RAD51 and BRCA2 Enhance Oncolytic Adenovirus Type 5 Activity in Ovarian Cancer
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Abstract
Homologous recombination (HR) function is critically important in high-grade serous ovarian cancer (HGSOC). HGSOC with intact HR has a worse prognosis and is less likely to respond to platinum chemotherapy and PARP inhibitors. Oncolytic adenovirus, a novel therapy for human malignancies, stimulates a potent DNA damage response that influences overall antitumor activity. Here, the importance of HR was investigated by determining the efficacy of adenovirus type 5 (Ad5) vectors in ovarian cancer. Using matched BRCA2-mutant and wild-type HGSOC cells, it was demonstrated that intact HR function promotes viral DNA replication and augments overall efficacy, without influencing viral DNA processing. These data were confirmed in a wider panel of HR competent and defective ovarian cancer lines. Mechanistically, both BRCA2 and RAD51 localize to viral replication centers within the infected cell nucleus and that RAD51 localization occurs independently of BRCA2. In addition, a direct interaction was identified between RAD51 and adenovirus E2 DNA binding protein. Finally, using functional assays of HR competence, despite inducing degradation of MRE11, Ad5 infection does not alter cellular ability to repair DNA double-strand break damage via HR. These data reveal that Ad5 redistributes critical HR components to viral replication centers and enhances cytotoxicity.

Implications: Oncolytic adenoviral therapy may be most clinically relevant in tumors with intact HR function. Mol Cancer Res; 14(1): 44–55. ©2015 AACR.

Introduction
Aberrant DNA damage responses (DDR) are common in human malignancies (1). This is particularly true in high-grade serous ovarian cancer (HGSOC), where approximately 15% patients have germline mutations in BRCA1 or BRCA2 (2). Moreover, data from The Cancer Genome Atlas (TCGA) consortium inferred that homologous recombination (HR) defects may be present in 50% HGSOC, through a variety of additional mechanisms, including somatic BRCA1/2 mutation and epigenetic loss of BRCA1 expression (3). A separate study, which used functional assays of HR competence in primary ascites cells from women with advanced HGSOC, strikingly concurred with TCGA, with 52% (26/50) showing HR deficiency (4). There is great interest in the use of poly-(ADP ribose) polymerase (PARP) inhibitors in HR-defective HGSOC (5), but there are few therapeutic targets available for HR-competent tumors, which have a poorer prognosis (6) and are less likely to respond to platinum-based chemotherapy (4).

Oncolytic adenoviruses are a potential novel therapy for ovarian and other human cancers. These viruses infect malignant cells, multiply selectively within them and cause cell death with release of mature virions that infect neighboring cells. An understanding of the complex interplay between the virus and host cells is vital to increase efficacy, develop biomarkers, and improve patient selection in clinical trials. E1A CR2-deleted Ad5 vectors, such as dl922-947 (7) and Δ24 (8), replicate selectively within cells with a defective Rb pathway, a frequent abnormality in many malignancies, including HGSOC (3). We have previously shown that dl922-947 has considerable activity in ovarian cancer and is more potent than E1A wild-type adenoviruses and the E1B-55K deletion-mutant dl1520 (9, 10). dl922-947 induces death via a necrosis-like mechanism (11), but the sensitivity of ovarian cancer cells to dl922-947 varies considerably, even between cells with similar infectivity (12).

Infection by many DNA viruses, including adenovirus, triggers a DDR, which viruses seek to circumvent through a series of mechanisms. A major target following adenovirus type 5 (Ad5) infection is the MRN complex (MRE11, RAD50, NBS1) and components of the non-homologous end-joining (NHEJ) pathway. Immediately following infection, before E1A is expressed, core protein VII protects the viral genome from recognition by MRN (13). As early proteins are expressed, MRN is inactivated by several mechanisms, including proteosomal degradation (14, 15) and mislocalization to PML-containing “nuclear tracks” (16, 17). Specific mechanisms that inhibit NHEJ include proteosomal degradation of DNA Ligase IV (18) and inactivation of DNA-dependent protein kinase (DNA-PK; ref. 19).
Beyond the observation that the BLM helicase is also degraded following Ad5 infection (20), there has been little specific investigation into the role of components of the HR pathway in adenovirus biology. Previously, we showed that oncolytic adenovirus activity is associated with profound deregulation of cell-cycle checkpoints and cell-cycle progression (21), inducing cellular DNA damage, with subsequent activation of DDR pathways, including ATR-Chk1 (22). Here, we have investigated the relationship between homology-mediated DSB repair and oncolytic adenovirus activity in ovarian cancer further. Our data indicate for the first time that key HR components BRCA2 and RAD51 interact with viral DNA replication centers and promote both virus replication and cytotoxicity.

