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Brief communication

BRCA2 is epistatic to the RAD51 paralogs in response to DNA damage

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ABSTRACT

Homologous recombination plays an important role in the high-fidelity repair of DNA double-strand breaks. A central player in this process, RAD51, polymerizes onto single-stranded DNA and searches for homology in a duplex donor DNA molecule, usually the sister chromatid. Homologous recombination is a highly regulated event in mammalian cells: some proteins have direct enzymatic functions, others mediate or overcome rate-limiting steps in the process, and still others signal cell cycle arrest to allow repair to occur. While the human BRCA2 protein has a clear role in delivering and loading RAD51 onto single-stranded DNA generated after resection of the DNA break, the mechanistic functions of the RAD51 paralogs remain unclear. In this study, we sought to determine the genetic interactions between BRCA2 and the RAD51 paralogs during DNA DSB repair. We utilized siRNA-mediated knockdown of these proteins in human cells to assess their impact on the DNA damage response. The results indicate that loss of BRCA2 alone imparts a more severe phenotype than the loss of any individual RAD51 paralog and that BRCA2 is epistatic to each of the four paralogs tested.

1. Introduction

The RAD51 paralogs were originally identified by both database searches for sequence similarity to RAD51 and screens designed to identify novel genes involved in DNA cross-link sensitivity in hamster cell lines [1–5]. Five RAD51 paralogs were discovered, which are RAD51B, RAD51C, RAD51D, XRCC2, and XRCC3 and they were found to exist *in vivo* in two major complexes: RAD51B/RAD51C/RAD51D/XRCC2 and RAD51C/XRCC3 [6,7]. Despite several years of investigation, the functional significance of the two *in vivo* RAD51 paralog complexes in homologous recombination remains unclear. Each individual paralog has substantially diverged from the others at the sequence level, demonstrates less ATPase activity than RAD51, and does not self-associate as does RAD51 [8–11]. There are many parallels between cells deficient in BRCA2 and each of the five RAD51 paralogs: (1) knockout of BRCA2 or any of the individual RAD51 paralogs in mice results in embryonic lethality; (2) cells derived from tumors or hamster mutants exhibit extreme sensitivity to cross-linking agents such as mitomycin C or cisplatin, yet only mild sensitivity to ionizing radiation; (3) loss of RAD51 foci; (4) and genomic instability [10,12–20]. Additionally, the RAD51 paralogs have emerged as breast and ovarian cancer susceptibility genes in several recent clinical studies [21–25]. Because of these similarities and the enigmatic role of the RAD51 paralogs in homologous recombination, we decided to closely examine the relationship between BRCA2 and paralog function in human cells using a reverse genetic (siRNA) approach.

In this study, we utilize human cells to examine the epistatic interactions between BRCA2 and the RAD51 paralogs in response to DNA damaging agents. Genetic analysis of BRCA2 and the RAD51 paralogs in mammalian cells has been difficult due to the lack of an isogenic cell system in which to measure the contribution of each to homologous recombination and cell survival. Human tumor cell lines and hamster cells have been established possessing mutant forms of both BRCA2 and the RAD51 paralogs; however, these cells suffer from chronic genomic instability, and hence, interpretations derived from these cell lines suffer from a similar uncertainty. Furthermore, mouse knockout cell lines cannot be established due to embryonic lethality further hampering progress in understanding these proteins. To overcome the above limitations, we utilized RNA interference to transiently deplete BRCA2 and the paralogs in human cells that have not undergone prior selection for survival in the absence of these proteins and to assess their response to DNA damaging agents. We were able to deplete BRCA2 expression in combination with four of the five RAD51 paralogs using a single siRNA oligonucleotide targeted against each respective mRNA. Our results demonstrate that loss of BRCA2 imparts the greatest

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sensitivity to DNA damaging agents in comparison to loss of any individual RAD51 paralog. We also show that loss of a single RAD51 paralog, in combination with BRCA2 loss, results in a similar level of survival to that of BRCA2 loss alone supporting the idea that BRCA2 and the RAD51 paralogs work together during DNA break repair.

