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Analysis of DNA repair and recombination responses in mouse cells depleted for Brca2 by SiRNA

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ABSTRACT

The tumor suppressor BRCA2 is considered to play an important role in the maintenance of genome integrity through the repair of DNA lesions by homologous recombination. A mechanistic understanding of BRCA2 has been complicated by the embryonic lethality of mice bearing allelic knockouts of *Brca2*, and by variation in the DNA damage response in cells bearing BRCA2 deficiencies. It would be advantageous to develop approaches that avoid the cell lethality associated with complete inactivation of the gene, or the use of established tumor cell lines in which other genes in addition to *BRCA2* may be mutant. In this study, SiRNA was used in stable transformation assays to knockdown Brca2 in mouse hybridoma cells by at least 75%. The Brca2-depleted cells were analyzed with respect to cell growth, sensitivity to DNA damaging agents (mitomycin C, methylmethane sulfonate, or ionizing radiation), intrachromosomal homologous recombination and gene targeting. Although the effect of Brca2-depletion on cell growth and sensitivity to DNA damaging agents was modest, the Brca2-depleted cells did show a significant shift in homologous recombination from gene conversion to single-strand annealing and a significant decrease in the efficiency of gene targeting. Both of these phenotypes are consistent with the proposed role of Brca2 in DNA repair and recombination.

1. Introduction

Inherited mutations in the breast cancer susceptibility 2 gene (*BRCA2*) confer high risk for breast, ovarian, and pancreatic cancers ([1]; reviewed in [2]). BRCA2-deficient human cells display gross chromosomal instability manifest as translocations, deletions and changes in chromosome number [3–6]. Chromosome aberrations in BRCA2-deficient cells have been attributed to loss of normal control by BRCA2 of the eukaryotic strand exchange protein RAD51, that is required during S phase for DNA repair and homologous recombination ([7,8]; reviewed in [2]). Abnormal cytokinesis has been suggested to explain chromosomal instability and aneuploidy in cells deficient for BRCA2 [9].

The analysis of individuals bearing *BRCA2* mutations suggests that inactivation of both *BRCA2* alleles is a prerequisite for tumor development (reviewed in [10]). In contrast, mice bearing knockouts of *Brca2* alleles display embryonic lethality [11]. These results suggest that different tissues might respond differently to the levels of BRCA2, and that loss of *BRCA2* alleles may be accompanied by other genetic alterations that contribute to tumorigenesis [11]. In cultured cells, the capacity for DNA repair and response to DNA-damaging agents appears to depend on the nature of the BRCA2 defect

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Abbreviations: IR, ionizing radiation; MMC, mitomycin C; MMS, methyl methane sulfonate; SiRNA, small inhibitory RNA; TNP, 2,4,6-trinitrophenyl; TNP-specific PFC, TNP-specific plaque forming cells

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and the cell line studied. Brca2-deficient mouse cells [12,13], hamster cells [8,14] and chicken DT-40 cells [15] are reported to have a reduced, but not deficient capacity to perform homology-directed DNA break repair. With respect to the cellular response to DNA damaging agents, methyl methane sulfate (MMS) treatment leads to increased sensitivity of mouse and human cells with BRCA2 truncations beginning at exon 11 [4,16], but not chicken DT-40 cells in which one *Brca2* allele has been disrupted [17]. In another example, DT-40 cells heterozygous for Brca2 [17] and murine ES cells homozygous for a Brca2 exon 27 deletion [18] reveal an increased sensitivity to mitomycin C (MMC) treatment, whereas mouse cells bearing a more extensive Brca2 truncation beginning at exon 11 do not [4]. Similarly, treatment with ultraviolet (UV) light has no effect on chicken DT-40 Brca2 heterozygotes [17], but UV hypersensitivity is observed in mouse cells with the Brca2-exon 11 truncation [4]. Fibroblasts and immortalized human lymphoblastoid lines with germline mutations in BRCA2 show increased sensitivity to ionizing radiation (IR), suggesting haplotype insufficiency [19,20], whereas a human pancreatic adenocarcinoma line that is heterozygous for BRCA2 showed no hypersensitivity to IR [21]. Reduced cell growth and increased sensitivity to MMC and IR is characteristic of chicken DT-40 Brca2 heterozygotes [17], whereas mouse cells in which one *Brca2* allele has been truncated at exon 11 show no significant increased sensitivity to MMS, MMC, IR or UV [4]. It would be advantageous to develop approaches that avoid the cell lethality associated with complete inactivation of this gene or the use of established tumor cell lines in which other genes in addition to *BRCA2* may be mutant.

An alternative approach to investigating BRCA2 function is to specifically reduce or eliminate BRCA2 protein in cultured cells, which can then be compared to parental cells with respect to DNA repair and recombination responses. Small inhibitory RNA (SiRNA) technology results in degradation of the mRNA for the target gene, and can be used to specifically reduce eukaryotic gene expression [22]. In this study, SiRNA was used in stable transfections to knockdown *Brca2* expression in mouse hybridoma cells, which were then tested for their response to DNA damaging agents and capacity to perform intrachromosomal homologous recombination and gene targeting.

