad51-foci positive cells in PD20 cells following MMC treatment or IR treatment was very similar to that in PD20 cells reconstituted with wild-type FANCD2 (Fig. 2*D*). In contrast, FANCD2 foci were detected only in FA-D2 + FANCD2 cells and not in FA-D2 cells (data not shown). Thus, it appears that FANCD2 is not required for the localization of Rad51 to nuclear DNA damage foci.

BRCA2 Is Not Required for FANCD2 Mono-ubiquitination or FANCD2 Focus Formation following DNA Damage—Although FANCD2 is not required for Rad51 localization to nuclear DNA damage foci, it is possible that the BRCA2·Rad51 complex may

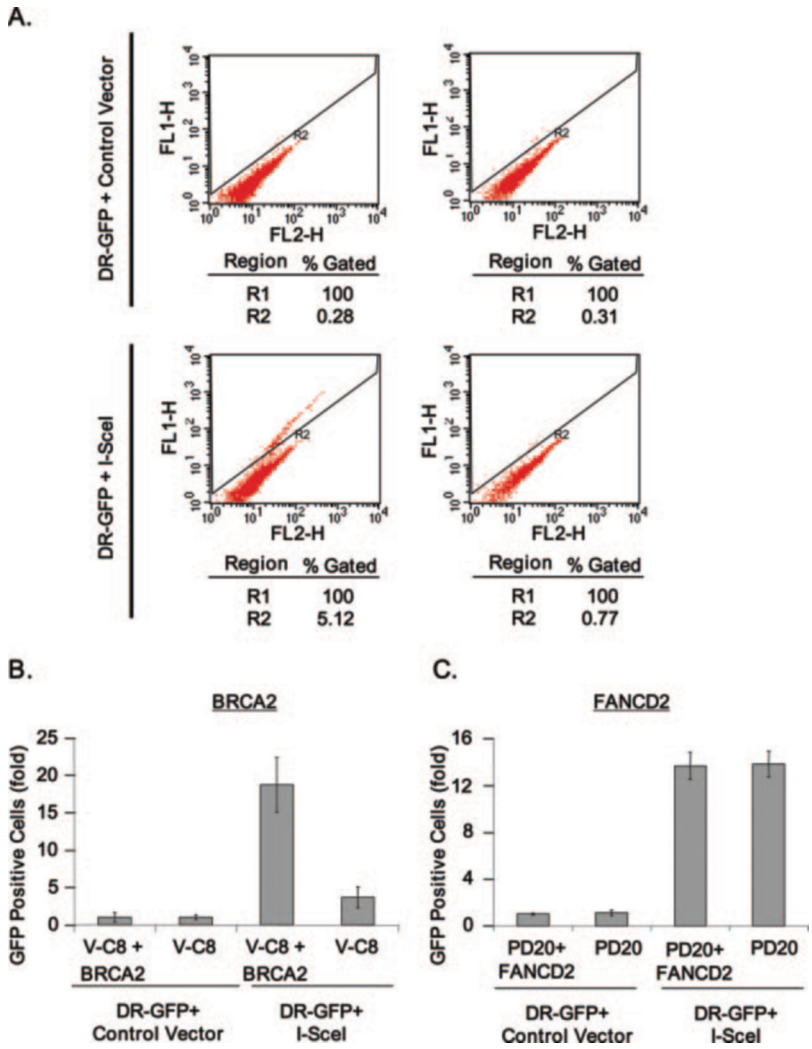

FIG. 3. **BRCA2 but not FANCD2 mediates HR repair.** *A*, fluorescence-activated cell sorting analysis for the detection of GFP positive cells following repair of I-SceI-induced DNA breaks in BRCA2-reconstituted V-C8 cells. V-C8 and V-C8-reconstituted cells transfected with I-SceI and/or DR-GFP reporter plasmids were evaluated for GFP positive cells by fluorescence-activated cell sorting. *Upper panels* show the cells transfected with DR-GFP and control vector (negative control). *Lower panels* show cells transfected with DR-GFP and I-SceI. *Left* and *right panels* show V-C8 + BRCA2 and V-C8 cells, respectively. GFP-expressing cells are located above the diagonal lane. *FL1-H* represents GFP intensity, and *FL2-H* represents cell size. The percentage of GFP positive cells is shown below each panel as *R2 % Gated*. *B*, quantification of HR repair in V-C8 + BRCA2 and V-C8 cells. The -fold increase in GFP positive cells relative to vector control in V-C8 + BRCA2 cells is shown. *C*, quantification of HR repair in PD20 + FANCD2 and PD20 cells. The -fold increase in GFP positive cells relative to vector control in PD20 + FANCD2 cells is shown.

