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ORIGINAL PAPERS

Hypersensitivity of Brca1-deficient MEF to the DNA interstrand crosslinking agent mitomycin C is associated with defect in homologous recombination repair and aberrant S-phase arrest

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Hypersensitivity of Brca1-deficient cells to interstrand crosslinking (ICL) agents such as cisplatin and mitomycin C (MMC) implicates an important role for Brca1 in cellular response to the ICL DNA damage repair. However, the detailed mechanism of how Brca1 is involved in the ICL response remains unclear. In this study, we analysed the cellular response to MMC treatment using isogenic mouse embryonic fibroblasts (MEFs) including wild type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$. Marked hypersensitivity of $p53^{-/-}Brca1^{-/-}$ MEFs to MMC was found, and the reconstitution of Brca1 expression in these cells restored resistance to MMC. Upon MMC treatment, wild-type MEF was temporarily arrested at G2/M phase but subsequently resumed a normal cell cycle progression. In contrast, Brca1-deficient MEF exhibited a marked time-dependent accumulation of cells arrested at S phase and a prolonged increase in the G2/M population, followed by extensive cell deaths. Importantly, DNA damage-induced Rad51 foci were not formed in these cells, suggesting a defect in homologous recombination. Such defects are fully rescued by reconstitution of Brca1 expression in Brca1-deficient MEF, suggesting that Brca1 directly plays an essential role in ICL repair, which depends on homologous recombination during S phase.
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Introduction

DNA interstrand crosslinks (ICLs) are among the most toxic of all the DNA lesions. ICL prevents DNA strand separation, blocks essential cellular processes such as DNA replication, transcription and recombination, and eventually induces cell cycle arrest, chromosomal rearrangement and cell death. ICL agents such as cisplatin, mitomycin C (MMC) and nitrogen mustard are widely used as potent anticancer therapeutics (Metzler, 1986); however, the molecular detail of ICL repair is still not well understood in higher mammalian cells. Although the detailed sequential events remain to be determined, previous studies suggest that nucleotide excision repair (NER) and homologous recombination (HR) are involved in ICL repair (reviewed in Dronkert and Kanaar, 2001).

In contrast to procaryotes, cumulative evidence suggests that ICLs elicit double-stranded breaks (DSBs) in eucaryotes (Dardalhon and Averbeck, 1995; De Silva *et al*., 2000; McHugh *et al*., 2000). DSBs are produced as repair intermediate following treatment with ICL agents (De Silva *et al*., 2000) and can also be generated near the sites of ICL during S phase of the cell cycle when encountered by replication fork (Akkari *et al*., 2000). Thus, it has been proposed that in mammalian cells, the repair of ICL proceeds via the formation of a DSB and followed by DSB repair via HR (Rothfuss and Grompe, 2004). Consistent with this model, cells deficient in Rad51 paralog or other proteins involved in HR-mediated DSB repair are sensitive to DNA damage induced by ICL agents (De Silva *et al*., 2000; Dronkert and Kanaar, 2001). Rad51, a mammalian homolog of the bacterial protein RecA, is essential for repair of DNA DSB by HR (Sonoda *et al*., 1998).

The importance of understanding ICL repair is also highlighted by the hypersensitivity to ICL agents among Fanconi anemia (FA) patients (Sasaki and Tonomura, 1973) and in cells lacking breast cancer tumor suppressor genes, *BRCA1* and *BRCA2* (Bhattacharyya *et al*., 2000; Yu *et al*., 2000; Moynahan *et al*., 2001). Interestingly, recent studies suggest interactions between FA gene products and BRCA1 and BRCA2 (Garcia-Higuera *et al*., 2001; D'Andrea and Grompe, 2003). Following DNA damage, FANCD2 protein colocalizes in nuclear foci with BRCA1 and Rad51 in S phase (Taniguchi *et al*., 2002). These studies indicate the involvement of the FA/BRCA pathway in HR-mediated repair of DNA damage induced by ICL agents.

Substantial evidence exists to support a role for BRCA1 in mediating the cellular response to DNA lesions, especially, DNA DSBs (reviewed in Zheng *et al*.,

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2000a; Ting and Lee, 2004). The direct association of BRCA1 with the Rad50 complex and the subnuclear colocalization of BRCA1 with Rad50 in IR-induced foci suggest a role for BRCA1 in DSB repair (Zhong *et al*., 1999). DSBs are repaired either by HR or nonhomologous end joining (NHEJ). The presence of BRCA1 is required for the efficient formation of IR-induced Rad51 foci (Scully *et al*., 1997), and embryonic stem (ES) cells expressing exon 11-deleted Brca1 only show reduced HR activity compared to the wild type in studies using a defined chromosomal substrate (Moynahan *et al*., 1999, 2001). These data suggest that Brca1 is necessary for HR-mediated repair via Rad51. Moreover, BRCA1 also plays a critical role in NHEJ as shown in studies using both *in vitro* and *in vivo* substrates (Zhong *et al*., 2002a, b), supporting the notion that Brca1 directly participates in DSB repair. In addition, BRCA1 is involved in transcriptional regulation, cell cycle checkpoint control and cell death (reviewed in Zheng *et al*., 2000a; Ting and Lee, 2004). Although the hypersensitivity of Brca1-deficient cells to ICL agents has been observed, the detailed mechanism of how Brca1 participates in this process remains unclear.