2. Materials and methods

2.1. *Hybridomas*

The cell lines that were used in this study are based on the wildtype Sp6/HL mouse hybridoma cell line, which has its origins in a B cell derived from the spleen [23], a tissue shown previously to express Brca2 [24,25]. The Sp6/HL hybridoma bears a single copy of the trinitrophenyl (TNP)-specific chromosomal immunoglobulin μ heavy chain gene and makes normal, TNP-specific IgM (κ-chain) [23,26]. The igm482 hybridoma cell line is an Sp6/HL-derived mutant that bears a 2-bp deletion in the third constant region exon of the chromosomal μ gene (Cμ3), which results in synthesis of a truncated μ chain that when assembled into IgM is unable to activate complement-dependent lysis of TNP-coupled sheep red blood cells [26]. This difference provides a convenient means of distinguishing the IgM produced by wildtype and mutant igm482 cells. In addition, the 2-bp mutant igm482 deletion creates a *Tfi*I restriction enzyme site that replaces a wildtype *Xmn*I site permitting wildtype and mutant igm482 μ genes to be distinguished at the DNA level. Hybridoma cell lines Im/RCμ482-3/1 (abbreviated 3/1) and Im/RCμ-19/2 (abbreviated 19/2) are based on the Sp6/HL hybridoma [27,28], and were used as recipients for SiRNA transfection. In 3/1 and 19/2, the single copy TNP-specific chromosomal immunoglobulin μ gene has been modified by gene targeting and contains a pair of Cμ region heteroalleles that undergo high frequency intrachromosomal homologous recombination [27–29]. The conditions for hybridoma cell growth in DMEM are described elsewhere [23,26].

2.2. *Stable transformants expressing anti-Brca2 SiRNA*

Established criteria were used in the design of anti-*Brca2* SiRNA ([22]; Ambion Inc.). Four different regions of the mouse *Brca2* mRNA were targeted; SiRNA A spans nucleotide positions 820–838 (5′ tgttaggagattcatctgg 3′), SiRNA B spans nucleotide positions 2722–2740 (5′ ggcctagtctcaagaactc 3′), SiRNA C spans nucleotide positions 5449–5467 (5′ ggaattgtaaggtaggctc 3′), and SiRNA D spans nucleotide positions 8423–8441 (5′ agaccacgaagaagacaca 3′). Each SiRNA was inserted separately into a derivative of pSUPER (Oligoengine Inc.) bearing a dominant selectable hygromycin resistance gene (pSUPER-*hyg*) to permit selection of stable HYG-resistant (HYG^R) transformants. Fifty-micrograms of SiRNA-containing pSUPER-*hyg* were linearized by *Xmn*I cleavage and transferred into 2×10^7 hybridoma 3/1 or 19/2 cells by electroporation [30]. Selection for HYG^R transformants was performed in batch culture and limited dilution cloning in 96-well tissue culture plates (0.1 cell/well) was used to recover independent transformants, which were saved for analysis of *Brca2* expression.

2.3. *Assessment of Brca2 expression*

RNA was prepared by Trizol extraction (Invitrogen Inc.), treated with DnaseI, reverse-transcribed using Superscript (Invitrogen Inc.) and amplified using Biotools *Taq* polymerase according to manufacturer specifications (Interscience Inc.). Screening of mouse *Brca2* expression in HYG^R transformants relied on RT-PCR and multiplex RT-PCR procedures [31,32]. Forward and reverse primers, 5′ GTGAGGAGACTGTGGTAG 3′ and 5′ GTTGTGGTCACCAAGAGG 3′, respectively, were used to amplify a specific 0.9-kb segment from the well-conserved exon 11 of mouse *Brca2* [33]. In multiplex RT-PCR, the same *Brca2* primers were used in conjunction with forward and reverse primers, 5′ CAACACTGAAGTCATCCAGG 3′ and 5′ CTGTGTCAGACATGATCAGG 3′, respectively, specific for a 1.2-kb mRNA fragment encoding the Cμ region of the TNP-specific chromosomal μ gene. Standard procedures were used to optimize the RT-PCR assay with respect to primer concentration and cycle number. The dependence of the reaction on RNA was verified by elimination of the specific products following treatment with RNase but not DNaseI. General conditions used

for PCR have been described previously [28]. PCR amplification products were quantified by densitometry using BioRad Gel Doc software (BioRad Inc.).

Northern analysis was performed on RNA prepared from the various cell lines. A 4-kb cDNA fragment was PCR-amplified from the well-conserved *Brca2* exon 11 using forward and reverse primers, 5′ CAGTGGAACTCCAGGAAGAAGACC 3′ and 5′ CTGCGTCAACAGTCACTCCTCCTC 3′, respectively, and used as a hybridization probe. DNA sequencing verified that the probe fragment was specific for mouse *Brca2* (data not shown). To control for loading, the level of immunoglobulin μ gene mRNA in each sample was examined using a 993-bp *SmaI*/*PstI* restriction fragment from the wildtype cDNA clone μ17 [34]. To determine the threshold for detection of the *Brca2* message, the level of *Brca2* hybridization signal in 2.5, 5, 7.5, 10, 20 and 40 μg of hybridoma 3/1 RNA was determined, and compared to that observed in 40 μg of RNA extracted from the various knockdown and control cell lines.

For Western blot analysis of Brca2, hybridoma cells were lysed in RIPA buffer, and 100 μg of total protein was loaded onto a 4–12% gradient SDS-PAGE gel and transferred to a PVDF membrane. The membrane was probed with anti-Brca2 pep-3 antibody [35], followed by horse radish peroxidase (HRP)-conjugated secondary antibody. Immunoblot signals were detected using ECL reagent as recommended by the manufacturer (Amersham Biosciences). As a control for sample loading, the level of β-actin in each sample was determined using rabbit anti-β-actin and goat anti-rabbit IgG-HRP as recommended by the manufacturer (Abcam).