Materials and Methods

Cell lines, viruses, and chemotherapy

Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) or RPMI (both Sigma) supplemented with 10% heat-inactivated FBS (Biosera), 100 units/mL penicillin and 100 mg/mL streptomycin (PAA Laboratories). Cell lines were maintained at 37°C in a humidified atmosphere with 5% CO2 and routinely passaged twice a week using 0.5% trypsin in PBS. All cell lines were routinely tested for mycoplasma and underwent 10-loci STR profiling to verify their authenticity, most recently in July 2014. SKOV3 and HeLa were obtained from Cancer Research UK Cell Services (Clare Hall, Hertfordshire, UK), TOV21G from Prof. F. Balkwill (Barts Cancer Institute, UK) and IGROV1 from NCI. PEO1 and PEO4 were kindly provided by Dr. Simon Langdon (University of Edinburgh, UK). Of these cell lines, IGROV1, PEO1, PEO4, and SKOV3 cells were maintained in RPMI, HeLa, and TOV21G cells in DMEM.

All viruses, including wild-type Ad5, Ad11 and Ad35, were originally obtained from Dr. Y. Wang (Barts Cancer Institute, UK). The Ad5 mutant d922-947 is deleted in the region encoding amino acids 122 to 129 of E1A CR2. It also contains a 745-bp deletion in E3B (nt 30,050–30,750) that is substituted by a 642-bp non-coding DNA fragment (7). d309 has the same E3B deletion as d922-947 but is E1A wild-type. Ad GFP is deleted in E1 and E3B and has green fluorescent protein (GFP) in the E1 position under control of the cytomegalovirus (CMV) immediate early promoter.

Cisplatin (Accord Healthcare) was obtained for the chemotherapy pharmacy, St Bartholomew’s Hospital, London. Ruca-parib was provided by Clovis Oncology.

Cell survival assays

For adenovirus and cisplatin experiments, cell survival was measured using the MTT assay (23) using a Wallac1420 Multi-label reader (PerkinElmer Life and Analytical Sciences). A total of 10^4 cells were infected in 24-well plates in serum-free medium. Cell viability was measured after 120 hours. CellTiter Glo (Promega) and sulforhodamine B assays were used in assays involving PARP inhibitors. For clonogenic assays, cells were infected in 24-well plates as above. Then, 72 hours after infection, cells were trypsinized and 100 to 200 cells were plated onto 6-well plates in triplicate. Colonies were stained with Crystal Violet 10 days thereafter and counted.

Virus infectivity assay

Cells (5 x 10^6) were infected with Ad GFP. GFP fluorescence was assessed 24 hours after infection on a FACSCaliber (Becton Dickinson). All conditions were repeated in triplicate and analyzed using FlowJo software.

Viral replication assays: TCID_{50} and quantitative PCR

Cells (2 x 10^4) were infected with d922-947 in serum-free medium and re-fed 2 hours later with serum containing medium. Up to 72 hours after infection, cells were harvested in 0.1 mol/L Tris pH 8.0 and subjected to 3 rounds of freeze/thawing (liquid N_2/37°C); all time points were harvested in triplicate. The supernatant was titred on JH293 cells.

Cells (2 x 10^5) were harvested at 24 to 72 hours following infection, washed twice in ice-cold PBS, and scrapped into 500 μL PBS. Extraction of viral DNA was performed using the QIAamp DNA Blood Mini Kit (Qiagen). Real-time PCR was performed using ABI Prism7500. Oligonucleotides and probes were as follows:

E1A: sense: 5’-CCACCTACCCCTACGAGCTG-3’; antisense: 5’-GCCTCTCTGTGGCATCTC-3’

PCR conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. A standard curve using 10^4 to 10^9 d922-947 genomes was used for quantification.

Reverse transcriptase PCR

For analysis of BRCA2 expression, 1 μg DNase-treated RNA was reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies). The following primers and probes were used to assess BRCA2 transcription.