In this communication, we examined the role of Brca1 in response to treatment with ICL agents by analysing cell cycle progression of isogenic wild-type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ mouse embryonic fibroblasts (MEFs). The marked hypersensitivity of $p53^{-/-}Brca1^{-/-}$ MEFs to MMC treatment and the restoration of MMC resistance by reconstitution of Brca1 expression in $p53^{-/-}Brca1^{-/-}$ MEFs substantiated the essential role of Brca1 in ICL repair. Wild-type MEFs were temporarily arrested at G2/M following MMC treatment; however, they resumed a normal cell cycle progression subsequently. Conversely, Brca1-deficient MEFs exhibited a time-dependent accumulation of S-phase arrest, following a severe apoptosis, after MMC treatment. Reconstitution of Brca1 expression in Brca1-deficient MEFs restored efficient Rad51 foci formation, rescued S-phase arrest and prevented cell death, suggesting that Brca1 plays an important role in ICL repair during S phase.

Results

Brca1-deficient MEFs are hypersensitive to MMC

To address the role of BRCA1 in the repair process of ICL DNA damage, we first examined the cellular response to the ICL agent MMC using previously generated isogenic wild-type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs (Zheng *et al*., 2000b). First, cell survivals after treatment with various doses of MMC were measured by colony forming assay. As shown in Figure 1, wild-type and $p53^{-/-}$ MEFs showed considerable resistance up to the level of 0.8 μg/ml of MMC treatment. $p53^{-/-}$ MEFs showed similar or higher resistance, suggesting that p53 itself is not critical to cellular resistance at least in this range of MMC concentration. However, $p53^{-/-}Brca1^{-/-}$ MEFs showed marked hypersensitivity to MMC treatment compared to the wild-type and $p53^{-/-}$ MEFs. With 0.4 μg/ml of MMC treatment, $p53^{-/-}Brca1^{-/-}$ MEFs were about 100-fold more sensitive than wild-type and $p53^{-/-}$ MEFs, suggesting Brca1 is critical for proper ICL repair. Consistently, the profound MMC hypersensitivity of $p53^{-/-}Brca1^{-/-}$ MEFs may be attributed to the absence of the full-length Brca1 because ES cells expressing the exon 11-deleted isoform also exhibit a similar hypersensitivity (Moynahan *et al*., 2001), irrespective of a difference in cell types. Nevertheless, these results indicated that our isogenic MEFs provide an ideal system for examining the roles of Brca1 in the cellular response to ICL agents.

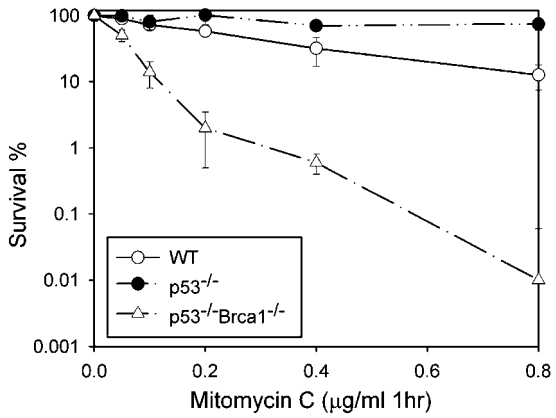

Figure 1 $Brca1^{-/-}$ MEFs are hypersensitive to MMC. Clonogenic survival of wild-type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs was examined in 100 mm dish after 1 h treatment with various doses of MMC. Colonies were stained after 10–12 days with methylene blue and counted. Error bars indicate the standard deviation

Brca1-deficient MEFs showed a prolonged increase of G2/M and extensive cell death in response to MMC

To understand the molecular basis for the hypersensitive phenotype of $p53^{-/-}Brca1^{-/-}$ MEFs, we examined the cell cycle response upon MMC treatment by flow cytometric analysis of propidium iodide (PI)-stained nuclei. In wild-type MEFs, cells arrested at G2/M phase emerged 12 h after MMC treatment and the number peaked after 24 h (Figure 2a and b). However, this arrest was released after 36 h and the cells re-entered a normal cell cycle progression, suggesting that MMC-induced DNA damage was completely repaired. In $p53^{-/-}$ MEFs, G2/M-arrested population was not accumulated after MMC treatment, suggesting the importance of p53 for the initiation of G2/M arrest upon MMC treatment, as previously described (Innocente *et al*., 1999). On the other hand, unlike these two types of MEFs, the proportion of G2/M population in the $p53^{-/-}Brca1^{-/-}$ MEFs was increased 12 h after MMC treatment and maintained until 48 h after treatment (Figure 2a and b). Consequently, the G1 fraction was diminished and sub-G1 fraction, indicative of cell death, emerged after 24 h and gradually increased in the later time course. These results suggest that Brca1-deficient MEFs cannot efficiently repair DNA damage induced by MMC, and