2.4. *Cell growth and response to DNA damaging agents*

For cell growth determinations, 2×10^6 exponentially growing hybridoma cells were resuspended in 10 ml of culture medium in triplicate tissue culture flasks. At 24-h intervals, duplicate samples were removed from each flask, and the number of viable cells/millilitre in each culture was determined by trypan blue dye exclusion. The same methods were used to determine the effect of DNA damaging agents on cell growth except that cultures were treated with ionizing radiation (IR) (range: 0–5 Gy doses of ^{137}Cs gamma-irradiation) (1.2 Gy/min), methyl methane sulfonate (MMS) (range: 0–75 μM) or mitomycin C (MMC) (range: 0–0.025 μM).

2.5. *Measurement of homologous recombination*

For measurement of intrachromosomal homologous recombination between Cμ region heteroalleles, aliquots of the indicated cell lines containing ~10–50 cells were inoculated into culture medium in separate flasks in the presence or absence of 0.6 mg/ml G418, grown to a density of ~10^5 cells/ml and assayed for TNP-specific plaque forming cells (TNP-specific PFC) [30].

For gene targeting, 2×10^7 of the indicated recipient cells were electroporated with 50 μg of pCμEn$^-$ vector linearized within the Cμ region at the unique *XbaI* site [36], and the frequency of TNP-specific PFC determined 48 and 72 h post-electroporation [30]. To calculate transfection efficiencies, the same experiment was repeated but transfected cells were distributed in 24-well tissue culture plates at densities of 10^4 and 10^5 cells/well in 0.6 mg/ml G418. Culture wells were scored for colony growth approximately 14 days post-electroporation. The mean transfection frequency was calculated from the fraction of growth-negative wells and the Poisson distribution.

3. Results

3.1. *SiRNA depletion of mouse Brca2*

The 3/1 or 19/2 mouse hybridoma cell lines used as recipients for SiRNA transfection are based on the wildtype Sp6/HL mouse hybridoma cell line, which has its origins in a B cell derived from the spleen [23], a tissue shown previously to express Brca2 [24,25]. In the recipient cell lines, the single copy, TNP-specific chromosomal immunoglobulin μ heavy chain gene has been modified by gene targeting such that it now bears a pair of closely linked Cμ region heteroalleles that serve in the analysis of intrachromosomal homologous recombination as described below [27,28]. In brief, the upstream (5′) Cμ region contains the 2-bp mutant igm482 Cμ3 deletion [26], and is separated from the downstream (3′) wildtype Cμ region by the backbone of the pSV2neo vector (Fig. 1A). Homologous recombination corrects the 5′ mutant igm482 Cμ region, allowing recombinant cells to synthesize TNP-specific IgM(κ) and to be detected as TNP-specific plaque-forming cells (TNP-specific PFC) in a sensitive TNP-specific plaque assay [29,30]. TNP-specific PFC consist of G418-resistant (G418^R) gene conversion products (Fig. 1B) and G418-sensitive (G418^S) deletion products (Fig. 1C) [29,36,37].

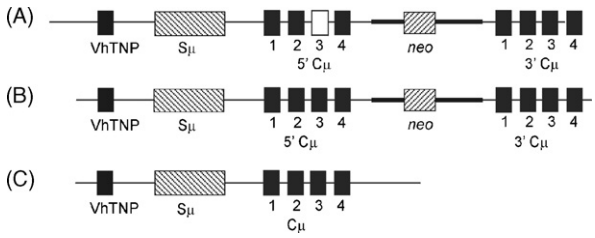

Fig. 1 – Structure of the chromosomal immunoglobulin μ heavy chain genes. In the hybridoma cell lines 3/1 and 19/2, the haploid TNP-specific immunoglobulin μ heavy chain gene contains a pair of closely linked Cμ region heteroalleles [27,28]. The upstream (5′) Cμ region contains a 2 bp deletion in exon Cμ3 (referred to as the 2 bp mutant igm482 Cμ3 deletion) (denoted by the open rectangle), and is separated from the downstream (3′) wild-type Cμ region by the backbone of the pSV2neo vector. Intrachromosomal homologous recombination can correct the 5′ Cμ region 2-bp mutant igm482 deletion, allowing recombinant cells to make normal TNP-specific IgM and to be detected TNP-specific PFC [30]. The products of such intrachromosomal homologous recombination can be (B) G418^R gene conversion products or (C) G418^S deletion products. The diagrams are not drawn to scale. *Abbreviations*: VhTNP, TNP-specific heavy chain variable region; Sμ, μ gene switch region; Cμ, μ gene constant region; *neo*, neomycin phosphotransferase gene of pSV2neo [39].

