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Inhibition of non-homologous end joining in Fanconi Anemia cells results in rescue of survival after interstrand crosslinks but sensitization to replication associated double-strand breaks

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Abstract

When Fanconi Anemia (FA) proteins were depleted in human U2OS cells with integrated DNA repair reporters, we observed decreases in homologous recombination (HR), decreases in mutagenic non-homologous end joining (m-NHEJ) and increases in canonical NHEJ, which was independently confirmed by measuring V(D)J recombination. Furthermore, depletion of FA proteins resulted in reduced HR protein foci and increased NHEJ protein recruitment to replication-associated DSBs, consistent with our observation that the use of canonical NHEJ increases after depletion of FA proteins in cycling cells. FA-depleted cells and FA-mutant cells were exquisitely sensitive to a DNA-PKcs inhibitor (DNA-PKi) after sustaining replication-associated double strand breaks (DSBs). By contrast, after DNA interstrand crosslinks, DNA-PKi resulted in increased survival in FA-deficient cells, implying that NHEJ is contributing to lethality after crosslink repair. Our results suggest FA proteins inhibit NHEJ, since repair intermediates from crosslinks are rendered lethal by NHEJ. The implication is that bone marrow failure in FA could be triggered by naturally occurring DNA crosslinks, and DNA-PK inhibitors would be protective. Since some sporadic cancers have been shown to have deficiencies in the FA-pathway, these tumors should be vulnerable to NHEJ inhibitors with replication stress, but not with crosslinking agents, which could be tested in future clinical trials.

INTRODUCTION

The genome is constantly under threat from both exogenous and endogenous sources, with the double strand break (DSB) inducing a particular pattern of genome instability and a greater likelihood of cell death (1). To prevent cancer development, the cell uses multiple DSB repair pathways. Homologous recombination (HR) is usually an error-free mechanism

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LE carried out the experimental work, with significant support from AB. SNP conceived and developed the scientific ideas. All authors contributed to the writing, figure preparation and completion of the paper.

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of DSB repair, since it uses homologous undamaged duplex DNA as a template for repair of the damaged DNA duplex. Since the most readily available homologous template is the sister chromatid, HR occurs predominantly in the S/G₂ phases of the cell cycle. In contrast to HR, non-homologous end joining (NHEJ) is active throughout the cell cycle, but is error prone during the repair of DSBs, most frequently producing small deletions at the break site.

Despite known details of DSB repair by HR and NHEJ, it's less clear how each DSB repair mechanism is engaged, both with respect to the type of DNA damage and the phase of the cell cycle. If a broken duplex gets resected on one strand, then canonical NHEJ cannot be engaged and alternative NHEJ can be utilized to anneal the two partially resected ends together, using some degree of micro-homology, followed by strand gap filling with a specialized polymerase, such as POLQ (Polθ). There is increasing evidence that mutagenic-NHEJ (m-NHEJ; also known as alternative NHEJ or microhomology-mediated end-joining (MMEJ)) is a repair pathway in its own right, rather than simply a back-up pathway when canonical Ku-dependent NHEJ (c-NHEJ) is unavailable (2). M-NHEJ is more error prone than c-NHEJ and is particularly prone to translocation due to the increased likelihood of inappropriate joining of any two partially resected DNA ends, which can occur in proliferating cells.

Fanconi Anemia (FA) is a complex disorder caused by biallelic mutations in any one of the 22 genes currently known to be FA genes. The disorder is autosomal recessive and is characterized by diverse congenital skeletal abnormalities, progressive pancytopenia and predisposition to malignancy (3–5). The repair pathway mediated by FA proteins is responsible for removal of interstrand crosslinks (ICL), and as a result FA-defective cells are characterized by sensitivity to agents such as Mitomycin C. Upon recognition of ICL in replicating cells by FANCM, the FA core complex, which comprises FANC-A, B, C, E, F, G, and L, is assembled at or near the site of damage. Once assembled, the core complex protein FANCL ubiquitylates FANCD2, in the FANCD2/I complex, which allows its recruitment to the chromatin. All members of the core complex must be present for correct assembly and subsequent ubiquitylation of the FANCD2/I complex (6,7). The ubiquitylated complex is then able to localize with HR proteins and initiate downstream repair. Therefore, FANCD2 is one key player of the FA pathway due to its critical ubiquitylation and its increased abundance relative to other FA proteins (8,9).