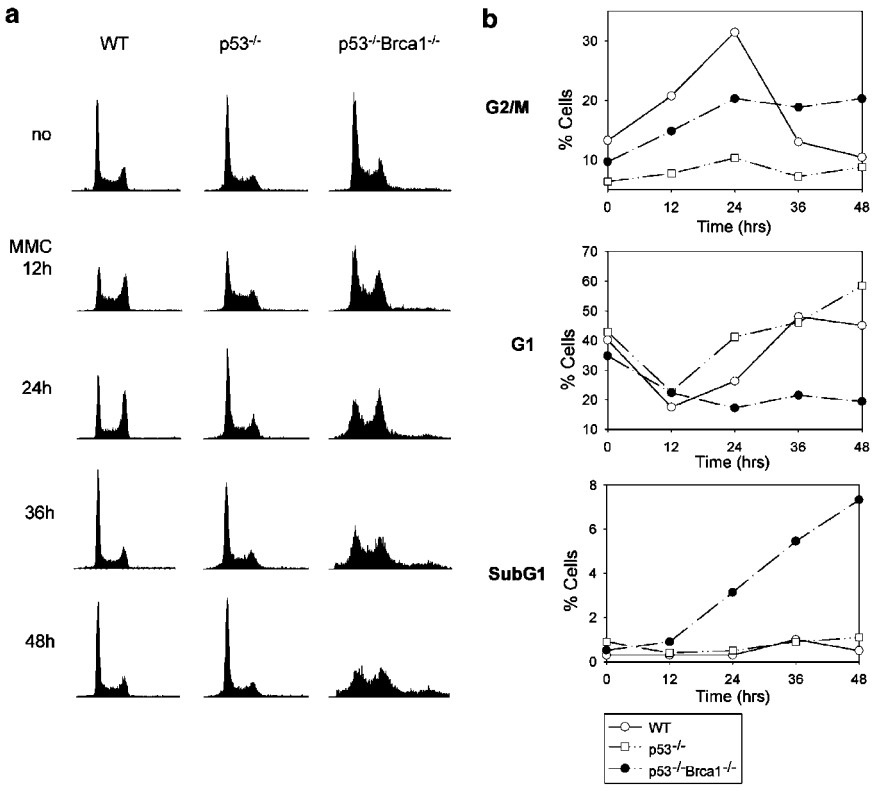

Figure 2 Brca1$^{-/-}$ MEFs are defective to MMC-induced cell cycle response. (**a**) Wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs were treated with 400 ng/ml of MMC for 2 h and harvested at the indicated time points. Cells were fixed, stained with PI and then analysed by flow cytometry as described in Materials and methods. (**b**) The percentage of each cell cycle phase of wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs was determined with CellQuest software and plotted

are subjected to cell death. This finding provides an explanation for the hypersensitivity of p53$^{-/-}$Brca1$^{-/-}$ MEFs to MMC treatment.

Aberrant S-phase arrest in Brca1-deficient MEFs upon MMC treatment

Recent studies proposed that ICL repair mainly occurs in S phase by the DNA DSB repair pathway (Rothfuss and Grompe, 2004). However, our initial one-dimensional FACS analysis failed to distinguish these expected differences in S-phase progression (Figure 2). We then employed a two-dimensional FACS analysis by BrdU pulse-chase labeling and DNA content distribution (Sala-Trepat *et al*., 2000). As shown in Figure 3a, this methodology allowed us to distinguish between the replicating (box *b*) and arrested S-phase cells (box *c*).

By this analysis, we observed a time-dependent accumulation of the S-phase-arrested cells in Brca1-deficient MEFs, but not in the wild-type and p53$^{-/-}$ MEFs (Figure 3b). The appearance of S-phase-arrested cells in p53$^{-/-}$Brca1$^{-/-}$ MEFs began at 12 h, and after 24 h the number continuously increased in parallel with the time course of the increase in apoptosis (Figure 2), implying that the delayed S-phase progression, perhaps caused by an inefficient ICL repair, eventually resulted in cell death. Such a unique role of Brca1 for the ICL repair in S phase is distinctive from the previous report that Brca1 is critical for G2/M checkpoint control, which attributes to the ICL repair (Bhattacharyya *et al*., 2000; Yu *et al*., 2000; Moynahan *et al*., 2001).

Reconstitution of Brca1 expression restored MMC resistance

To further substantiate the critical role of Brca1 in mediating MMC resistance, we reintroduced the full-length Brca1 cDNA into Brca1-deficient MEFs using a retrovirus-mediated gene transfer as described in Materials and methods. After infection with Brca1 retrovirus, cells were selected with puromycin and the individual colonies were isolated. Brca1 expression in the individual colonies was confirmed by Western blot analysis showing that the Brca1-reconstituted cell lines expressed comparable levels of Brca1 protein to either wild-type or p53$^{-/-}$ MEFs (Figure 4a).