2. Materials and methods

2.1. Cell culture

All cell lines were obtained from ATCC. HT-1080, U2OS, and HEK-293 cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum, used at low passage number, and maintained in humidified, 5% CO_2, 37 degree incubators.

2.2. siRNA transfections

BRCA2, RAD51C, RAD51B, RAD51D, and XRCC3 siRNAs were purchased from Dharmacon as individual siRNAs derived from their siGENOME SmartPool© siRNA reagents. A single siRNA oligonucleotide for each gene was selected from the set of four provided based on maximum achievable knockdown by western blotting. A non-targeting siRNA control #2 (Dharmacon #D-001210-02-05) was used as the mock control. The individual siRNAs were used to transfect HT1080, U2OS, and HEK-293 cells using the DHARMAFect1 transfection reagent at a final concentration of 100 or 200 nM. For transfections with two siRNAs in the same cell population, the same concentrations of oligonucleotides were used while the concentration of DHARMAFect1 was increased 2-fold. Cells were harvested 64 h post-transfection in 50 mM HEPES (pH 7.5), 250 mM NaCl, 1% NP-40, 5 mM EDTA, and protease inhibitor cocktail (Roche). 50–150 μg of total cellular protein samples (measured by Bradford Assay) were analyzed on Bio-Rad TGX gradient 4–15% SDS-PAGE gels. Western blotting was then performed using antibodies against BRCA2 (Ab-2, EMD), Rad51C (Novus), RAD51B (Novus), XRCC3 (Santa Cruz Biotechnology), RAD51D (Novus), ATM S1981-P (Cell Signaling), NBS1 S343-P (Cell Signaling), and gamma-H2AX S139-P (Upstate Biotechnology). Equal loading of protein was verified by stripping blots and re-probing with a tubulin antibody (Sigma); this was further confirmed by quantification of total protein staining using Bio-Rad stain-free gels and image analysis on a Bio-Rad ChemiDocMP system. Quantification of band intensity in western blots was performed using Image Lab Version 4.0 (Bio-Rad).

2.3. Clonogenic survival assays

HT-1080, HEK-293, or U2OS cells were seeded in 6 well plates at $3–5 \times 10^5$ cells/well. Twenty-four hours after seeding, cells were transfected with siRNAs against BRCA2, RAD51B, RAD51C, RAD51D, or XRCC3 at a final concentration of either 100 or 200 nM using DharmaFect1 (Dharmacon) at a 1:3 ratio (siRNA:DharmaFect reagent). At 64 h post-transfection, cells were treated with an acute concentration of mitomycin C (Sigma). Mitomycin C (MMC) stock was made by diluting 2 mg powder in 4 mL dH_2O giving a 1.5 mM solution. For treatment of cells, MMC was diluted in 2 mL DMEM with no serum at the indicated concentrations and placed on cells for 1 h. After 1 h incubation with drug, the media was aspirated off, cells were washed two times with PBS, then trypsinized and resuspended in 2 mL DMEM + 10% FBS. For each sample, cells were counted using a hematocytometer or a Bio-Rad TC10 cell counter, serially diluted, and plated out in triplicate into 6 well plates. Cells were fed with DMEM + 10% FBS + penicillin/streptomycin (50 U/mL) to prevent any contamination during 12–14 days of cell growth. After colony formation, the cells were removed from the incubator, washed one time with 0.9% NaCl (saline solution), and stained with a 0.25% crystal violet solution in 3.5% formaldehyde, 72% methanol. The cells were stained for 1 h in crystal violet followed by destaining in water. Plates were dried overnight and then colonies containing 50 or more cells were counted on each plate and the surviving fraction was determined for each drug treatment.