BRCA2-sense 1: 5’-CAGAAGCCCTTTGAGAGTGGA-3’

BRCA2-antisense 2: 5’-AGAAACCGCAAGGGAACCTTG-3’

BRCA2-probe: 5’-GACCGCCCTCCCTCG-3’

PCR conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 60 seconds. A standard curve using 10^4 to 10^9 d922-947 genomes was used for quantification.

Immunoblotting

Protein lysates were electrophoresed on precast gels (Invitrogen) and transferred onto nitrocellulose membranes (GE Healthcare) by semi-dry blotting. Antibody binding was visualized using enhanced chemiluminescence (GE Healthcare) by semi-dry blotting. Antibody binding was visualized using enhanced chemiluminescence (GE Healthcare). Antibodies were obtained as follows: E1A (mouse, Becton Dickinson 554155), Ad5 (goat, Abcam Ab36851), RAD51 (rabbit, Santa-Cruz sc-8348), mec3 (rabbit, Abcam ab36851), Ku70 (goat, Santa-Cruz, sc-1486), and (rabbit, Cell Signaling, 4895). All HRP-conjugated secondary antibodies were obtained from Dako.

Southern blotting

Cells (10^6) plated and infected with d922-947 (MOI 10 for PEO4, MOI 100 for PEO1 cells). Forty-eight and 72 hours later, cells were trypsinized and washed in TBS. Extraction of DNA was performed using the QIAamp DNA Blood Mini Kit (Qiagen). DNA concentration was measured using Nanodrop ND-1000 spectrophotometer and 10 μg genomic DNA was electrophoresed on a 1% agarose gel. The gel was soaked in 200 mL 0.2 mol/L HCl for 10 minutes, washed and then soaked in alkaline transfer buffer (0.4 mol/L NaOH, 1 mol/L NaCl). DNA was transferred onto a Hybond N+ membrane (GE Healthcare Life Sciences) by
conventional capillary transfer over 48 hours. Probe labeling against viral DNA, hybridization, and detection was performed using the Amersham ECL Direct Nucleic Acid Labeling and Detection systems.

**Flow cytometry**
For cell-cycle analyses, cells were trypsinized, washed twice in ice-cold PBS, and fixed in ice-cold 100% ethanol. Cells were then washed with PBS and resuspended in 200 µL propidium iodide (PI) and 100 µg/ml RNase A (MP Biomedicals). For γH2AX analysis, cells were harvested, washed, and fixed in ice-cold 70% ethanol. After incubation with primary anti-γH2AX mAb or IgC control, cells were washed and then incubated with FITC-conjugated anti-mouse secondary antibody (Invitrogen) for 1 hour in the dark and analyzed using a FACSCaliber flow cytometer. Results analyzed using FlowJo software.

**Immunofluorescence and coimmunoprecipitation**
Cells were seeded on poly-L-lysine-coated coverslips and treated with rucaparib (10 µmol/L for 24 hours) or db922-947 for up to 48 hours. Following treatment, medium was aspirated and 0.04% Triton (Sigma) in PBS added for 1 minute. Cells were then fixed in 3% paraformaldehyde and 2% sucrose for 10 minutes. The cells were stained with anti-γH2AX antibody (Millipore) or a rabbit anti-Ad5 E2-DNA binding protein (E2-DBP, a kind gift of Dr. David Ornelles, Wake Forest Medical Center, NC) and costained with anti-RAD51 or anti-BRCA2 (E2-DBP, a kind gift of Dr. David Ornelles, Wake Forest Medical Center, NC) and costained with anti-RAD51 or anti-BRCA2 antibody (Millipore) or a rabbit anti-Ad5 E2-DNA binding protein (E2-DBP, a kind gift of Dr. David Ornelles, Wake Forest Medical Center, NC) and costained with anti-RAD51 antibody (Santa Cruz, H-92, sc8349) or control antibody (anti-HA, Sigma) for 1 hour at room temperature and washed with PBS and resuspended in 200 µL PBS. Immuno-fluorescence and co-precipitation were counted using ImageJ software.