To test whether reconstitution of Brca1 expression restores the resistance to MMC, two reconstituted clones together with control cells were subjected to colony forming assay. These two Brca1-reconstituted cell lines were indeed resistant to MMC treatment to a degree comparable with the wild-type and p53$^{-/-}$ MEFs (Figure 4b). These results confirm that the

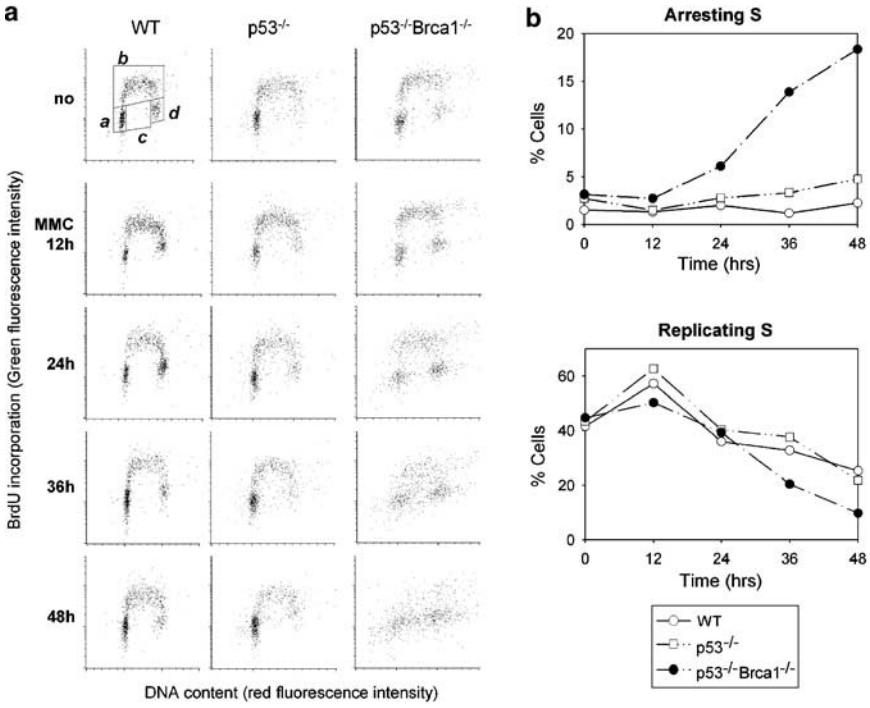

Figure 3 Brca1$^{-/-}$ MEFs are arrested in S phase following MMC treatment. (**a**) Wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs were treated with MMC and the BrdU pulse-chase analysis was carried out as described in Materials and methods. The region for the G1 phase (box *a*), replicating S phase (box *b*), arrested S phase (box *c*) and G2/M phase (box *d*) is indicated. (**b**) The percentages of arrested and replicating S-phase cells of wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs were determined with CellQuest software and plotted

hypersensitivity of p53$^{-/-}$Brca1$^{-/-}$ MEFs to MMC is solely due to the absence of Brca1 function.

Ectopic Brca1 expression in Brca1-deficient cells restored Rad51 foci formation and normal cell cycle response to MMC treatment

Since the reconstitution of Brca1 expression sufficiently restores MMC resistance, it is likely that ICL repair activity is also recovered. To test this possibility, we monitored the DNA damage-induced Rad51 foci formation, the hallmark event for HR-mediated DSB repair, in Brca1-reconstituted cells together with wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs. As shown in Figure 5a, Rad51 foci formation was impaired in p53$^{-/-}$Brca1$^{-/-}$ MEFs following MMC treatment, while the wild type and p53$^{-/-}$ displayed a dramatic increase in the number of nuclear Rad51 foci in response to MMC. As expected, the number of Rad51 foci in the two Brca1-reconstituted clones sharply increased upon MMC treatment, indicating that HR-mediated repair was restored (Figure 5a). Consistently, Brca1$^{-/-}$ ES cells are defective in Rad51 foci formation after treatment with the ICL agent cisplatin (Bhattacharyya *et al*., 2000). Thus, these results suggest that Brca1 is required for Rad51 foci formation in response to ICL DNA damage.

Accordingly, it was expected that the reconstitution of Brca1 expression would reduce the aberrant S-phase arrest and apoptosis in response to MMC. We then examined cell cycle response of the two Brca1-reconstituted clones following MMC treatment by two-dimensional FACS analysis as described above. The results shown in Figure 5b revealed that the time-dependent accumulation of S-phase arrest was reduced in these reconstituted clones, albeit not to the same level as the wild-type MEFs, while extensive apoptosis completely disappeared. Taken together, these results suggest that the absence of Brca1 is solely responsible for the hypersensitivity to ICL agents in Brca1-deficient MEFs, which is associated with the reduction in HR repair of the damaged DNA and the increase in the aberrant S-phase arrest.

Discussion

The hypersensitivity of Brca1-deficient cells to ICL agent has been reported previously using ES cells expressing an exon 11-deleted Brca1 isoform (Bhattacharyya *et al*., 2000; Moynahan *et al*., 2001). Our p53$^{-/-}$Brca1$^{-/-}$ MEF, which is a genuine Brca1-null cell with no Brca1 protein expression, also showed a marked hypersensitivity to MMC treatment (Figure 1) and reconstitution of Brca1 expression at a physiological level fully restored the resistance (Figure 4b), confirming that Brca1 is an essential component for the cellular