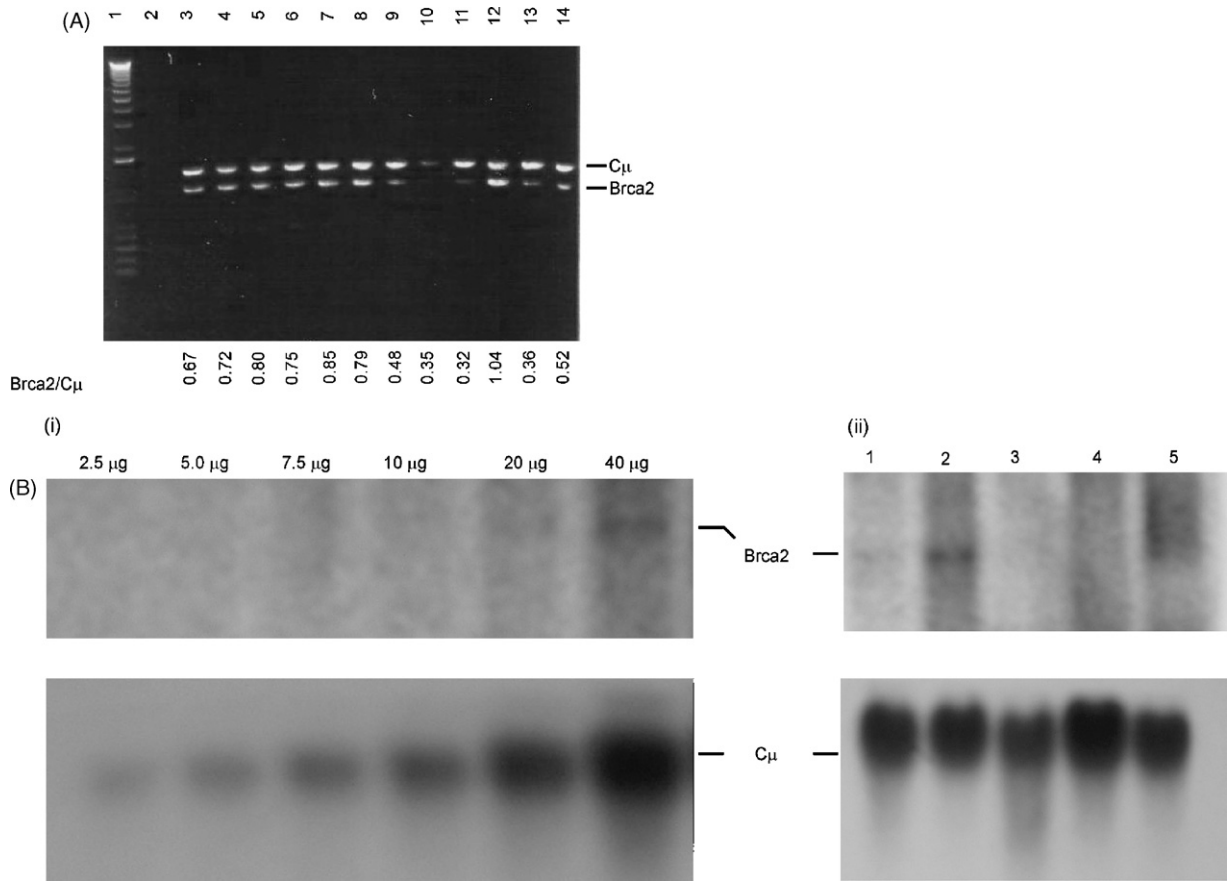

Fig. 2 – Analysis of Brca2 expression. (A) Multiplex RT-PCR analysis of anti-Brca2 knockdowns. In each sample, the knockdown in *Brca2* expression was assessed relative to that of the control μ gene constant (Cμ) region. Densitometric scanning of band intensity was used to compute the ratio, Cμ/*Brca2*, presented at the bottom of each lane. For further details, refer to the text. (B) Northern blot analysis of anti-*Brca2* knockdowns. (Panel i) Titration of RNA from hybridoma cell line 3/1 hybridized to *Brca2*-specific (top) and Cμ-specific (bottom) probe fragments. The quantity of RNA analyzed in each lane is indicated above the figure. (Panel ii) Forty micrograms of RNA from the various cell lines was analyzed to quantify the level of *Brca2* expression (top) relative to the Cμ loading control (bottom). Cell lines: lane 1, recipient 3/1; lane 2, mock-transfected 3/1 cells; lane 3, *Brca2*-depleted hybridoma 5 cells; lane 4, *Brca2*-depleted hybridoma 9 cells; lane 5, *Brca2*-depleted hybridoma 12 cells.

Initial transfections involved the use of hybridoma cell line 19/2 and pSUPER-*hyg* containing anti-Brca2 SiRNA C from which 40 HYG^R transformants were saved from two different electroporations. Routine screening by RT-PCR suggested that six HYG^R transformants were altered in their level of *Brca2* expression (data not shown). These cell lines were re-examined by multiplex RT-PCR (Fig. 2A). Lanes 3–7 present five separate RT-PCR reactions of the parental 19/2 hybridoma cell line. Densitometric analysis of the *Brca2*/Cμ ratio in each sample (presented below the figure) reveals a mean of 0.76 ± 0.03 ($\pm$ the standard error of the mean, $S\bar{x}$) suggesting reproducibility of the RT-PCR procedure. This value contrasts to the absence of signal in the sample lacking template (lane 2), but compares well to that obtained for mock-transfected (vector only) 19/2 cells (lane 8; *Brca2*/Cμ ratio of 0.79). When the *Brca2*/Cμ ratios in five HYG^R transformants (lanes 9–11, 13 and 14) are expressed as a fraction of the *Brca2*/Cμ ratio in the parental 19/2 hybridoma cells (from above, 0.76), the results reveal reductions in *Brca2* expression ranging from 32% to 58%. The greatest reduction (58%) was observed in hybridoma cell line 9 (lane 11). One HYG^R transformant (lane 12) appeared to have a slightly elevated level of *Brca2* expression. To test whether it was possible to recover transformants with further reductions in *Brca2* expression, the recipient 3/1 hybridoma cell line was transfected with the combination of pSUPER-*hyg* vectors containing all four SiRNAs (A–D). Independent HYG^R transformants were screened by RT-PCR and putative knockdowns were re-examined by multiplex PCR (data not shown). This analysis identified transformants 5 and 12 in which the mean reduction in *Brca2* expression is 55% and 63%, respectively. The above results suggest that hybridoma cell lines recovered following transfection with SiRNA C, or a combination of the four SiRNAs are not markedly different with respect to *Brca2* expression.