3. Results

3.1. BRCA2 depletion leads to a more severe phenotype in response to DNA damage than depletion of any individual RAD51 paralog in response to DNA damage

In the first set of experiments, we utilized siRNA oligonucleotides targeted against RAD51B, RAD51C, RAD51D, XRCC3, and BRCA2 (Fig. 1A) to transiently knockdown protein expression either alone or with each paralog in combination with BRCA2 to determine whether the double knockdowns resulted in: (1) a more severe phenotype (synergistic), (2) the same phenotype as either single knockdown alone (epistatic), or (3) perhaps a more complex relationship. The advantage of using siRNA for these particular genes, as opposed to complemented mutant cell lines, is that it permits an analysis in an isogenic system where neither pre-existing mutations nor selection for survival in an environment of genomic instability has occurred. Given the known extreme sensitivity of BRCA2 and RAD51 paralog mutant cells to cross-linking agents [1,7,26], we utilized mitomycin C survival curves as an endpoint to our analyses.

We first examined the ability of the siRNA oligonucleotides to knockdown protein expression in human HT-1080 cells. We chose these cells because they possess wild type copies of p53, an important regulator of the DNA damage response, and previous experiments had shown they are amenable to colony formation assays and siRNA transfection [27]. Initially, we established that we could detect siRNA-mediated knockdown of all proteins in HT-1080 cells using western blot analysis, with the exception of XRCC2. The commercial antibodies against XRCC2 that we attempted to utilize in this study were unable to detect the endogenous XRCC2 protein in all three human cell lines tested. We were able to visualize and knockdown the endogenous levels of the remaining four RAD51 paralogs (Fig. 1A). We also verified knockdown of BRCA2 and individual paralogs in cells transfected with siRNA oligonucleotides targeted against both proteins simultaneously, although we noted a slight decrease in the efficiency of BRCA2 knockdown when BRCA2 was targeted in combination with any of the RAD51 paralogs (Supplementary Fig. 1). As shown in Fig. 1B, we examined the effects of siRNA-mediated knockdown of BRCA2 (magenta diamonds) in HT-1080 cells in response to mitomycin C, relative to cells transfected with a non-targeting control siRNA ("Mock", blue squares). As anticipated, depletion of BRCA2 resulted in a significant decrease in survival (as much as 6-fold reduced) compared to the "mock" treated cells in response to mitomycin C. Knockdown of RAD51B, RAD51C, RAD51D, or XRCC3 individually resulted in an intermediate level of sensitivity compared to the most severe effect elicited by depletion of BRCA2 (Fig. 1B). There was a range of sensitivities for each paralog knockdown in HT-1080 cells, with RAD51C knockdown tracking close to the survival of BRCA2 knockdown, and RAD51B and XRCC3 displaying a more mild sensitivity; however, further studies demonstrated these responses were dependent on cell type (see Fig. 1D, and Fig. 3B and C). The variable responses in the different cell lines can result from either cell-type specific alternative splicing of the RAD51 paralogs or differences in DNA repair capacity [22,28–30].