**DCB damage (28), we con**

**Results**

**Adenovirus cytotoxicity is greater in HR-competent ovarian cancer cells**
To investigate the link between cellular HR function and adenovirus activity, we first utilized the PEO1/PEO4 cell pair (26). These cells originate from the same ovarian cancer patient: PEO1 was derived at the time of first, platinum-sensitive relapse and contains a deleterious BRCA2 mutation; PEO4 was derived at subsequent relapse, when platinum resistance had developed, and contains a secondary BRCA2 mutation that restores the open reading frame (27). Using a previously described assay of HR competence, based upon formation of RAD51 foci in response to DSB damage (28), we confirmed that PEO4 cells demonstrate functional HR, while PEO1 are HR defective (Fig. 1A and Supplementary Fig. S1). We also confirmed that BRCA2-mutant PEO1 are more sensitive to both BRCA2 wild-type PEO4 to both cisplatin and the PARP inhibitor rucaparib (Supplementary Fig. S2).

We found PEO4 to be significantly more sensitive to cytotoxicity induced by the E1A CR2-deleted Ad5 vector db922-947 (Fig. 1B) as well as Ad5 WT and db309 (E1A wild-type; Fig. 1C). PEO4 were slightly more infectable with Ad5 vectors than PEO1 (data not shown). However, even when MOI was adjusted to ensure equal levels of infection (hereafter called iso-infection), PEO4 remained significantly more sensitive to both db922-947 and db309 (Fig. 1D). This increased sensitivity in PEO4 was confirmed by the clonogenic assay (Supplementary Fig. S3). By immunoblot, there was comparable early (E1A) and late viral protein expression in iso-infected cells (Fig. 1E).

Assessing viral replication, there was no significant difference in the number of infectious virions generated following iso-infection (Fig. 1F). However, quantitative PCR indicated that there was more viral DNA generated in PEO4 (Fig. 1G), which was
Figure 1.
Greater efficacy and viral DNA replication in HR-competent than HR-defective ovarian cancer cells. A, competence of HR was assessed in PEO1 and PEO4 cells. Cells were treated with rucaparib (10 μmol/L, 24 hours), permeabilized, fixed in 4% PFA, and stained for RAD51 and γH2AX. RAD51 foci were counted in at least 30 nuclei per treatment condition. Bars, mean (± SD) number of RAD51 foci per cell. Dotted line, 2× number of foci in untreated cells. B, 10^4 PEO1 and PEO4 cells were infected in triplicate with dl922-947. Cell survival was measured 120 hours after infection by the MTT assay. Mean (± SD) IC50 for four experiments are shown: *, P = 0.01. C, 10^4 PEO1 and PEO4 cells were infected in triplicate with Ad5 WT (left) or dl309 (right) (MOI 0.001–1000 pfu/cell). Cell survival was measured 120 hours after infection by the MTT assay. D, PEO1 and PEO4 cells were infected with Ad CMV-GFP (left) at MOI 60 and 100 (PEO1) and 6 and 10 (PEO4). GFP positivity was assessed 24 hours after infection by flow cytometry. PEO1 and PEO4 cells were also infected with dl922-947 (right) at MOI 60 and 100 (PEO1) and 6 and 10 (PEO4). Cell survival was assessed 120 hours after infection by the MTT assay. Data, mean (± SD); n = 3. **, P < 0.001. E, PEO1 and PEO4 cells were infected with dl922-947 MOI 100 (PEO1) or 10 (PEO4). Protein was harvested up to 72 hours after infection. Expression of E1A and adenovirus 5 structural proteins was assessed by immunoblot. E1A band density was assessed from three separate exposures: 24, 48, and 72 hours; mean (±SD). E1A:KU70 ratio was 1.3 ± 0.2, 1.4 ± 0.1, and 1.4 ± 0.2 for PEO1, and 0.7 ± 0.4, 1.2 ± 0.3, and 1.6 ± 0.2 for PEO4. F and G, PEO1 and PEO4 cells were infected with dl922-947 MOI 100 (PEO1) or 10 (PEO4) for up to 72 hours. Virus replication was assessed by TCID50 (F) or quantitative PCR (G). ***, P < 0.001. H, PEO1 and PEO4 cells were infected with dl922-947 MOI 100 (PEO1) or 10 (PEO4) for up to 72 hours. DNA was extracted and subjected to neutral pulsed-field gel electrophoresis, probed with HRP-labeled adenovirus type 5 probe. 100 ng purified dl922-947 DNA was run as a positive control (+).
confirmed by Southern blotting (Fig. 1H). There were no obvious abnormalities in viral DNA processing on the Southern blot, and specifically no obvious concatemers in either cell line (Fig. 1H and Supplementary Fig. S4 for long exposure). The increased sensitivity appeared to be Ad5 specific, as there was no difference between PEO1 and PEO4 in sensitivity to the group B adenoviruses Ad11 and Ad35 (Supplementary Fig. S5).