The FA proteins also function outside of a restricted role in ICL repair. It has been shown that FANCG and FANCD2 form complexes with various HR proteins, including BRCA2 and the Rad51 paralogs (10–12). The exact function of such complexes is not clear, although a role in HR is proposed (13). FANCD2 has also been previously shown to actively promote alt-EJ mechanisms through its recruitment of polymerase *theta* to both stalled and collapsed replication forks due to HR-deficiency (14). Several HR proteins were recently identified as FA proteins, such as FANCD1/BRCA2, FANCO/Rad51C, FANCP/Slx4, FANCR/Rad51, and FANCS/BRCA1 (15–19). In addition, the FANCD2 gene is a nucleotide excision repair protein ERCC4, also involved in DNA flap repair (20). FANCG is considered to be the scaffold protein for the FA core complex and phosphorylation of this protein is essential for FANCD2-BRCA2 interaction (13).

Many FA proteins are conserved across evolution allowing them to be studied in various organisms, particularly *C. elegans*, where the essential components of the FA pathway remain intact (21–23). This whole organism system was successfully used to identify the role of FANCD2 in prevention of inappropriate engagement of DNA-PKcs to one-ended DSBs (24). Blocking NHEJ has also been shown to suppress the ICL sensitivity of FA-deficient cells (24,25), since inhibition of NHEJ prevents improper binding of NHEJ proteins and promotes error-free repair by HR. Together, these studies lead to questions of the involvement of FA proteins in wider role in DNA damage repair, specifically DSB repair pathway choice.

In order to determine the effect of depleting FA proteins on the choice of DSB repair pathway, we utilized a human cell reporter assay (26) in addition to the V(D)J recombination assay (27). After depleting FA proteins, we identified a significant decrease in both HR and m-NHEJ, while the use of canonical, Ku-dependent NHEJ (c-NHEJ) significantly increased. Supporting this observation, exposure of FA-depleted cells to replication-associated DSB-inducing agents increased the recruitment of NHEJ proteins, and decreased the recruitment HR proteins. Additionally, FA-depleted cells were shown to be uniquely sensitive to inhibition of DNA-PKcs in combination with replication-associated DSB-inducing agents, but the use of crosslinking agents (to which FA-depleted cells are sensitive) with DNA-PK inhibitors resulted in paradoxically improved survival. The interplay of DNA damaging agents, genetic status of tumors and repair inhibitory drugs needs to be understood for the optimal treatment of Fanconi Anemia and Fanconi Anemia-defective human tumors.

MATERIALS AND METHODS

DSB repair reporter assays

HR and mutagenic NHEJ—U2OS cells were stably integrated with both pDR-GFP and EJ-RFP plasmids as previously described (26). 0.75×10^6 cells were transiently transfected with 1.5 μ g siRNA and seeded into two wells of a 6-well plate. After 24 h, cells were transfected with 2 μ g of pCMV-ISceI-3xNLS or pCMV-3xNLS empty vector and 1 μ g siRNA using Lipofectamine 2000 (Invitrogen). After 96 h, cells were trypsinized and the percentage of green fluorescent protein (GFP) versus red fluorescent protein (RFP) was assessed using flow cytometry (FACS Calibur, Becton Dickinson) as previously described (26).

Canonical NHEJ— 2×10^6 HEK293T cells were transiently transfected with 4 μ g NT, FANCD2 or FANCG siRNA. After 24 h, cells were transfected with 2 μ g each CMV6-RAG1 (Origene), CMV6-RAG2 (Origene), and pSVD3-320, in addition to 1 μ g siRNA using Lipofectamine 2000 (Invitrogen). After 48 h, cells were trypsinized, pelleted, and plasmids were harvested using the method as previously described (28). DNA was extracted and purified with phenol: chloroform: isoamyl alcohol (pH 8.0; Sigma) and isopropanol/sodium acetate precipitation. 2 μ g DNA was digested with 20 U DpnI (New England Biolabs) using standard method to remove unreplicated plasmids. DNA was purified again, and 250 ng DNA was transformed into 25 μ l electrocompetent DH5 α cells (Invitrogen). After incubation, the culture was spread onto ampicillin selective agar plates, with the

remaining culture being spread on ampicillin chloramphenicol selective agar plates and incubated at 37°C overnight. Colonies were counted and the ratio of ampicillin and chloramphenicol resistant colonies to ampicillin-only resistant colonies was recorded as the measure of c-NHEJ.