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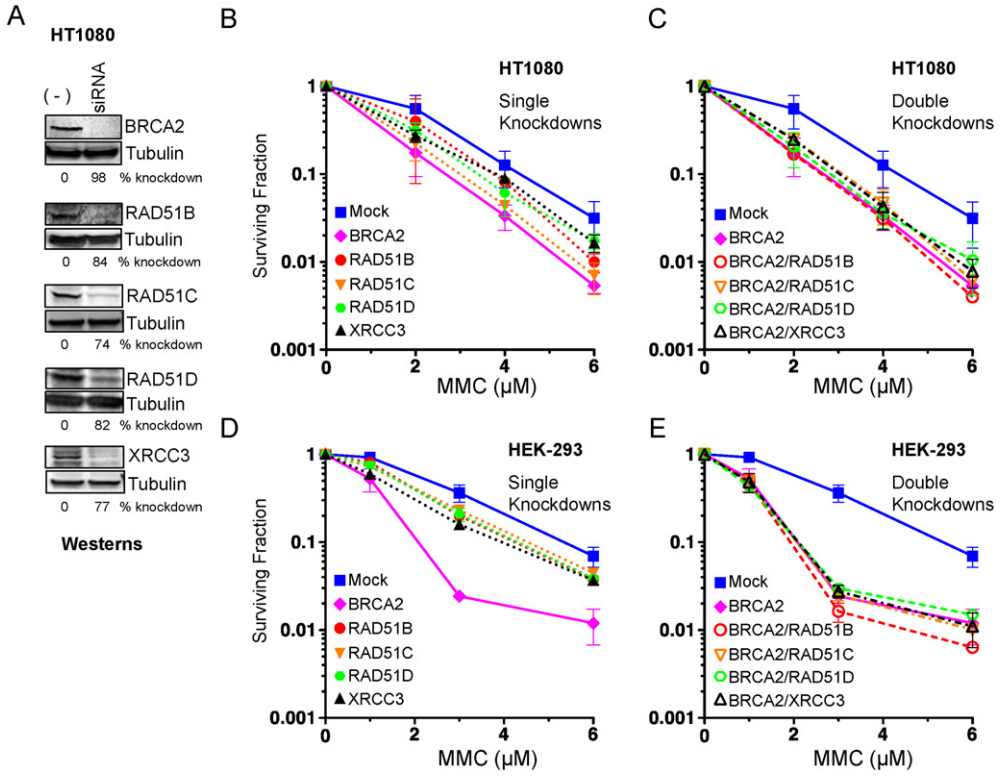

Fig. 1. Loss of BRCA2 results in a phenotype more severe than loss of a RAD51 paralog in human cells. (A) Western blots showing siRNA mediated knockdown of BRCA2, RAD51B, RAD51C, RAD51D, and XRCC3 in human HT1080 cells. Tubulin was used as an internal loading control. (B) Clonogenic survival of HT1080 cells depleted of BRCA2 or paralogs individually, and exposed to increasing concentrations of mitomycin C. (C) Mitomycin C clonogenic survival of HT1080 cells depleted of either BRCA2 alone or BRCA2 in combination with RAD51B, RAD51C, RAD51D, or XRCC3. (D) Clonogenic survival of HEK-293 cells depleted of BRCA2 or paralogs individually, and exposed to increasing concentrations of mitomycin C. (E) Mitomycin C clonogenic survival of HEK-293 cells depleted of either BRCA2 alone or BRCA2 in combination with RAD51B, RAD51C, RAD51D, or XRCC3. Data were analyzed using Prism 5.0c (GraphPad Software). All experiments were repeated a minimum of three times and error bars represent the standard deviation (S.D.).

3.2. *BRCA2 is epistatic to the RAD51 paralogs*

To assess the response of the double knockdowns, we targeted both BRCA2 and an individual paralog in the same cell population. Each of the double knockdowns (BRCA2/RAD51 paralog) displayed a very similar level of MMC sensitivity as cells depleted of BRCA2 alone (Fig. 1C). A similar result was recently shown in chicken DT40 cells where BRCA2 and XRCC3 null cells were examined for sensitivity to cisplatin, camptothecin, and olaparib [31]. To verify that the epistatic relationship between BRCA2 and the RAD51 paralogs was not due to any particular characteristics of HT-1080 cells, we performed the same experiment in another human cell line, HEK-293 (Fig. 1D and E). Knockdown of proteins, both individually and in combination, was assessed in HEK-293 cells by western blotting as shown in Supplementary Fig. 2. In agreement with the results in HT-1080 cells, we found that single paralog knockdowns in combination with BRCA2 resulted in a level of survival similar to the single BRCA2 knockdown in response to MMC (Fig. 1D and E). In HEK-293 cells depleted of BRCA2, we did note a modest added effect of RAD51B knockdown; however, the difference was less than 2-fold. The epistatic genetic interaction between BRCA2 and the RAD51 paralogs in two independent human cell lines, HT-1080 and HEK-293, supports the general validity of our findings. The greater sensitivity of the BRCA2 knockdown in both cell lines compared to the individual paralog knockdowns likely places BRCA2 in a hierarchical role in orchestrating recombination due to its key function in directly loading RAD51 onto single-stranded DNA (ssDNA) prior to the DNA strand invasion step. The epistatic relationship between BRCA2 and the paralogs differs from the independent genetic behavior of BRCA2 and RAD52 established recently [32].