Northern blot analysis was performed as an additional measurement of the knockdown in *Brca2* mRNA expression. Fig. 2B (panel i) presents the titration of 3/1 recipient cells performed to determine the threshold of detection for *Brca2*

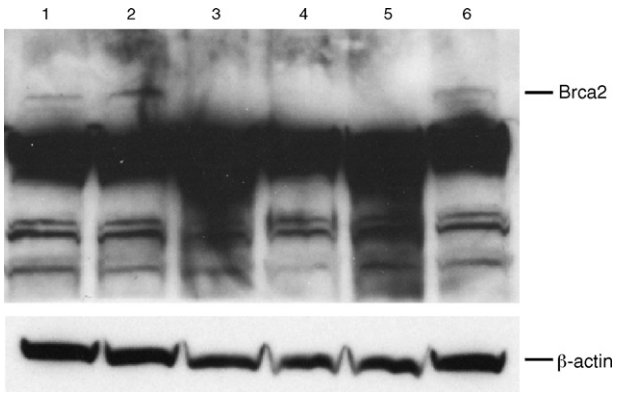

Fig. 3 – Analysis of Brca2 protein. Western blot analyses for mouse Brca2 and β-actin expression in control and Brca2-depleted hybridoma cells. The position of the 370 kb Brca2 protein is distinguishable from other cross-hybridizing bands in the gel. As a loading control, samples were examined for β-actin. Cell lines: lanes 1 and 6, recipient 3/1 cells; lane 2, mock-transfected 3/1 cells; lane 3, *Brca2*-depleted hybridoma 5 cells; lane 4, *Brca2*-depleted hybridoma 9 cells; lane 5, *Brca2*-depleted hybridoma 12 cells.

expression. The level of Cμ mRNA serves as a loading control. *Brca2* message was only detected when 20 and 40 μg of RNA was used. Therefore, the Northern analysis presented in Fig. 2B (panel ii) was performed using 40 μg of RNA. In non-transfected and mock-transfected cells (lanes 1 and 2, respectively), the specific 11 kb *Brca2* mRNA is visible [24]. Lanes 3–5 represent hybridomas 5, 9 and 12 respectively, shown from the multiplex RT-PCR analysis to have a quantitative reduction in *Brca2* expression. The absence of signal in lanes 3–5 suggests that the level of *Brca2* reduction approaches approximately 75% in all three hybridoma cell lines, since between 25% and 50% of the *Brca2* mRNA levels in the parent 3/1 cell line should be visible based on the titration experiments (panel i). Thus, the extent to which stably expressed SiRNA reduces Brca2 expression in the various hybridoma cell lines as determined by both PCR and Northern analysis is in approximate agreement.

Western analysis using the pep-3 antibody against murine Brca2 [35] is presented in Fig. 3. Recipient (lanes 1 and 6) and mock-transfected (lane 2) hybridoma cell lines reveal the 370 kDa mouse Brca2 protein, whereas no mouse Brca2 is observed in cell line 9 generated with SiRNA C (lane 3), or cell lines 5 and 12 generated with SiRNAs A-D (lanes 4 and 5). The results suggest that the level of Brca2 protein is reduced by even more than the ~75% that would be expected based on the *Brca2* RNA analysis.

3.2. *Effect of Brca2 depletion on cell growth*

The effect of the Brca2 depletion on growth of the hybridoma cells was examined (Fig. 4A). The results show that hybridoma cell lines 5 and 12 that are significantly depleted for Brca2 display approximately the same growth kinetics as the recipient or mock-transfected control cell lines over all 3 days. Repeated measures ANOVA reveals no significant difference in growth kinetics between Brca2-depleted lines and controls ($F = 1.01$, $p = 0.47$).

3.3. *Sensitivity of Brca2-depleted cells to DNA damaging agents*

To investigate whether Brca2 depletion affects cell recovery following treatment with the DNA damaging agents MMS, MMC or IR, the hybridoma cell lines were subjected to graded doses of each mutagen and cell viability, expressed as the fraction of treated to untreated cells, was determined over time. Initial experiments revealed gradual death of untreated recipient cells over MMS and MMC concentration ranges of 0–75 and 0–0.025 μM, respectively, and over IR doses between 1 and 5 Gy (data not shown), and these were used to assess the effect of Brca2 depletion on cell viability.

Fig. 4B and C present hybridoma cell viability measurements at the 48 h time point of continual MMS and MMC exposure, respectively, while Fig. 4D presents the response to IR (similar results were obtained at the 72 h time point) (data not shown). A two-way ANOVA was performed on all data sets to determine the significance of any differences observed. The response to MMS shows a significant interaction between cell line and dosage ($F = 3.60$, $p = 0.0034$). To determine the nature of the differences, the means were compared using Tukey honestly significant difference (HSD) analysis, which revealed that only hybridoma cell line 12 displays a significantly higher level of survival at MMS concentrations of 25 and 50 μM (Fig. 4B). In contrast, no significant interaction between cell line and treatment was revealed for MMC ($F = 0.70$, $p = 0.70$, Fig. 4C) or IR ($F = 0.46$, $p = 0.95$, Fig. 4D) exposure.