be involved in the regulation of FANCD2. To investigate whether BRCA2 affects FANCD2 regulation following DNA damage, we evaluated FANCD2 DNA damage focus formation in the absence of functional BRCA2 using BRCA2 mutant FA-D1 cells (VU423/PMMP) cells. Following MMC or IR treatment, the percentage of FA-D1 cells with FANCD2 foci was similar to that in FA-D1 + BRCA2 cells (Fig. 2, *E* and *F*). Similarly to V-C8 cells, these FA-D1 cells showed defective Rad51 focus formation after MMC or IR treatment, which could be restored upon expression of exogenous human BRCA2 (data not shown). This suggests that BRCA2 is not required for the formation of FANCD2 foci following DNA damage.

Previous studies have shown that FANCD2 is mono-ubiquitinated following DNA damage and that this modified form of FANCD2 can be detected as an upward mobility shift on SDS-PAGE (19, 22). Therefore, we further evaluated the influence of BRCA2 on FANCD2 function by examining the effect of the absence of functional BRCA2 on FANCD2 ubiquitination in BRCA2 mutant FA-D1 cells. As shown in Fig. 2*G*, the mono-ubiquitinated form of FANCD2 was prominent in both FA-D1 cells and FA-D1 cells reconstituted with human BRCA2 (A913 423/2-33; FA-D1 + BRCA2) following MMC or IR treatment. Importantly, the expression levels of FANCD2 in these cells remained the same (data not shown). Taken together these data strongly suggest that BRCA2 is not required for FANCD2 activity in response to DNA damage.

FANCD2 Is Not Required for Homologous Recombination Repair of DNA Breaks—To confirm the requirement for BRCA2 in HR repair and to investigate whether FANCD2 is involved in the same HR repair pathway as BRCA2, we used an I-SceI-dependent GFP reporter assay to measure homologous recombination activity in BRCA2- or FANCD2-deficient cells (29). This assay depends on reconstitution of the GFP signal as a consequence of repair of an I-SceI-induced double strand break, as described under "Experimental Procedures." We transfected V-C8, V-C8 + BRCA2, PD20, and PD20 + FANCD2 cells with the DR-GFP reporter plasmid and an I-SceI expression plasmid (pcBASce). 48 h after transfection the proportion of cells expressing GFP following homologous recombination repair of the I-SceI-induced double strand break was measured by flow cytometry. As shown in the example in Fig. 3*A*, GFP positive cells were rare in both V-C8 + BRCA2 and V-C8 cells transfected with DR-GFP and control vector, whereas the combination of DR-GFP and I-SceI resulted in a substantial increase in GFP positive cells in V-C8 + BRCA2 cells (*left panel*) but not in V-C8 cells (*right panel*). When taking the mean of three experiments, we detected an 18.6-fold increase in GFP cells in BRCA2-reconstituted V-C8 cells but only a 3.9-fold increase in GFP cells among V-C8 cells, relative to the controls (Fig. 3, *A* and *B*). These results indicate that BRCA2 is required for recombination repair of I-SceI-induced DNA double strand breaks.

In FANCD2-deficient PD20 cells, we observed a 13.9-fold increase of GFP positive cells following DNA breaks when compared with controls (Fig. 3*C*). Moreover, this increase is very similar to the one observed in PD20 cells reconstituted