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3.3. *BRCA2 and RAD51 paralog depletion leads to reduced survival in the absence of exogenous DNA damage in human U2OS cells*

To further verify the epistatic relationship between BRCA2 and the RAD51 paralogs, we performed siRNA knockdowns in another human cell line with wild type p53 function, U2OS (Figs. 2 and 3). As shown in Fig. 3A and Supplementary Fig. 3, we were able to knockdown BRCA2 and the paralogs both individually and in pairwise combinations. Unexpectedly, siRNA knockdown of BRCA2 and the paralogs in U2OS cells resulted in a significant loss of cell viability in the absence of any exogenous DNA damage (Fig. 2). This loss of viability upon siRNA depletion alone was not observed in HT-1080 or HEK-293 cells. The depletion of BRCA2 in U2OS cells resulted in the most dramatic loss of clonogenic survival compared to the individual depleted paralogs. Nonetheless, the epistatic nature of BRCA2 to the paralogs was again revealed in the absence of exogenous DNA damage, as the surviving fraction of cells in the double knockdowns was very similar to the BRCA2 knockdown alone. We did observe that the BRCA2/RAD51D double knockdown displayed a slightly more severe loss of viability than BRCA2 alone. Endogenously generated lesions (*e.g.*, reactive oxygen species) leading to unrepaired DNA breaks or gaps may account for the reduced survival observed in cells depleted of BRCA2 and the RAD51 paralogs. As shown in Fig. 2C, we analyzed the levels of gamma-H2AX S139 phosphorylation as a surrogate marker for induction of DNA double-strand