Adenovirus DNA replication triggers the DDR and interacts with core HR machinery
We first investigated whether the difference in sensitivity to Ad5 vectors between HR-proficient and HR-deficient cells was reflected in their accumulation of DNA damage. In keeping with their germline BRCA2 mutation and genomic instability (29), uninfected PEO1 cells demonstrated greater basal levels of DNA damage (γH2AX positivity) and a higher proportion of the cells with >4N DNA content on flow cytometry than PEO4 (Fig. 2A and Supplementary Figs. S1 and S6). However, following iso-infection with d922-947, there were significantly greater increases in both γH2AX positivity and >4N DNA in PEO4 (Fig. 2A), consistent with our previous observations that virus-induced DNA damage correlates with sensitivity (22, 30).

Inhibition of NHEJ using the DNA-PKcs inhibitor NU7026 had no effect on cytotoxicity (data not shown), and, as previously noted (14), there was a reduction in expression of MRE11 following d922-947 infection in both PEO1 and PEO4 (Fig. 2B), and the reduction was similar in both cell lines. However, expression of RAD51 did not diminish following infection in either PEO1 or PEO4 (Fig. 2C). By confocal microscopy, BRCA2 foci was maintained in PEO4 following d922-947 infection. Interestingly, there was clear colocalization between BRCA2 and viral replication centers (VRC), as indicated by expression of viral E2 DNA binding protein (E2 DBP; Fig. 2D). Furthermore, we also saw clear colocalization between RAD51 and E2 DBP in PEO4 (Fig. 2E). It is widely known that, in BRCA2-deficient cells, RAD51 is unable to form foci at the site of DNA damage (Supplementary Fig. S1; ref. 28). However, to our surprise, we observed RAD51 foci colocalized with E2 DBP in PEO1 cells, despite the absence of BRCA2 (Fig. 2E). These findings were confirmed in two other lines that demonstrated HR competence, TOV21G, and HeLa (Fig. 3A), as well as in IGROV1 cells, which are hypermutated and contain mutations in both BRCA1 and BRCA2 (ref. 31; http://cancer.sanger.ac.uk/). These results were recapitulated in other malignant cell lines, HeLa and TOV21G, that are BRCA2 wild-type and HR competent (Fig. 3B and C). Taken together, our data suggest that recruitment of RAD51 and BRCA2 to VRC augments viral replication and cytotoxicity, and is independent of its role in the response to DNA damage.