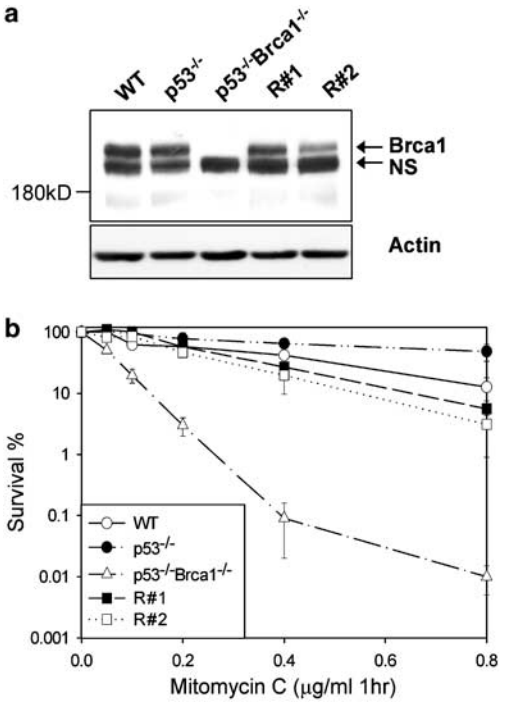

Figure 4 MMC resistance is restored by Brca1 reconstitution. (**a**) A 50 μg portion of cell lysate from wild-type, p53$^{-/-}$ and p53$^{-/-}$ Brca1$^{-/-}$ MEFs and two Brca1-reconstituted clones (R#1, R#2) was separated by SDS–PAGE and subjected to Western blotting using anti-murine Brca1 antibodies and anti-actin antibodies. The nonspecific band (NS) is indicated. (**b**) Clonogenic survivals of wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs and two Brca1-reconstituted clones (R#1, R#2) were examined as described in Figure 1

response to ICL agent. However, the detailed mechanism of how Brca1 mediates such response has never been examined. In the present communication, we analysed cell cycle response to the ICL agent MMC using isogenic wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs. We observed that p53$^{-/-}$Brca1$^{-/-}$ MEFs displayed prolonged accumulation in G2/M and extensive cell death following MMC treatment, while wild-type MEFs were temporarily arrested in G2/M but later re-entered a normal cell cycle progression (Figure 2b). Although we only monitored cell cycle response up to 48 h after MMC treatment, a sharp increase in sub-G1 fraction of Brca1-deficient MEFs indicates that the proportion of cell death would further increase at later time points and that apoptosis is the major reason for the hypersensitivity to MMC.

In wild-type MEFs, increase of G2/M cells after MMC treatment is attributed to the activation of G2/M cell cycle checkpoint control. In p53-null MEFs, the G2/M checkpoint is defective and the cells are not accumulated in G2/M phase. However, in p53$^{-/-}$ Brca1$^{-/-}$ MEFs, there is a significant accumulation of G2/M cells 24 h after MMC treatment (Figure 2). This phenotype cannot be explained by the activation of the checkpoint control. It has been reported that Brca1 participates in G2/M checkpoint control in response to ionizing radiation (IR) (Xu *et al*., 1999, 2001). The Brca1-null cell should have its G2/M checkpoint control inactivated; therefore, little or no accumulation of G2/M cells should be seen in p53$^{-/-}$ Brca1$^{-/-}$ MEF upon MMC treatment. Then, an alternative explanation is that the prolonged increase of G2/M phase is only a

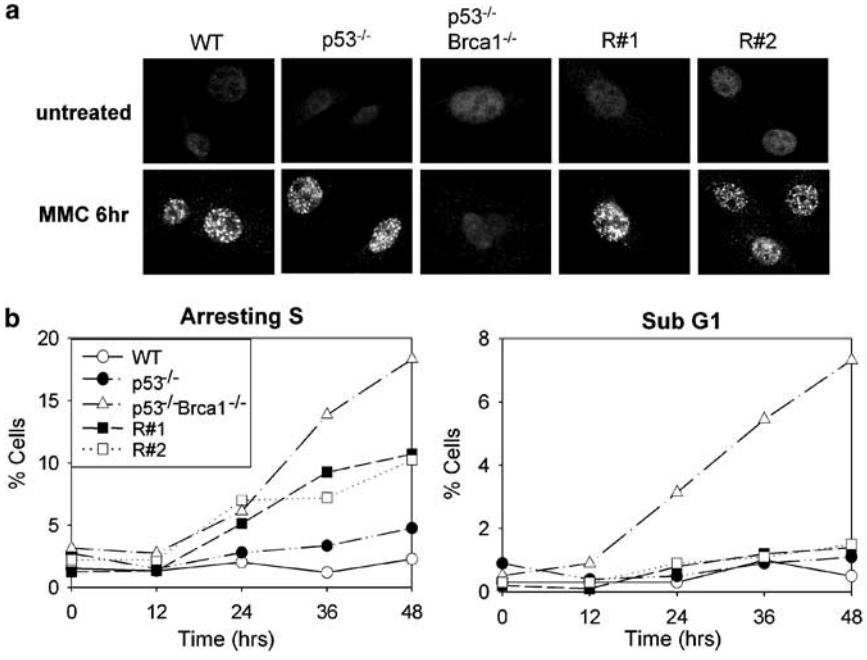

Figure 5 The Brca1 reconstitution restores Rad51 foci formation and normal cycle response following MMC treatment. (**a**) Wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs and two Brca1 reconstituted cells (R#1, R#2) were exposed to 400 ng/ml MMC for 2 h and Rad51 foci formation was examined 6 h later as described in Materials and methods. (**b**) The percentages of arrested and replicating S-phase cells of wild-type, p53$^{-/-}$ and p53$^{-/-}$Brca1$^{-/-}$ MEFs and two Brca1-reconstitution cells (R#1, R#2) were determined as described in Figure 3 and plotted