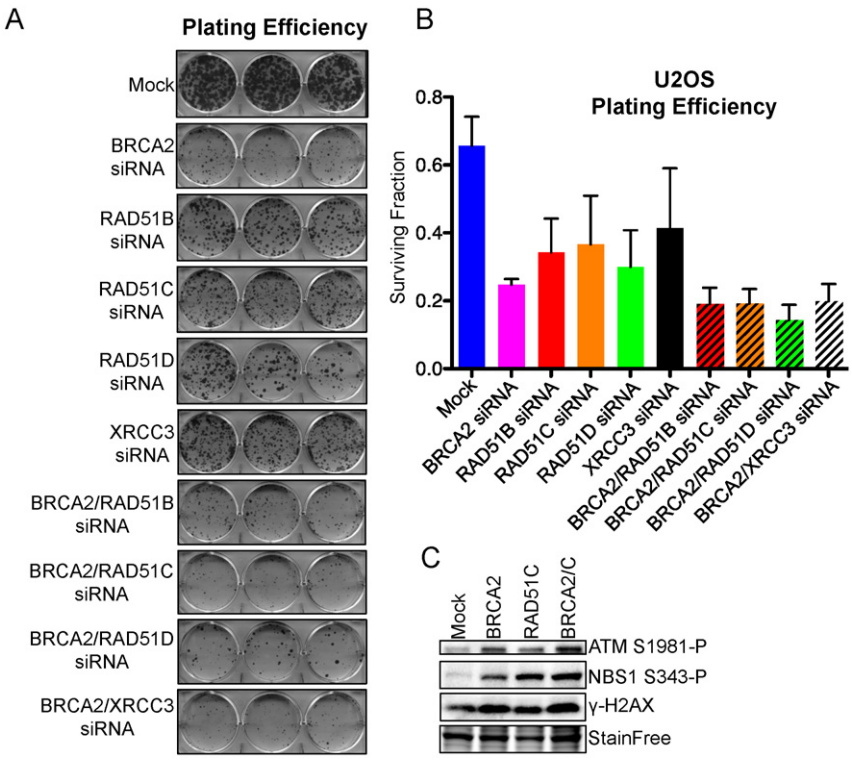

Fig. 2. BRCA2 is epistatic to the RAD51 paralogs in the absence of exogenous DNA damage. (A) Representative plates showing surviving colonies following depletion of BRCA2 and the paralogs by siRNA, both individually and as double-knockdowns. (B) Graph quantifying the fraction of surviving cells by clonogenic survival after siRNA transfections in U2OS cells. Mock treatment was a non-targeting siRNA. All experiments were repeated a minimum of three times and error bars represent the standard deviation (S.D). (C) Western blots showing induction of gamma-H2AX, NBS1 S343, and ATM S1981 phosphorylation in total cell lysates from U2OS cells 64 h after siRNA transfections. StainFree imaging of total protein in each lane was used as a loading control (cropped image of gel shown).