Colony survival assays

2.5×10^6 U2OS cells were transiently depleted of FANCD2, FANCG, or NT control and $\sim 0.25 \times 10^6$ cells per well were seeded into 6-well plates. After 48 h cells were treated with indicated doses of the damaging agent. After 4 h cells were reseeded at various densities of cells per well of a 6-well plate. DNA-PKcs inhibitor (NU7441; 1 μ M) was added to the cells 1 h prior to treatment and PARP inhibitor (ABT888 or veliparib; 40 μ M Selleck Bio) was added 24 h prior to treatment. Colonies were stained 14 days later.

Imaging protein recruitment by confocal microscopy

U2OS cells were transfected with NT, FANCD2 or FANCG siRNA. Cells were incubated overnight and then treated with the appropriate damaging agent. Cells were fixed, blocked, and permeabilized. Cells were stained using the following antibodies: anti-BRCA1 (sc-6954) from Santa Cruz Biotechnologies, anti-53BP1 (#4937) from Cell Signalling, anti-phospho DNA-PKcs (ab18192) from AbCam, or anti-Mre11 (GTX118741) from GeneTex. Secondary antibodies AlexaFluor 596-labeled donkey anti-rabbit IgG, AlexaFluor 488-labeled chicken anti-mouse IgG (Molecular Probes) were used. Images were obtained using a Zeiss LSM 510 Meta scanning confocal microscope and were analyzed using ImageJ software. At least 250 nuclei were counted per experiment.

RESULTS

Depletion of FA proteins disrupts DSB pathway choice

Using the dual DSB repair reporter system, U2OS EJ-DR, developed within our laboratory, we are able to assess the levels of repair of an I-SceI-induced DSB in the same cell by HR and m-NHEJ, at two separate loci (26). HR is measured via the previously reported DR-GFP plasmid (29) stably integrated into human osteosarcoma U2OS cell line, where repair of an I-SceI-induced DSB by HR, using a downstream GFP fragment as the homologous template, results in expression of GFP quantifiable by FACS. M-NHEJ is similarly measured by FACS; here, disruption of the Tet repressor loop following mis-repair of the I-SceI-induced DSB allows RFP expression. Validation of the assay is shown in Supplementary Figure S1, where depletion of HR protein BRCA2 causes loss of measurable HR as expected, and depletion of c-NHEJ proteins ligase IV and XRCC4 leads to a significant increase in m-NHEJ. Similarly, decreased competition between the two pathways of NHEJ suggests that a decrease in m-NHEJ is indicative of an increase in c-NHEJ.

In order to investigate the effect of the FA pathway on DSB repair pathway choice, we depleted various FA proteins using siRNA. Supplementary Figure S2 shows efficient knockdown by western blot analysis and by lack of FANCD2 foci formation after MMC exposure. Following depletion of FANCA, FANCG, or FANCL from the core complex, or depletion of FANCD2, we induced a DSB by transfection of I-SceI and assessed levels of HR and m-

NHEJ repair in the U2OS EJ-DR cells. Depletion of any of the FA proteins studied resulted in a significant reduction in both HR and m-NHEJ by approximately 50% (representative FACS plots in Figure 1A and quantification in Figure 1B), irrespective of whether the FA protein depleted belongs to the core complex or FANCD2. As siRNA depletion of all five FA proteins studied led to similar results, off-target effects from each siRNA are unlikely to be contributing to the loss of HR or m-NHEJ activity. For FANCA, FANCD2 and FANCG, independent siRNAs were utilized with similar results, again supporting on target effects of the siRNAs used.