3.4. *Effect of Brca2 depletion on intrachromosomal homologous recombination*

As indicated above, intrachromosomal homologous recombination between the Cμ heteroalleles positioned in the chromosomal immunoglobulin μ locus of the mouse hybridoma cells (Fig. 1A) corrects the 2 bp mutant igm482 Cμ3 deletion allowing recombinants to be detected as TNP-specific PFC. In the absence of G418 selection, TNP-specific PFC generated by intrachromosomal homologous recombination consist of both gene conversion (Fig. 1B) and deletion (Fig. 1C) events, whereas, in the presence of G418 selection, only gene conversion (Fig. 1B) events are detected [29,36,37]. This system is convenient for assessing the effect of Brca2 depletion on the mechanism of intrachromosomal homologous recombination. Control cells and Brca2-depleted hybridoma 5 and 12 cells display a similar frequency of intrachromosomal homologous recombination under non-G418 selected conditions (F_{TOTAL}) (Table 1). In contrast, in the presence of G418 selection, a significant decrease in the frequency of homologous recombination is observed in the Brca2-depleted hybridoma 5 and 12 cell lines compared to control cell lines ($F_{GENE\ CONVERSION}$). Two-way ANOVA on log transformed data revealed a significant cell line/treatment interaction ($F = 3.57$, $p = 0.02$). A comparison of means using Tukey HSD analysis showed that

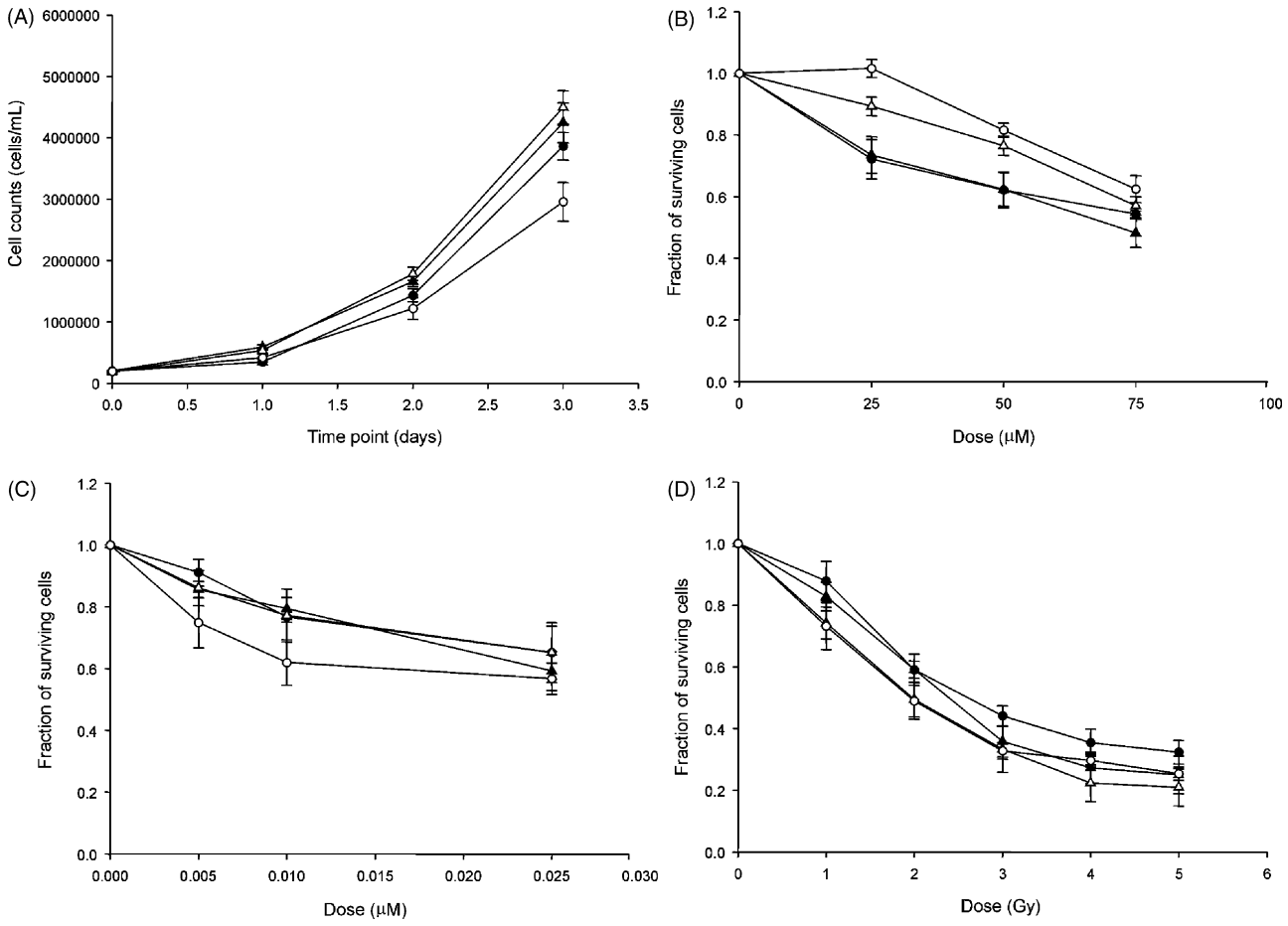

Fig. 4 – DNA damages response in Brca2-depleted mouse hybridoma cells. The various graphs present the growth of (A) untreated hybridoma cells, or those treated with (B) MMS, (C) MMC or (D) IR. Each point represents the mean ± standard error of the mean of three independent samples. Cell lines: (●) recipient 3/1 cells; (▲) mock-transfected 3/1 cells; (△) *Brca2*-depleted hybridoma 5 cells; (○) *Brca2*-depleted hybridoma 12 cells.

while there is no significant difference between F_{TOTAL} and $F_{GENE\ CONVERSION}$ values in the control lines, the Brca2-depleted hybridoma cell lines 5 and 12 both have $F_{GENE\ CONVERSION}$ values that are significantly lower than their F_{TOTAL} values. On average, the magnitude of the shift in recombination frequency in the Brca2-knockdown cell lines is approximately five-fold as judged by the $F_{GENE\ CONVERSION}/F_{TOTAL}$ (F_{GC}/F_T) ratios. Thus, the lower F_{GC}/F_T ratio in the Brca2-depleted hybridoma 5 and 12 cells suggests a shift in the mechanism of intrachromosomal homologous recombination from gene conversion to deletion.