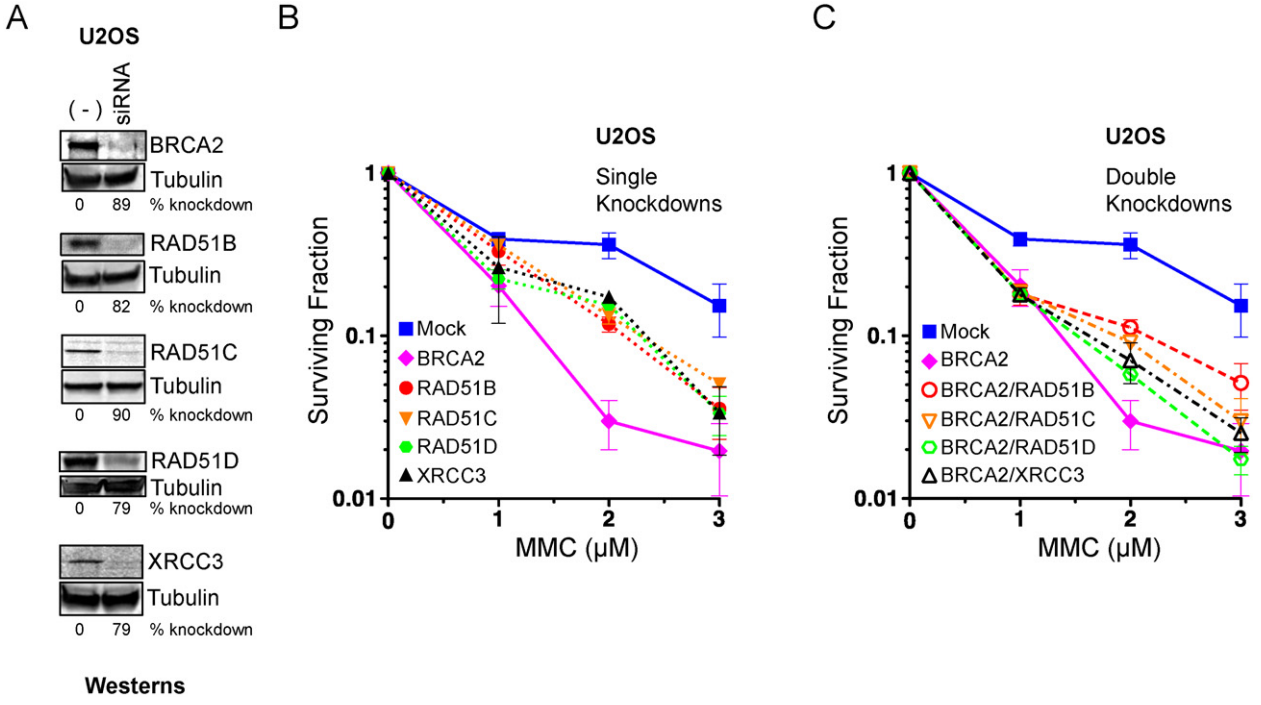

Fig. 3. BRCA2 is epistatic to the RAD51 paralogs in response to DNA damage. (A) Western blots showing siRNA-mediated knockdown of BRCA2, RAD51B, RAD51C, RAD51D, and XRCC3 in human U2OS cells. Tubulin was used as an internal loading control. (B) Clonogenic survival of U2OS cells depleted of BRCA2 or paralogs individually, and exposed to increasing concentrations of mitomycin C. (C) Clonogenic survival of U2OS cells depleted of either BRCA2 alone or BRCA2 in combination with RAD51B, RAD51C, RAD51D, or XRCC3; cells were exposed to increasing concentrations of mitomycin C. Each experiment was independent and repeated a minimum of three times. Error bars represent S.D.

breaks (DSBs) in cells depleted of BRCA2 and/or RAD51C. Additionally, we observed increased phosphorylation of S343 on NBS1 and S1981 on ATM; both are known phosphorylation events in response to DNA DSBs (Fig. 2C). We are currently pursuing the underlying mechanisms that result in these DNA breaks.

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3.4. Epistasis analysis in response to DNA damage

In agreement with the previous results obtained in HT-1080 and HEK-293 cells exposed to exogenous DNA damage, knockdown of BRCA2 in U2OS cells resulted in a more severe sensitivity to MMC compared to any RAD51 paralog alone. This result was found consistently even when normalized to the fraction of cells surviving in the absence of exogenous DNA damage (Fig. 3B). The depletion of each single RAD51 paralog resulted in an intermediate sensitivity to MMC compared to BRCA2 alone, and comprised a varied range of sensitivities with marginal differences between each individual RAD51 paralog. Surprisingly, some of the double knockdowns in U2OS cells resulted in decreased sensitivity to MMC, most notably at the 2 μM MMC concentration (Fig. 3C). The enhancement in survival was specific to exogenous DNA damage, as we saw no survival advantage in untreated U2OS cells that were doubly depleted by siRNA relative to their singly depleted counterparts (Fig. 2B). The survival advantage seemed to decrease at the higher 3 μM MMC concentration for some of the pairwise combinations.

4. Discussion

4.1. Epistatic relationship between BRCA2 and the RAD51 paralogs

From all human cell lines examined in this study, as well as results derived from *brca2* null mutants in DT40 cells [31], it is clear that BRCA2 exhibits a more severe defect than loss of any one RAD51 paralog. Our work and the study by Qing et al. have established that combined depletion of both BRCA2 and individual RAD51 paralogs result in a similar level of sensitivity to DNA damage as loss of BRCA2 alone. We observed the epistatic nature of BRCA2 knockdown both in the absence or presence of exogenous DNA damage. One interpretation of this analysis is that BRCA2 and the RAD51 paralogs are working together, possibly by direct interaction, in a similar sub-pathway to promote homologous recombination.

One limitation of our study is the inability to completely knock out BRCA2 and RAD51 paralog expression, as it was difficult to effectively deplete all cellular protein using RNA interference in human cells. While the siRNA-mediated knockdowns resulted in significant loss of expression, we cannot exclude the possibility that the functions of the residual BRCA2 or the RAD51 paralogs could lead to a different phenotypic outcome when compared to cells functionally null for these proteins. Indeed, inconsistent results from two separate studies regarding the genetic interplay between BRCA2 and RAD52 may be attributed to fundamental differences between chicken DT40 and human cells, or to differences resulting from RNA interference-mediated knockdown *versus* targeted knockout [31,32]. In any case, our manipulations of gene expression by siRNA resulted in significant decreases in survival as measured by clonogenic assay, and allowed us to directly compare both single- and double-knockdown scenarios within the same cell type. Furthermore, the epistatic nature of BRCA2 and the RAD51 paralogs was conserved across multiple human cell types reinforcing the observed relationship between these proteins.