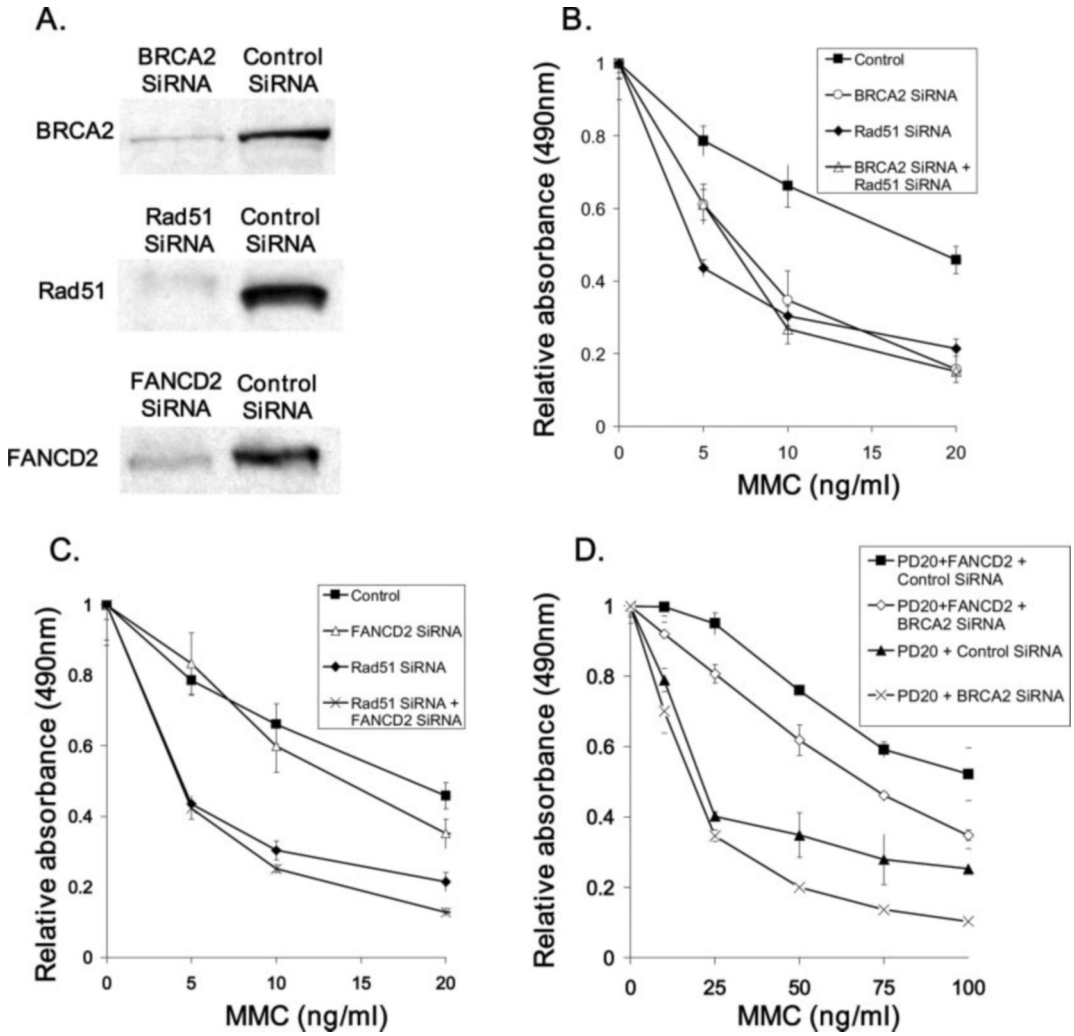

FIG. 4. **FANCD2 functions independently of BRCA2 and Rad51 in cell survival following MMC treatment.** *A*, depletion of BRCA2, Rad51, and FANCD2 by siRNAs. 293T cells were transfected with BRCA2 siRNA oligonucleotides and immunoblotted with anti-BRCA2 antibodies. Similarly, HeLa cells were transfected with Rad51 or FANCD2 siRNAs and immunoblotted with anti-Rad51 or anti-FANCD2 antibodies. *B*, depletion of BRCA2 and Rad51 reduces cell survival in response to DNA damage. HeLa cells were transfected with control siRNA (*filled square*), BRCA2 siRNA (*open circle*), Rad51 SiRNA (*filled diamond*), or both BRCA2 and Rad51 siRNAs (*open triangle*) and analyzed for MMC sensitivity using MTT assay. Data are presented as the mean ± S.E. ($n = 3$). *C*, depletion of FANCD2 enhances MMC sensitivity in Rad51-deficient cells. HeLa cells transfected with control siRNA (*filled square*), FANCD2 siRNA (*open triangle*), Rad51 siRNA (*filled diamond*), or both FANCD2 and Rad51 siRNAs (× *sign*) were analyzed for MMC sensitivity using MTT assay. Data are presented as the mean ± S.E. ($n = 3$). *D*, depletion of BRCA2 enhances MMC sensitivity in FANCD2-deficient cells. PD20 cells (*filled triangle*), PD20 FANCD2-reconstituted cells (*filled square*), PD20 cells with BRCA2 siRNA (× sign), and PD20 FANCD2 cells with BRCA2 siRNA (*open diamond*) were analyzed for MMC sensitivity using MTT assay. Data are presented as the mean ± S.E. ($n = 3$).

with wild-type FANCD2 (13.7-fold increase), suggesting that FANCD2 does not make a substantial contribution to HR repair, whereas BRCA2 is required for this process.