Table 1 – Intrachromosomal homologous recombination in Brca2-depleted mouse hybridoma cell lines

Cell line[a]	Brca2 expression[b]	F_{TOTAL} (F_T)[c] ($\times 10^{-3}$)	$F_{GENE\ CONVERSION}$ (F_{GC})[d] ($\times 10^{-3}$)	F_{GC}/F_T
Parent	Normal	2.6 ± 0.6	1.0 ± 0.1	.38
Mock-transfected	Normal	7.2 ± 2.5	5.8 ± 2.1	.81
5	Knocked-down	2.4 ± 0.6	2.6 ± 0.3	.11
12	Knocked-down	5.6 ± 1.5	5.9 ± 0.4	.11

[a] The parent hybridoma cell line is 3/1 [27]. The mock-transfected cell line refers to hybridoma 3/1 that has received the pSUPER-hyg vector devoid of SiRNA, while hybridomas 5 and 12 are HYG^R transformants of 3/1 generated following stable transfection with pSUPER-hyg containing SiRNA A-D.

[b] Determined from multiplex RT-PCR and Western blotting analysis.

[c] Frequency of recombination in cells grown in the absence of G418 selection. The mean ± standard error of the mean of six subclones is presented for each cell line.

[d] Frequency of recombination in cells grown in the presence of G418 selection. The mean ± standard error of the mean of six subclones is presented for each cell line.

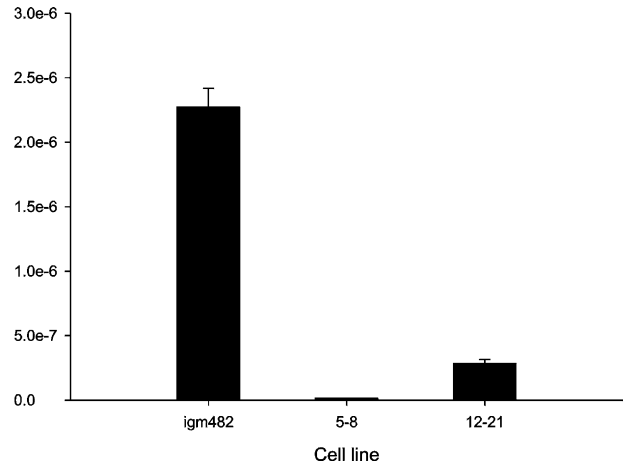

Fig. 5 – Effect of Brca2 depletion on gene targeting. Control igm482 hybridoma cells, and Brca2-depleted 5–8 and 12–21 hybridoma cells were transfected with the Cμ-linearized gene targeting vector, pCμEn$^-$ [36]. For each hybridoma cell line, the frequency of gene targeting was determined by dividing the number of TNP-specific PFC by the total number of cells plated, and is presented as the mean ± standard error of the mean (S$\bar{x}$) of two replicate experiments.

3.5. *Effect of Brca2 depletion on gene targeting*

Determination of the effect of Brca2 depletion on gene targeting is based on the ability of transfected plasmid DNA bearing the wildtype Cμ region to correct the 2 bp mutant igm482 Cμ3 deletion in the chromosome of recipient cells generating TNP-specific PFC [30]. To generate igm482 derivatives depleted for Brca2, G418^S deletion recombinants arising by intrachromosomal homologous recombination between the Cμ region heteroalleles were recovered from the independent Brca2-depleted hybridoma cell lines 5 and 12 reported in the studies above. Analysis of DNA and IgM from candidate G418^S cell lines served to identify the independent hybridoma cell lines 5–8 and 12–21 from the parental Brca2-depleted hybridomas 5 and 12, respectively, as bearing a single copy of the mutant igm482 chromosomal μ gene (data not shown). RT-PCR analysis on cell lines 5–8 and 12–21 revealed levels of Brca2 reduction that were similar to the parental hybridomas 5 and 12, respectively (data not shown). Recipient 5–8 and 12–21 hybridoma cells were electroporated with Cμ-linearized pCμEn$^-$ vector [36] and the frequency of TNP-specific PFC determined in replicate experiments. As shown in Fig. 5, Brca2-depleted hybridoma cell lines 5–8 and 12–21 display a significant, greater than eight-fold decrease in the frequency of gene targeting compared to the control igm482 hybridoma cell line as judged by one-way ANOVA ($F = 100.31$, $p < 0.0001$). In these experiments, the transfection efficiencies for hybridoma cell lines igm482, 5–8 and 12–21 were 4.70×10^{-5}, 2.34×10^{-5} and 3.40×10^{-5}, respectively, which according to one-way ANOVA are not significantly different ($F = 5.08$, $p = 0.11$). Thus, the decreased gene targeting was not the result of reduced transfection efficiency in the Brca2-depleted hybridoma cell lines.