secondary consequence of excessive unrepaired DNA damage leading to cell cycle delay. It has been reported that FA cells also showed a hypersensitivity to ICL agents and a marked increase of G2/M population after exposure to ICL agents (Kubbies *et al*., 1985; Berger *et al*., 1993). Many investigators have speculated that the observed G2/M accumulation of FA cells after MMC treatment is caused by a defective cell cycle checkpoint control and that the FA genes may directly regulate one or more checkpoints (Kupfer and D'Andrea, 1996; Kruyt *et al*., 1997). However, a recent study revealed that the G2/M arrest in FA cells reflects a secondary response to the increase in the damaged DNA following exposure to ICL agents (Heinrich *et al*., 1998). Heinrich *et al*. compared the cell cycle response of wild-type, FA cells and reconstituted FA cells to ICL agents and found that all the cells showed an equivalent accumulation in the G2/M phase. We also observed an increase of G2/M population in Brca1-reconstituted cells similar to that of the wild-type cells (data not shown); therefore, the prolonged G2/M accumulation in Brca1-deficient MEFs most likely resulted from inefficient repair of the damaged DNA induced by MMC, which may activate G2/M cell cycle checkpoint through a BRCA-independent pathway.

Accumulating evidence suggests that ICL DNA damage induces DNA DSB in a cell cycle-dependent manner (Dardalhon and Averbeck, 1995; De Silva *et al*., 2000; McHugh *et al*., 2000). First, rapidly dividing cells are more sensitive to ICL agents than slowly growing cells (Mu *et al*., 1995; Hartmann *et al*., 1999). Second, ICL-induced DSB occurs in *Saccharomyces cerevisiae* only when proliferating (McHugh *et al*., 2000). Finally, MMC-induced γ-H2AX foci formation, which is specifically formed at the DSB site in the chromosome, requires cell cycle progression (Niedernhofer *et al*., 2004). Moreover, the recognition and repair of ICL in human cells primarily occur during the S phase. Human primary fibroblast becomes arrested in the late S phase when ICL DNA damage is introduced during the cell cycle (Akkari *et al*., 2000). It is recently reported that DSB is formed exclusively in the S phase during ICL repair and activates the FA/Brca1 pathway (Rothfuss and Grompe, 2004). Thus, ICL recognition, formation of DSB intermediate and repair of DSB are likely to proceed in the S phase of the cell cycle. Consistent with this notion, Brca1-deficient MEFs showed time-dependent accumulation of S-phase-arrested cells following MMC treatment (Figure 3). However, ectopic expression of the full-length Brca1 reduced, although did not completely eliminate, the S-phase-arrested cells (Figure 5), suggesting that other Brca1 isoforms may also contribute to this function. It is likely that BRCA1-IRIS, an BRCA1 isoform, may also be important for this process because of its potential role in regulating DNA replication machinery (Elshamy and Livingston, 2004). Nevertheless, this finding is in odd with a previous report that BRCA1 has an S-phase checkpoint activity based on the observation that S-phase checkpoint was diminished in HCC1937 cells, which contain a C-terminal truncated BRCA1 (Xu *et al*., 2001). Increase of the S-phase arrest in Brca1-null MEFs upon MMC treatment (Figure 3b) cannot be explained by the activation of S-phase checkpoint function of Brca1. Instead, Brca1 may directly participate in the DNA damage repair process, and failure in this repair delays S-phase progression and leads to cell death (Figure 2).

Recent studies suggest that DSB induced by ICL in S phase is subsequently repaired by HR, the major pathway for the accurate and error-free repair of DNA DSB (reviewed in van den Bosch *et al*., 2002). It has been shown that HR is the predominant pathway for ICL agent-induced DSB repair in *S. cerevisiae* (McHugh *et al*., 2000) and cells with defects in HR are hypersensitive to ICL agents (Collins, 1993). A substantial amount of evidence also indicates that Brca1 plays a critical role in DSB repair including HR pathway (Ting and Lee, 2004) where Rad51 plays a major role. Brca1 is colocalized with Rad51 to form IR-induced foci (Scully *et al*., 1997; Chen *et al*., 1999b). Consistently, Rad51 foci formation is delayed in the FA-like cell in response to ICL agents (Larminat *et al*., 2004), and Brca1-null ES cell shows a reduced level of Rad51 foci after cisplatin treatment (Bhattacharyya *et al*., 2000). Our results also show that Rad51 foci formation following MMC treatment is significantly impaired in Brca1-deficient MEFs but is restored by reconstitution of Brca1 expression (Figure 5). Thus, our results and those by others suggest that Brca1 is required for Rad51 foci formation in response to ICL agent and ICL-induced DSB repair by HR.