Discussion
In this article, we show for the first time that components of the HR pathway of DNA double-strand break repair significantly influence the activity of Ad5 vectors. Using matched BRCA2-mutant and wild-type ovarian cancer cells, we show that the activity of both E1A wild-type (Ad5 WT and d9309) and E1A CR2-deleted (d922-947) Ad5 viruses is greater in the presence of functional BRCA2, with increased cytotoxicity and viral DNA replication, and that BRCA2 colocalizes with VRC within the nucleus. These results were recapitulated in other malignant cell lines, HeLa and TOV21G, that are BRCA2 wild-type and HR competent. Moreover, we were able to demonstrate that RAD51, a key partner of BRCA2, also influences Ad5 activity. Strikingly, we
Figure 2. dl922-947 replication induces genomic DNA damage; RAD51 and BRCA2 colocalize with sites of adenovirus replication. A, PEO1 and PEO4 cells were harvested 48 hours following infection with dl922-947 (MOI 100 and 10, respectively) or mock infection, fixed in 70% cold ethanol, incubated with an anti-γH2AX Ab, counterstained with PI, and analyzed by flow cytometry (left). Increase in γH2AX-positive cells and cells with >4N DNA are plotted (right); bars, mean ± SD; n = 3. P < 0.001. B and C, PEO1 and PEO4 cells were harvested following infection with dl922-947 (MOI 100 and 10, respectively) or mock; expression of E1A, MRE11 (B), and RAD51 (C) was detected by immunoblot. D and E, PEO1 and PEO4 cells were fixed in 4% PFA following infection with dl922-947 (MOI 300 and 30, respectively). Expression of adenovirus E2 DNA binding protein, BRCA2 (D), and RAD51 (E) was assessed by confocal microscopy.
Figure 3.
RAD51 and BRCA2 colocalize with sites of adenovirus replication in multiple malignant cell lines. A, HR competence was assessed in TOV21G, HeLa, and IGROV1 cells as for Fig. 1A. Bars, mean (=SD) number of RAD51 foci per cell. Dotted line, 2× number of foci in untreated cells. TOV21G and HeLa demonstrate HR competence, while IGROV1 are HR defective. B and C, cells were fixed in 4% PFA following infection with δ922-947 (MOI 10). Expression of adenovirus E2 DNA binding protein, BRCA2 (B), and RAD51 (C) was assessed by confocal microscopy. D, RAD51 was immunoprecipitated from TOV21G infected with δ922-947 (MOI 10), and the presence of E2 DNA binding protein was detected by immunoblotting.
Figure 4.
RAD51 knockdown decreases adenovirus efficacy and replication. A, using two different siRNA pools, RAD51 was knocked down in both PEO1 and PEO4 cells. B and C, 24 hours following siRNA-mediated RAD51 knockdown, PEO1 (MOI 300) and PEO4 cells (MOI 30) were infected with dl922-947 (left) and dl309 (right, MOI 500 and 50). Survival was assessed 96 hours after infection by the MTT assay (B). Viral replication was also assessed 48 hours after infection by quantitative PCR (C). *, P < 0.05; ***, P < 0.001. D–F, 24 hours following siRNA-mediated RAD51 knockdown, TOV21G (D), HeLa (E), and IGROV1 (F) cells were infected with dl922-947 (MOI 1, 8, and 5 respectively). Survival was assessed 96 hours after infection by the MTT assay. RAD51 knockdown was confirmed by immunoblot. Viral replication was also assessed in TOV21G 48 hours after infection. **, P < 0.01; ***, P < 0.001.
show that RAD51 influences adenovirus activity and locates to VRC in the absence of functional BRCA2.

HR is vitally important in the biology of HGSOCl. Tumors with intact HR are less likely to respond to platinum-based chemotherapy (4) and have a worse overall prognosis (6). HR is a complex process involving multiple proteins. However, BRCA2 is particularly important, as it mediates the loading of RAD51 onto 3'-single-stranded DNA overhangs (created by MRE11 nuclease activity), creating a RAD51 nucleoprotein filament. The nucleoprotein filament then catalyzes the critical step of HR, namely strand invasion and homology search on the sister chromatid.

The interaction between adenoviral infection and the DDR is complex; in contrast to other forms of DNA damage, such as irradiation, where damage occurs almost instantaneously, viral infection represents a dynamic onslaught to the cell, which makes analysis challenging. However, the DDR is clearly activated following viral infection—here and previously (22), we show robust phosphorylation of H2AX following infection with dl922-947 and other Ad5 viruses, which others have also observed (32). We have also shown that Ad5 infection activates replication-dependent ATR/Chk1 signaling (22). Ad5 inhibits DDR using a variety of mechanisms, prime among which is proteasome-mediated degradation of key cellular proteins, including MRE11 and DNA Ligase IV. Degradation is largely orchestrated by E1B55K and E4orf6, in concert with cellular proteins Cul5, Rbx1, and elongins B and C (15, 33), while infection with E4-deleted viruses results in concatemer formation (34). Consistent with these previous findings, we show here that MRE11 expression diminishes following Ad5 infection in ovarian cancer cells, regardless of their HR competence, and that inhibition of DNA-PK has no effect on overall cytotoxicity in both HR-competent and HR-defective cells. In addition, our Southern blot showed no concatemer formation in either PEO4 or PEO1 cells, suggesting that viral DNA can be processed correctly regardless of the state of cellular HR competence.

The relocation of other DDR proteins, including RPA32 (35), ATR, ATRIP, Rad9, TOPBP1, Rad17, and hnRNPUL1 (36–38), to VRC following Ad5 infection has been described previously. However, it has been unclear whether this relocation inhibits DNA damage repair function or whether it is required for viral replication (reviewed in 39)—in the case of BRCA2 and RAD51, our data suggest the latter, as loss of either protein reduces Ad5 replication. In addition, using three different techniques to assess HR function, our results suggest that the ability of cells to repair DNA DSB damage via HR is not inhibited following Ad5 infection. This reinforces the idea that Ad5 utilizes components of the HR pathway rather than degrading and inhibiting them, as is the case with NHEJ.