As the depletion of FA proteins results in decreased levels of HR and m-NHEJ, we surmised that c-NHEJ should be increased over wild type. To test this directly, we used the V(D)J recombination assay first developed by Gellert and colleagues (27) as a direct measure of canonical NHEJ, as shown schematically in Figure 2A. HEK293T cells were depleted of FANCD2 or FANCG and transiently transfected with RAG1 and RAG2 expression plasmids (Figure 2B), in addition to the pSVD3-320 plasmid containing the target cleavage sites for RAG1/2 proteins. In this system, the RAG proteins are expressed constitutively throughout the cell cycle. The pSVD3-320 plasmid also contains an ampicillin resistance cassette and replication is driven by SV40 derived sequences. Chloramphenicol resistance is acquired following successful V(D)J recombination, therefore, c-NHEJ can be measured by the ratio of chloramphenicol and ampicillin resistant bacterial colonies to ampicillin resistant colonies formed after harvesting low molecular weight DNA from HEK293T cells and subsequent transformation into bacteria. Using this well-established technique, we showed that c-NHEJ is indeed increased in both FANCD2- and FANCG-depleted cells over the control non-target (NT), shown in Figure 2C. Taken together, these results show that depletion of FA proteins decreases HR and m-NHEJ by ~50%, and there is a proportional and significant increase in the use of c-NHEJ for repairing DSBs.

Inactivation of the FA pathway renders cells specifically sensitive to replication-associated DSB-inducing agents and inhibition of DNA-PK

Inhibition of DNA-PKcs has little effect on FA-depleted cells after irradiation—

Since the reduction in FA proteins decreased the use of HR and m-NHEJ, we reasoned that these cells would become hyper-dependent upon c-NHEJ for cell survival. Inhibition of the essential c-NHEJ protein DNA-PKcs, should therefore sensitize them to DSB-inducing agents. Cells were pre-treated for 1 h with DNA-PKcs inhibitor NU7441 before being irradiated at various doses, followed by plating at appropriate cell densities to assess cell survival over 10–14 days. As shown in Supplementary Figure 3, inhibition of DNA-PKcs resulted in increased sensitivity to IR in all cells. Interestingly, no difference was seen between control and FA-depleted cells suggesting that FA proteins have little effect on the repair of DSB induced by IR.

DNA-PKcs inhibition renders FA-depleted cells increasingly sensitive to replication-associated DSBs—IR produces DNA damage throughout the cell cycle, so by challenging the cells with etoposide (ETO), a topoisomerase II inhibitor (30), which at low concentrations selectively induces DSBs in a replication-associated manner (31), we could determine whether the role of FA proteins on DSB repair was more significant in

replication and post-replication phases of the cell cycle. A slight, but not significant, increase in sensitivity to ETO was found after depletion of either FANCD2 or FANCG (Figure 3A). However, after inhibition of DNA-PKcs this effect is significantly enhanced such that FA-depleted cells show >5-fold increased sensitivity to ETO (Figure 3B and C). Camptothecin (CPT), a topoisomerase I inhibitor and therefore specifically causing DSB during S phase (32), was found to have a similar response (Figure 3D–F), further demonstrating the involvement of FA proteins in the repair of a specific subset of replication associated DSB.

We sought to examine further the idea that FA proteins play a role in the choice of DSB repair pathway in proliferating cells. We inhibited poly-ADP-ribose polymerase (PARP) in order to cause replication-associated DSB by an alternative mechanism. PARP inhibitors indirectly cause DSB by preventing repair of single strand breaks (SSB) resulting in conversion to DSB during replication (33). PARP inhibition has other significant impacts on the cell beside failure to repair SSB's. This inhibition can affect early recruitment of the nuclease MRE11 which contributes to DNA-end processing at DSBs. PARP has also been implicated in the control and recruitment of vital HR proteins such as BRCA1 and RAD51 (34). IR induces a plethora of DNA lesions, with a considerable yield of SSBs (35) that are less able to be repaired after PARP inhibition. Veliparib is a PARP inhibitor currently in clinical trials and was chosen over olaparib, another common PARP inhibitor, since olaparib was shown to arrest U2OS cells in S phase, whereas veliparib had no effect on cell cycle (36). Cells were treated for 24 h with veliparib before irradiation with varying doses X-rays and cell survival was assessed by clonogenic assay. As shown in Supplementary Figure 3 and Figure 4A, depletion of FANCD2/FANCG does not increase cell sensitivity to IR with respect to control NT cells. Inhibition of PARP causes a slight decrease in cell survival in response to IR treatment in control cells, but depletion of FA proteins significantly exacerbates this sensitivity (Figure 4B and C). Therefore, FA proteins function to promote HR repair and inhibit NHEJ for a specific subset of DNA DSB generated by DNA replication.