FANCD2 Mediates the Response to MMC Treatment in a BRCA2- and Rad51-independent Manner—To assess whether BRCA2·Rad51 and FANCD2 contribute independently to cell survival in response to MMC, we utilized RNA interference approaches. 293T cells or HeLa cells were transfected with siRNAs targeting BRCA2, Rad51, and FANCD2. Each of these siRNAs significantly reduced the expression of targeted proteins (Fig. 4*A*). In addition, treatment of cells with combinations of these siRNAs resulted in similar reductions in protein levels (data not shown). As expected, depletion of each of these proteins substantially reduced cell survival following MMC treatment. Specifically, depletion of BRCA2 or Rad51 resulted in hypersensitivity to MMC (Fig. 4*B*). As sensitivity to MMC was not increased when both proteins were depleted in unison, it appears that BRCA2 and Rad51 respond to MMC treatment through the same pathway. In contrast, we noted that depletion of FANCD2 had a small but nonetheless significant effect on cell survival following MMC treatment (Fig. 4*C*), suggesting that FANCD2 might not influence MMC sensitivity in the same manner as BRCA2 and Rad51. Importantly, when FANCD2 was depleted along with Rad51, we observed enhanced MMC sensitivity compared with cells with either Rad51 or FANCD2 depletion alone (Fig. 4*C*). This increase in MMC sensitivity following co-depletion of FANCD2 was also observed in the cells depleted of BRCA2 (data not shown). The additive effect of FANCD2 deficiency and either Rad51 or BRCA2 deficiency on MMC sensitivity suggests that FANCD2 functions through a BRCA2- and Rad51-independent repair pathway. However, it is formally possible that combinations of partial defects in the same functional pathway, as might be caused by combined siRNA treatments, could lead to a greater effect than for a single defect. To address this possibility, we evaluated the influence of reduced BRCA2 levels on MMC sensitivity in FANCD2-deficient PD20 cells and in FANCD2-reconstituted PD20 cells. As shown in Fig. 4*D*, depletion of BRCA2 in both

PD20 and PD20-reconstituted cells enhanced MMC sensitivity. Given that the FANCD2 pathway is inactive in these cells, the added MMC sensitivity following BRCA2 depletion provides strong evidence against cooperation of partial defects in the same pathway. Overall, these results suggest that FANCD2 and BRCA2·Rad51 function independently in cell survival following MMC treatment.

DISCUSSION

In this study, we investigated the relationship between BRCA2·Rad51 and FANCD2 in DNA damage-signaling pathway and DNA repair. We have shown that Rad51 focus formation following DNA damage depends on BRCA2 but not FANCD2. In addition, the regulation of FANCD2 following DNA damage is independent of BRCA2, suggesting that FANCD2 may function independently of BRCA2·Rad51. In agreement with this hypothesis, we have shown that FANCD2 is dispensable for a BRCA2·Rad51-dependent homology-directed recombination. Moreover, cells with deficiency in both FANCD2 and BRCA2·Rad51 pathways are more sensitive to MMC, supporting the observation that FANCD2 and BRCA2·Rad51 function in different DNA repair pathways.

BRCA2 mutant and deficient cells display extreme sensitivity to DNA damage. In this study, we confirm that the DNA damage hypersensitivity in V-C8 cells (Brca2-deficient) is due to the lack of full-length Brca2. In addition, we show that Rad51 focus formation in response to DNA damage depends on BRCA2 (31), as previously reported for CAPAN-1 cells and Brca2-deficient mouse embryonic fibroblasts (32, 33). Taken together, these results suggest that BRCA2 is directly involved in DNA repair by recruiting Rad51 to the sites of DNA damage. Our findings support recent studies showing that BRCA2 and Rad51 are recruited together to sites of double strand DNA breaks (34) to facilitate homologous recombination repair (29) as opposed to single strand annealing (35).

BRCA2 and Rad51 mutant and deficient cells display great sensitivity to DNA cross-linking agents such as MMC, a phenomenon similar to that observed in Fanconi anemia cells (10). As biallelic mutations of *BRCA2* have been identified in FA-D1 cells (14), it is now accepted that *BRCA2* mutations are responsible for the phenotype observed in FA-D1 cells. These observations led to the hypothesis that the FA pathway is linked with the BRCA2 and Rad51 homologous recombination DNA repair pathway. Data in support of this came from the observations by Digweed *et al.* (25) that Rad51 foci are attenuated in FA-A, FA-C and FA-G cells after IR exposure. In addition, it appears that FANCG interacts with BRCA2 and co-localizes with BRCA2 after DNA damage (36). It has also been reported that FANCD2 interacts with BRCA2 (37) and is required for BRCA2 focus formation (26). In addition, mono-ubiquitination of FANCD2 is needed for the assembly of IR-induced FANCD2 and BRCA2 foci (26). However, Godthelp *et al.* (27) reported that normal Rad51 focus formation is observed in all FA cells except FA-D1 cells in response to DNA damage induced by MMC or X-rays, suggesting that the BRCA2-associated Rad51 homologous recombination repair pathway and the FA repair pathway may function independently in the response to these types of DNA damage.