4. Discussion

In the present study, anti-*Brca2* SiRNA was used in stable transfection assays to deplete Brca2 in mouse hybridoma cell lines, which were then examined for DNA repair and homologous recombination responses. The reductions in *Brca2* expression achieved with the single SiRNA C did not differ substantially from those obtained with the combination of all four SiRNAs A–D. Analysis of residual Brca2 mRNA and protein suggested that Brca2 expression was reduced by at least 75% in several cell lines stably expressing anti-Brca2 SiRNA. In this system, 75% depletion in cellular Brca2 may represent the maximum knock-down achievable without causing diminished cell survival.

Two independent Brca2-depleted hybridoma cell lines, 5 and 12, were chosen for analysis of DNA damage responses. In comparison to controls, hybridoma cell lines 5 and 12 showed similar growth rates and similar responses to MMC and IR exposure. Hybridomas 5 and 12 did appear to display enhanced survival to MMS-induced damage at 25 and 50 μM doses, but two-way ANOVA in combination with Tukey HSD analysis reveals significance only with hybridoma 12. The enhanced survival of hybridoma 12 in response to MMS exposure is consistent with the possibility of more efficient base-damage repair operating in the event that reduced Brca2 compromises homologous recombination. However, given that the response of hybridoma cell line 5 to MMS damage is not significantly different from controls, a link to Brca2 deficiency is not conclusive. The lack of a discernable effect of Brca2 depletion on cell growth or survival following MMC or IR exposure suggests that hybridoma cell lines 5 and 12 contain sufficient residual Brca2 for relatively normal cellular function. However, in view of the fact that the Brca2-depleted clones were recovered as stable HYG^R transformants, one might consider the possibility that a suppressor mutation has been selected for that permits the cells to respond normally in the various assays that were performed. Similarly, a suppressor that favors growth of Brca2-depleted hybridoma 12 cells in response to MMS damage might also be contemplated. During prolonged growth in culture, Brca2 knockdowns may accumulate suppressor mutants that eventually might comprise a substantial fraction of each population. However, the hybridoma cultures in this study were grown for 1 month, at most, before being discarded (~40 culture doublings), which makes significant accumulation of suppressor mutants highly unlikely. Suppressor mutations could arise spontaneously in the culture prior to transfection. However, the probability that such rare mutant cells would be the target of DNA transfection, especially in the several independent knockdown lines that were isolated in this study appears forbidding. While a suppressor mutation may arise following transfection, it would need to occur within the first few doublings in order for the mutant cells to comprise a significant proportion of the population. Again, this is unlikely in the several independent Brca2 knockdowns that were analyzed. Thus, residual levels of Brca2 in the Brca2-depleted hybridoma cell lines may be sufficient for the relatively normal growth rates and DNA repair responses that are observed.

To directly assess the requirement for Brca2 in homologous recombination, intrachromosomal recombination and gene targeting assays were utilized. In the case of intrachromosomal homologous recombination, the results suggested a bias favoring deletions over gene conversion events among the recombination products in the Brca2-depleted hybridoma 5 and 12 cell lines. In principle, deletion recombinants may arise by intrachromosome (or intrachromatid) reciprocal crossover or by single-strand annealing. However, single-strand annealing is considered a preferred mechanism of recombination between repeats on a chromosome [38]. Because single-strand annealing involves the net loss of a sequence repeat(s), it is regarded as a non-conservative mode of recombination, in contrast to the more conservative gene conversion mechanism in which there is no net loss of DNA. Accordingly, our results are consistent with the possibility of a shift to the non-conservative single-strand annealing pathway of intrachromosomal recombination in the Brca2-deficient hybridoma cell lines 5 and 12. This result is in agreement with previously published studies in which Brca2-defective mouse [13] and hamster [8] cells show a decrease in gene conversion with an accompanying increase in deletions, presumably arising from single-strand annealing. The shift in recombination outcomes is consistent with the interpretation that Brca2 is required for conservative homology-directed repair, and when it is limiting or absent, DNA repair defaults to non-conservative pathways such as single-strand annealing (reviewed in [10]).

The requirement for Brca2 in gene targeting was also investigated. The results reveal that the independent Brca2-depleted hybridoma cell lines 5–8 and 12–21 are greater than eight-fold reduced in gene targeting compared to igm482 control cells. The reduced gene targeting efficiency in the Brca2-depleted cells in this study is significantly greater than the previously reported 1.8-fold decrease in gene targeting in mouse embryonic stem (ES) cells expressing truncated Brca2 [12]. It is possible that this difference is due to the nature of the Brca2 deficiency. In the Moynahan et al. [12] study, the ES cells contained a Brca2 C-terminal truncation, and the milder effect may be due to the fact that the Brca2 truncation did not include the conserved BRC repeats in exon 11 [33]. In contrast, in this study, when the overall level of Brca2 is decreased by at least ~75%, a much larger effect is observed suggesting that depletion of cellular Brca2 is sufficient to significantly hinder homologous recombination through gene targeting.

In summary, this is the first study to successfully generate Brca2-depleted cell lines in stable transfections with anti-Brca2 SiRNA. The use of isogenic mouse hybridoma cell lines allowed us to compare the DNA repair and recombination responses of the Brca2-deficient cells directly to parental cells, thus avoiding the use of established tumor cell lines in which genes other than Brca2 may be mutant. In doing so, it was established that depletion of Brca2 by at least ~75% is sufficient to greatly reduce the efficiency of conservative homologous recombination as manifested both by the shift in intrachromosomal recombination from gene conversion toward single-strand annealing, and by the reduction in gene targeting. This data supports the role that the Brca2 tumor suppressor is considered to play in homologous recombination by controlling the availability, cellular localization and DNA binding capability of the strand exchange protein, RAD51 (reviewed [2]; [7,8]).

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