Inhibition of DNA-PKcs following MMC exposure, rescues sensitivity in FA-depleted cells—When a similar experiment was undertaken using the DNA interstrand crosslinking agent Mitomycin C, Fanconi Anemia cells are highly sensitive to these drugs and defines one of the characteristic features of Fanconi Anemia cells. However, the addition of the DNA-PK inhibitor in this experiment resulted in an improved survival in FA-deficient cells, whereas in wild-type cells, the inhibitor resulted in marginal sensitization (Figure 5). To confirm that the effect of the DNA-PK inhibitor was specific to cNHEJ, we used an alternative system to further validate this observation. HAP1 cells containing a knockout of ligase IV, then wild-type and NHEJ-deficient cells were depleted for FancD2 by siRNA. Survival after MMC exposure confirmed that the combination of FancD2 and LigIV inactivation resulted in improved survival compared to FancD2 inactivation alone (Figure S3C). The implication is that sensitivity to MMC in FA null background is mediated through inappropriate engagement of NHEJ factors, which when inhibited results in improved survival.

FA proteins promote the recruitment of HR repair proteins to, and inhibit c-NHEJ proteins from, the sites of replication-linked DSB

To further elucidate how the Fanconi anemia pathway inhibits NHEJ and promotes HR repair of replication-associated DSBs, we exposed cells to DSB-inducing agents and stained for HR or NHEJ proteins. DSBs were induced by treatment with ETO, aphidicolin (APH), MMC and IR. APH induces DSB through inhibiting the replicative DNA polymerase causing a replication fork stall and ultimately replication fork collapse. We first investigated the recruitment of BRCA1 to DSB caused by exposure to the DNA damaging agents. There was no difference in BRCA1 foci formation after irradiation in wild-type versus FA-depleted cells, supporting the previous evidence that radiation-induced breaks are processed independently of any effect of Fanconi anemia proteins. In contrast, BRCA1 recruitment to DNA damage sites was decreased in FANCD2 or FANCG-depleted cells in response to APH, ETO, and MMC. After APH treatment, BRCA1 recruitment is reduced by 1.9- and 1.7-fold with FANCD2 or FANCG depletion respectively (representative images depicted in Figure 6A with quantification in Figure 6B). Similarly, we see a significant decrease in Mre11 foci after FA depletion. The response to APH is the most striking, with a 3.0- and 2.3-fold reduction in Mre11 recruitment after FANCD2 or FANCG knockdown, respectively. In contrast, levels of phosphorylated DNA-PKcs (pDNA-PKcs) in response to DSB-inducing agents are increased after FA depletion, which is in keeping with the data showing an increase in c-NHEJ after depletion of FA. There is a 1.4-fold increase in pDNA-PKcs recruitment to DSBs induced by APH, ETO, or MMC after FANCD2/FANCG depletion, but as with BRCA1 and Mre11, pDNA-PKcs foci levels in response to irradiation are unaffected by loss of FA proteins. Recruitment of 53BP1 to DSB sites followed a comparable pattern to that of pDNA-PKcs, again with the largest effect seen after APH exposure where there is a 1.5- and 1.4-fold increase in foci after FANCD2 or FANCG siRNA knockdown, respectively. These data support the notion that FA proteins are required for promotion of HR repair of replication-associated DSBs, and loss of FA leads to the recruitment of NHEJ proteins to sites of damage.