4.2. Differential response of BRCA2 and RAD51 paralog knockdown in human U2OS cells

An unanticipated result from our study was the dramatic reduction in cell survival observed in U2OS cells after siRNA-mediated down-regulation of BRCA2 and the RAD51 paralogs. This effect was not observed in HT-1080 or HEK-293 cells perhaps due to deficits in cell cycle checkpoint or DNA damage signaling pathways in those particular cells. Our observations of increased phosphorylation of gamma-H2AX S139, S1981 on ATM, and S343 on NBS1, suggest that DNA DSBs are being generated in U2OS cells depleted of BRCA2 and RAD51C. One potential scenario is that U2OS cells rely on BRCA2 and the paralogs for repair of DNA DSBs during DNA replication. Another possible explanation for the increased cell death specific to U2OS cells is their reliance on BRCA2 and the RAD51 paralogs for maintenance of telomeres through the ALT pathway [33–37].

Unexpectedly, in response to MMC-induced DNA damage, we observed an increase in survival for certain BRCA2/paralog pairwise combinations specifically in U2OS cells. We did note less robust BRCA2 knockdown in U2OS cells depleted of both proteins simultaneously (see Supplementary Figs. 1–3) compared to individual protein knockdowns; however, we observed the same effect in HT-1080 and HEK-293 cells with no apparent increase in survival. The differential response in U2OS cells may relate to how these cells repair MMC induced cross-links. Perhaps, in the absence of BRCA2 and the RAD51 paralogs, repair is shunted into an alternate recombination pathway that can more efficiently remove the damaged DNA. Alternatively, if BRCA2 and the RAD51 paralogs are working together to remove inter-strands cross-links, the RAD51 paralogs may engage a futile repair pathway or stabilize a lesion that cannot be removed by homologous recombination. In this scenario, a forced alternate route to repair utilizing an error prone pathway may allow for more cell survival at the expense of mutagenic consequences for the cells.

4.3. Potential functions of RAD51 paralogs in homologous recombination

The exact mechanistic role each individual RAD51 paralog plays in recombination is currently unknown. It is perhaps surprising that depletion of RAD51C, the only paralog that is common to both of the RAD51 complexes B/C/D/X2 and C/X3 identified previously [6], did not result in the most sensitive behavior, relative to the other individual paralogs, with respect to cell survival in our assays. Reports in the literature ascribe a wide range of functions for the paralogs including: a mediator activity in assisting formation of the RAD51 filament, a role in telomere maintenance, and DNA melting activity [8,9,34]. Previous studies from our own laboratory have shown that BRCA2 plays a direct role in loading and stabilizing the RAD51 nucleoprotein filament by inhibiting the ATPase activity of RAD51 thereby maintaining the stable ATP bound form of RAD51 [38]. Perhaps, the paralogs allow for further stabilization of the RAD51 nucleoprotein filament in response to specific types of DNA damage. Another possibility is that the paralogs directly associate with the RAD51 filament and prevent its dissociation by motor proteins known to dislodge RAD51 from ssDNA, as is the case for Srs2 in yeast [39–41]. Because all RAD51 paralog and BRCA2 mouse knockouts studied to date result in embryonic lethality, it is likely they serve a required function necessary for growth and development that relates to chromosomal instabilities. Further studies utilizing the purified components will hopefully soon clarify the specific function of all five RAD51 paralogs in mediating homologous recombination.

Contributions

R.B.J. and S.C.K. conceived the general ideas for the study. R.B.J., A.O., A.E., and S.C.K. planned experiments and interpreted data; R.B.J., A.O., T.K., and A.E. performed the experiments. R.B.J. and S.C.K. wrote the manuscript.

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