Taken together, our results suggest that Brca1 functions as a molecular determinant in the cellular response to the ICL agent MMC and the hypersensitivity of Brca1-deficient cells is associated with impaired ICL repair, in particular, DSB repair by HR in S phase. It remains to be explored what is the precise biochemical function of Brca1 required for ICL repair and this will provide valuable information to address whether the Brca1 pathway serves as a better target for a more effective cancer treatment.

Materials and methods

Cell lines and MMC treatment

Wild-type, $p53^{-/-}$ and $p53^{-/-}Brca1^{-/-}$ MEFs were generated as previously described (Zheng *et al*., 2000b). All MEF cell lines used were cultured in DMEM high-glucose media containing 5% FCS. MMC (Sigma) was dissolved in distilled water and freshly diluted with cell culture media at the indicated concentration. After incubation with MMC, cells were washed three times with PBS and incubated with culture media for the indicated time.

Generation of Brca1 reconstitution cell line and Brca1 protein detection

A retroviral vector carrying the murine Brca1 expression unit was generously provided by Dr Olga Aprelikova (NCI, Bethesda, MD, USA) (Aprelikova *et al*., 2001). This plasmid was transfected into Phoenix ampho cells for a rapid production of high-titer, helper-free retroviruses (Pear *et al*.,

1993). Infectious retrovirus was produced within 48 h following transfection of the Brca1 construct. The retroviral supernatant was harvested at 48 h post-transfection and stored at −80°C.

A 1 ml portion of freshly thawed retroviral supernatant combined with polybrene at a final concentration of 2 μg/ml was used for infection of $p53^{-/-}Brca1^{-/-}$ MEFs. The infected cells were selected with 4 μg/ml of puromycin, and 10–14 days later the resistant clones were picked up and tested for Brca1 expression. The Brca1 protein expression was examined by immunoblotting with anti-murine Brca1 polyclonal antibodies and actin protein was also monitored using anti-actin antibodies serving as an internal loading control (C-2, Santa Cruz Inc.). Fusion protein containing amino acids 788–1135 of mouse Brca1 fused to glutathione transferase (GST) in-frame was used as antigen for preparation of mouse polyclonal antisera.

Cell cycle analysis

To analyse cell cycle distribution in response to MMC, the BrdU pulse-chase method was used. About 0.5×10^6 cells were seeded onto a 100-mm dish, exposed to 400 ng/ml of MMC for 2 h and then incubated for the indicated times. BrdU was added to the culture media to 30 μM and incubated for 30 min before harvest. Cells were trypsinized and fixed with ethanol at −20°C. The fixed cells were incubated with 2 N HCl/0.5% Triton X-100 solution for 30 min at room temperature to partially denature DNA and then neutralized with 0.1 M $Na_2B_4O_7$ (pH 8.5). After resuspending in PBS/0.5% Tween 20, the cells were incubated with 20 μl of FITC-conjugated anti-BrdU antibodies (BD Biosciences) for 1 h. Cells were collected and stained for 30 min with PI staining solution (50 μg/ml PI, 0.1% sodium citrate, 50 μg/ml RNase A, 0.03% NP-40 in PBS).

Flow cytometric analysis was performed using a FACSCalibur flow cytometer and CellQuest software (Beckton Dickinson). A total of 10 000 events were analysed for each sample, and the experiment was repeated at least twice.

MMC clonogenic survival assay

For MMC survival assay, 1×10^3 or 4×10^3 cells were seeded in a 100 mm plate, exposed to various doses of MMC for 1 h and then rinsed three times with PBS. After 10–12 days, the colonies were fixed and stained with methylene blue, and the number was counted. The number of colonies obtained with untreated cells was corrected for plating efficiency and normalized to 100% survival. Survival experiments were performed in duplicate or triplicate and the data were presented as a mean value with standard deviation.

Rad51 foci formation assay

MEF cells were seeded onto coverslips and treated with 400 ng/ml of MMC for 2 h. After incubated for 6 h, Rad51 foci formation was examined as described previously (Chen *et al*., 1999a) using anti-mouse Rad51 antibodies (14B4) and Texas red-conjugated secondary antibody. Coverslips were mounted in PermaFlour (Lipshaw-Immunonon, Pittsburgh, PA, USA) and examined under a fluorescence microscope (Zeiss).