DISCUSSION

The function of the Fanconi anemia pathway is tightly linked to repair at the replication fork. DNA damage that results in blockage or collapse of the replication fork can create one or more broken DNA duplexes. In proliferating cells the priority is to preserve the genome, which is most effectively achieved by promoting homologous recombination. There have been numerous reports on the factors that promote the recruitment of HR or NHEJ proteins under the general concept of pathway choice (37,38). The precise mechanism of how the FA proteins inhibit NHEJ and promote HR is not yet fully understood, but we have shown that the FA pathway actively inhibits NHEJ in proliferating cells. The results from the episomally replicating VDJ recombination substrate strongly support the model that the FA pathway inhibits cNHEJ. Once resection is initiated at a DSB by CtIP and the MRN complex, a length of ssDNA is revealed and the DSB is no longer a substrate for NHEJ and therefore must be repaired via HR (39). However, 53BP1 may inhibit this initiation of resection (40) showing that there are mechanisms in cells to direct the repair pathway utilized for particular DNA lesions. In addition, the presence of Ku80 can suppress HR;

repair of an I-SceI-induced DSB by HR is increased by 6-fold after depletion of Ku80, suggesting that control of NHEJ can regulate the utilization of HR.

A number of previous studies have recorded a modest decrease in HR repair using FA null cells (41,42), but here we report the measurement of both HR and NHEJ in the same cell at the same time. Irrespective of whether the depleted FA protein was part of the core complex, or downstream such as FANCD2, we saw a decrease in HR and m-NHEJ. Previously, we found that contact-inhibited cells displayed this repair pattern, consistent with the hypothesis that c-NHEJ is the main pathway employed in non-proliferating cells. However, even in proliferating cells, we found a significant increase in Ku-dependent NHEJ after depletion of either FANCD2 or FANCG, suggesting that FA proteins specifically inhibit c-NHEJ in proliferating cells. Although endogenous V(D)J recombination occurs during G1, the episomal vectors are replicating de-synchronized from the host cell and undergo V(D)J recombination independently of cell cycle. The effect of FA proteins on NHEJ must be working at the level of pan-nuclear function of one or more NHEJ proteins, since there is no reduction in the levels of any of the NHEJ proteins. One report has suggested that FA proteins inhibit Ku binding to DNA ends in vitro (43), but we are now investigating evidence for this in-vivo using iPOND (44) or eCHIP (45).

We focused on replication-associated breaks, which only occur in proliferating cells. The significant decrease in cell survival seen after treatment with etoposide and DNA-PKcs inhibitors supports the idea that FA proteins suppress NHEJ in relation to DNA replication. To further support this observation, we used the PARP inhibitor veliparib to induce DSB caused by replication fork collapse at persistent SSB. Ashworth and colleagues have shown that FANC-depleted cells show a mild sensitivity to PARP inhibitors, presumably due to unrepaired endogenous SSBs that are converted to DSBs, as no external stress was applied (46). We did not see any significant sensitivity to veliparib as a single agent, but after exposure of the FA-depleted cells to IR and veliparib, a significant reduction in survival is observed. This result is of particular interest as it suggests that tumors with a functional FA deficiency are vulnerable to lesions that require HR for their repair, and the de-repressed c-NHEJ pathway cannot help the cells survive.

It has been previously noted that Rad51 foci are decreased in response to MMC after FANCD2 depletion, but not in response to IR (47). Following MMC exposure and activation of the FA repair pathway, FANCD2 plays a role in recruitment of downstream proteins to the site of the ICL. We have built upon these observations by challenging cells with various DSB-inducing agents that would not typically be associated with Fanconi anemia sensitivity. Indeed, after treatment with both etoposide and aphidicolin, there is a decrease in BRCA1 recruitment to distinct foci when FA proteins are depleted. The localization of Mre11 to DSBs follows the same pattern as BRCA1, with a decreased response after FANCD2/FANCG knockdown. Both these proteins are early responders in the HR repair pathway and are upstream of Rad51, suggesting that promotion of HR by FA proteins occurs promptly after DSB formation. Additionally, loss of FA proteins leads to increased 53BP1 foci and phosphorylation of DNA-PKcs at the autophosphorylation Ser2056 site. We propose that FA proteins promote the use of HR for replication-associated DSB and prevent the engagement of c-NHEJ. It has previously been suggested that c-NHEJ downregulates m-NHEJ (26,48–

50), but how FA proteins prevent c-NHEJ recruitment is still under investigation (25). FANCD2 and BRCA1 have been shown to colocalize endogenously during S phase (51) in addition to after DNA damage in replicating cells. In contrast, if FA proteins are depleted then c-NHEJ becomes dominant in the repair of the DSB. If the initiating DNA lesion is a DNA crosslink, then the intermediates that are created in the absence of the FA pathway, can be acted on by the c-NHEJ proteins, but surprisingly they appear to produce a lethal lesion, since DNA-PKi actually rescues survival after MMC treatment. The implications of this work is that bone marrow hematopoietic stem cells in FA patients are destroyed by endogenously generated DNA crosslinks, then DNA-PKi may actually be beneficial in preventing the development of anemia.