Several key questions remain. First, how do cells retain the apparent capacity to repair DSB damage by HR following the proteasomal degradation of MRE11, which is critical for end resection? Although MRE11 is clearly critical to end resection, other molecules, in particular CtIP and Exo1, can also fulfill this role (40, 41). Thus, it is possible that other proteins substitute for the MRE11 end-resection function following Ad5 infection. Also, degradation of MRE11 is not complete (see Fig. 2B and ref. 42); thus, there may be a dosage effect whereby the remaining MRE11 retains sufficient end-resection capacity following adenovirus infection. Nonetheless, whatever residual HR capacity remains following infection is still unable to repair virus-induced genomic DNA damage. This may result from both the cell-cycle and DNA replication states induced by the virus. Adenovirus infection drives infected cells into an S phase–like state, with endoreduplication of genomic DNA (43). However, genomic DNA replication is clearly disorganized, which may preclude reliable generation of intact sister chromatids. In addition, adenovirus infection causes override of multiple cell-cycle checkpoints, with
appearance of multiple abnormal mitoses (21). Thus, cells may slip rapidly through S and G2 phases, thereby precluding HR repair. This reemphasizes the challenge of investigating cellular responses to adenovirus infection, where changes are dynamic and evolve over a period of 48 to 72 hours.

A second key question is how do BRCA2 and RAD51 relocate to VRC and which adenovirus proteins drive the process? And indeed what is their precise function within these VRC? Ad5E4orf3 is responsible for relocation of PML protein into cytoplasmic tracks, but whether it is also responsible for movement of cellular proteins into VRC is unknown. Our immunoprecipitation suggested a direct interaction between RAD51 and E2DBP; our attempts at immunoblot and immunoprecipitation of BRCA2 were unsuccessful, so we do not know whether there is also a similar direct interaction with E2DBP. Certainly, the RAD51 role in viral efficacy appears independent of its function in HR as knockdown in HR-competent cell lines also reduces viral efficacy. Our data, however, show that the presence of BRCA2 together with RAD51 results in more efficient viral replication. Adenovirus DNA replication generates a displaced single strand of parental DNA in addition to a duplex formed of a newly synthesized daughter strand plus the other parental strand, a structure that could resemble a replication fork. Both RAD51 and BRCA2 have recently been shown to protect newly replicated DNA strands at stalled replication forks from degradation (44, 45) and this role is independent of their function in HR. It is possible that adenovirus utilizes this function of RAD51 and BRCA2 resulting in more accurate and efficient viral DNA replication, and that RAD51 can execute this role alone. Certainly, no BRCA2 homolog has been identified in lower eukaryotes, including Saccharomyces cerevisiae, in which Rad51 alone can resolve replication stress. Clearly adenoviral replication and cytotoxicity can still occur in the absence of BRCA2 and RAD51, suggesting that these proteins are supportive rather than critical to the adenoviral lifecycle.

Third, why are there differences between adenovirus species in this effect? Here, we show that sensitivity to Ad11 and Ad35 does not vary between BRCA2 WT and mutant cells, and previous data demonstrate that DDR proteins vary in their targeting by different adenoviral serotypes (42). Nonetheless, it is clear that interaction with DDR machinery is a widespread phenomenon in DNA virus infection. ATM is required for optimal replication of SV40 (46) and HSV-1 requires ATM and the MRN complex for virus replication (47), while proteins involved in HR have been shown to localize to EBV VRCs (48).

In summary, we show for the first time that adeno- virus type 5 relocalizes components of the HR pathway to VRC and that viral replication is enhanced in the presence of functional HR. We have recently shown that oncolytic adenoviruses may be more effective in ovarian cancers with paclitaxel resistance (30). Data here show that these viruses may also have specific activity in another group.
of poor prognosis ovarian cancers, namely those with platinum and PARP inhibitor resistance through intact HR function. Given the importance of HR in the biology of HG SCT, an understanding of the interaction between HR and any novel therapy is particularly important in patient selection for clinical trials and identification of novel virus/drug combinations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.A. Tookman, A.K. Browne, C.K. Ingemarsdotter, S. Dowson, A. Shibata, I.A. McNeish, G. Bridge

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.A. Tookman, S. Dowson, M. Lockley, S.A. Martin, I.A. McNeish

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