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References

Akkari YM, Bateman RL, Reifsteck CA, Olson SB and Grompe M. (2000). *Mol. Cell. Biol.*, **20,** 8283–8289.
Aprelikova O, Pace AJ, Fang B, Koller BH and Liu ET. (2001). *J. Biol. Chem.*, **276,** 25647–25650.
Berger R, Le Coniat M and Gendron MC. (1993). *Cancer Genet. Cytogenet.*, **69,** 13–16.
Bhattacharyya A, Ear US, Koller BH, Weichselbaum RR and Bishop DK. (2000). *J. Biol. Chem.*, **275,** 23899–238903.
Chen CF, Chen PL, Zhong Q, Sharp ZD and Lee WH. (1999a). *J. Biol. Chem.*, **274,** 32931–32935.
Chen JJ, Silver D, Cantor S, Livingston DM and Scully R. (1999b). *Cancer Res.*, **59,** 1752–1756.
Collins AR. (1993). *Mutat. Res.*, **293,** 99–118.
D'Andrea AD and Grompe M. (2003). *Nat. Rev. Cancer*, **3,** 23–34.
Dardalhon M and Averbeck D. (1995). *Mutat. Res.*, **336,** 49–60.
De Silva IU, McHugh PJ, Clingen PH and Hartley JA. (2000). *Mol. Cell. Biol.*, **20,** 7980–7990.
Dronkert ML and Kanaar R. (2001). *Mutat. Res.*, **486,** 217–247.
ElShamy WM and Livingston DM. (2004). *Nat. Cell Biol.*, **6,** 954–967.
Garcia-Higuera I, Taniguchi T, Ganesan S, Meyn MS, Timmers C, Hejna J, Grompe M and D'Andrea AD. (2001). *Mol. Cell*, **7,** 249–262.
Hartmann JT, Kollmannsberger C, Kanz L and Bokemeyer C. (1999). *Int. J. Cancer*, **83,** 866–869.
Heinrich MC, Hoatlin ME, Zigler AJ, Silvey KV, Bakke AC, Keeble WW, Zhi Y, Reifsteck CA, Grompe M, Brown MG, Magenis RE, Olson SB and Bagby GC. (1998). *Blood*, **91,** 275–287.
Innocente SA, Abrahamson JL, Cogswell JP and Lee JM. (1999). *Proc. Natl. Acad. Sci. USA*, **96,** 2147–2152.
Kruyt FA, Dijkmans LM, Arwert F and Joenje H. (1997). *Cancer Res.*, **57,** 2244–2251.
Kubbies M, Schindler D, Hoehn H, Schinzel A and Rabinovitch PS. (1985). *Am. J. Hum. Genet.*, **37,** 1022–1030.
Kupfer GM and D'Andrea AD. (1996). *Blood*, **88,** 1019–1025.
Larminat F, Germanier M, Papouli E and Defais M. (2004). *Biol. Cell*, **96,** 545–552.
McHugh PJ, Sones WR and Hartley JA. (2000). *Mol. Cell. Biol.*, **20,** 3425–3433.
Metzler M. (1986). *J. Cancer Res. Clin. Oncol.*, **112,** 210–215.
Moynahan ME, Chiu JW, Koller BH and Jasin M. (1999). *Mol. Cell*, **4,** 511–518.
Moynahan ME, Cui TY and Jasin M. (2001). *Cancer Res.*, **61,** 4842–4850.
Mu D, Park CH, Matsunaga T, Hsu DS, Reardon JT and Sancar A. (1995). *J. Biol. Chem.*, **270,** 2415–2418.
Niedernhofer LJ, Odijk H, Budzowska M, van Drunen E, Maas A, Theil AF, de Wit J, Jaspers NG, Beverloo HB,

Hoeijmakers JH and Kanaar R. (2004). *Mol. Cell. Biol.*, **24,** 5776–5787.

Pear WS, Nolan GP, Scott ML and Baltimore D. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 8392–8396.

Rothfuss A and Grompe M. (2004). *Mol. Cell. Biol.*, **24,** 123–134.

Sala-Trepat M, Rouillard D, Escarceller M, Laquerbe A, Moustacchi E and Papadopoulo D. (2000). *Exp. Cell Res.*, **260,** 208–215.

Sasaki MS and Tonomura A. (1973). *Cancer Res*, **33,** 1829–1836.

Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T and Livingston DM. (1997). *Cell*, **88,** 265–275.

Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, Takata M, Yamaguchi-Iwai Y and Takeda S. (1998). *EMBO J.*, **17,** 598–608.

Taniguchi T, Garcia-Higuera I, Andreassen PR, Gregory RC, Grompe M and D'Andrea AD. (2002). *Blood*, **100,** 2414–2420.

Ting NS and Lee WH. (2004). *DNA Repair (Amst.)*, **3,** 935–944.

van den Bosch M, Lohman PH and Pastink A. (2002). *Biol. Chem.*, **383,** 873–892.

Xu B, Kim S and Kastan MB. (2001). *Mol. Cell. Biol.*, **21,** 3445–3450.

Xu X, Weaver Z, Linke SP, Li C, Gotay J, Wang XW, Harris CC, Ried T and Deng CX. (1999). *Mol. Cell.*, **3,** 389–395.

Yu VP, Koehler M, Steinlein C, Schmid M, Hanakahi LA, van Gool AJ, West SC and Venkitaraman AR. (2000). *Genes Dev.*, **14,** 1400–1406.

Zheng L, Li S, Boyer TG and Lee WH. (2000a). *Oncogene*, **19,** 6159–6175.

Zheng L, Pan H, Li S, Flesken-Nikitin A, Chen PL, Boyer TG and Lee WH. (2000b). *Mol. Cell*, **6,** 757–768.

Zhong Q, Boyer TG, Chen PL and Lee WH. (2002a). *Cancer Res.*, **62,** 3966–3970.

Zhong Q, Chen CF, Chen PL and Lee WH. (2002b). *J. Biol. Chem.*, **277,** 28641–28647.

Zhong Q, Chen CF, Li S, Chen Y, Wang CC, Xiao J, Chen PL, Sharp ZD and Lee WH. (1999). *Science*, **285,** 747–750.