In this study, we attempted to clarify these apparently inconsistent findings by systematically examining the contribution of FANCD2 to the BRCA2- and Rad51-dependent DNA damage response. Our data show the normal appearance of nuclear Rad51 foci in FANCD2-deficient PD20 cells. We did not detect any quantitative difference in Rad51 focus formation in FANCD2-deficient or proficient cells following MMC or IR treatment. We also noted that Rad51 focus formation appeared normal in FA-F cells following MMC or IR treatment (data not shown), whereas Rad51 foci failed to form in BRCA2-deficient cells. Our results are in keeping with those of Godthelp *et al.* (27) and strongly suggest that FANCD2 is not required for Rad51 focus formation following DNA damage. The results also appear to suggest that FANCD2 functions independently of BRCA2 and Rad51 in DNA repair.

To further address this possibility, we evaluated the influence of BRCA2 deficiency on FANCD2 function. We found that FANCD2 foci formed normally in BRCA2-deficient cells following exposure to DNA damaging agents. We also failed to detect any difference in damage-induced mono-ubiquitination of FANCD2 in BRCA2-defective or reconstituted cells, suggesting that BRCA2 is not involved in FANCD2 mono-ubiquitination after DNA damage. In support of our studies, Bruun *et al.* (38) have recently shown that BRCA2 depletion does not affect FANCD2 ubiquitination after DNA damage. Taken together, these findings establish that BRCA2 is not upstream of FANCD2 in the DNA damage-signaling pathway.

We next assessed the role of FANCD2 in homologous recombination repair of DNA breaks. It has been clearly established using a homology-directed repair reporter assay (29) that BRCA2 and Rad51 are required for HR repair of DNA double strand breaks. We used the same assay to confirm the requirement for BRCA2 in HR repair in V-C8 cells and to demonstrate that FANCD2 does not have a significant involvement in this BRCA2- and Rad51-dependent HR repair using FANCD2-deficient PD20 cells. Similarly, Nakanishi *et al.* (39) have reported that a deficiency in FANCD2 has a very mild effect on HR repair as measured by this assay (39). In addition, we used RNA interference to show that depletion of FANCD2 resulted in mild MMC sensitivity compared with the effects of BRCA2 or Rad51 depletion. Moreover, we observed that FANCD2 depletion or inactivation in combination with depletion of BRCA2 or Rad51 enhanced Rad51- and BRCA2-dependent MMC sensitivity, whereas double depletion of BRCA2 and Rad51 did not enhance MMC sensitivity.

On the basis of these data we propose a model in which FANCD2 mediates repair of IR-induced and other DNA damage through a pathway that is independent of the Rad51 homologous recombination repair pathway. Similarly, Nakanishi *et al.* (39) have proposed a role for FANCD2 in a second BRCA2-independent double strand break pathway involving homology. Likewise, based on the recent finding that FANCD2-disrupted chicken DT40 cells exhibit normal Rad51 repair foci and do not display decreased spontaneous sister chromatid exchange, Yamamoto *et al.* (40) have proposed that FANCD2 functions in a sub-DNA repair pathway that is independent of DNA crossover and sister chromatid exchange. Importantly these FANCD2-defective chicken cells were deficient in HR-mediated DNA repair. In contrast, studies in human cells have shown that deficiency in FANCD2 does not have a substantial effect on HR repair in human cells (39). The difference in these observations may be explained by the use of different HR assays to detect homology-directed repair. Certainly, a number of different recombination assays exist that appear to measure different HR and double stand break repair processes, some of which are yet to be fully understood. Alternatively, the contrasting results may derive from differences in double strand break repair between chicken DT40 cells and human cells. Nevertheless, it is clear that FANCD2 functions in a DNA repair pathway that is different from that of BRCA2 and Rad51. Future studies are needed to elucidate the molecular mechanisms of the FA- or FANCD2-dependent DNA repair pathway.

Acknowledgments—We thank Alan D. D'Andrea and Toshiyasu Taniguchi for valuable reagents and members of the Chen and Couch

laboratories for helpful discussions. We thank Tsuyoshi Ito for helping with fluorescence-activated cell sorting analyses.

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