There have been a number of reports of FA deficiencies in sporadic cancers (52), particularly loss of FANCF. Hypermethylation of FANCF has been shown to be involved, to varying degrees, in progression of sporadic ovarian, cervical and bladder cancers, in addition to granulosa cell tumors (53–57). Head and neck squamous cell carcinomas and non-small cell lung cancers also have a low incidence of FANCF promoter methylation (58). FANCF promoter methylation detected in adenocarcinomas of the lung is correlated with poor survival (58). Mutations have been noted in FANCF and also FANCC in pancreatic tumor samples (59,60). In addition, Pejovic and colleagues (61) identified a tissue-specific loss of FANCD2 in ovarian tumor samples. The overall prevalence of tumors with functional inactivation of the FA pathway still needs to be determined: the determination is difficult using cancer genomic data, since there appears to be overlap with homologous recombination deficient cancers. With respect to the effect of DNA-PK inhibitors, their impact is only observed in FA-deficient cells. These insights into the processing of DNA lesions in Fanconi Anemia have implications for cancer treatment suggesting that c-NHEJ inhibitors should work effectively with agents that produce replication stress in these cancers with FA defects. Conversely, use of DNA-PK inhibitors would be counterproductive in FA deficient tumors treated with DNA crosslinking agents. The new insights from this work should result in rational combinations of DNA damaging agents and DNA repair inhibitors in this specific subgroup of human tumors with FA-deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- DNA crosslinks are lethal in Fanconi anemia due to the action of NHEJ
- NHEJ is inhibited by actions of the Fanconi anemia pathway
- Replication fork collapse is sensitized by inhibition of NHEJ in Fanconi anemia
- DNA-PK inhibitors may rescue bone marrow attrition in Fanconi Anemia
- DNA-PK inhibitors will sensitize Fanconi anemia-deficient cells after replication stress

Figure 1.

Depletion of FA proteins decreases both HR and m-NHEJ repair of an I-SceI induced DSB. (A) representative flow cytometry profiles of GFP (Q3; HR) vs RFP (Q1; m-NHEJ) expression in U2OS cells transfected with siRNA targeting various FA proteins (FANCA, C, D2, G, or L), or NT control, following repair of an I-SceI induced DSB. (B) quantification of effect of FA protein depletion on HR, normalized to NT. (C), quantification of effect of FA protein depletion on m-NHEJ, normalized to NT. Error bars represent S.E.M. of at least 5 independent experiments. ** P<0.01 determined by student's t-test.

Figure 2.

Depletion of FA proteins leads to increased Ku-dependent NHEJ. **(A)** Schematic illustrating the experimental procedure. **(B)** Western blot confirming protein knock-down 48 h after siRNA treatment, and expression of complemented RAG1/RAG2. **(C)** RAG-induced NHEJ as measured by development of chloramphenicol resistance, normalized to NT. Error bars represent S.E.M. of at least 3 independent experiments. * $P < 0.05$ as determined by student's t-test.

Figure 3.

FA-depleted U2OS cells are uniquely sensitive to replication-associated DSB causing agents after DNA-PKcs inhibition. (**A and D**) FA depletion (FANCD2 or FANCG) does not render cells significantly sensitive to etoposide (ETO; **A**), or camptothecin (CPT; **D**). (**B and E**) FA depletion (FANCD2 or FANCG) makes cells uniquely sensitive to ETO (**B**) or CPT (**E**) after DNA-PKcs inhibition (PKi; NU7441, 1 μ M). (**C and F**) Sensitization of FA-depleted cells to ETO (**C**) or CPT (**F**) following DNA-PKcs inhibition, relative to NT. Error bars represent S.E.M of at least 3 independent experiments. n.s. not significant, * $P < 0.005$ determined by student's t-test.

Figure 4.

FA-depleted U2OS cells are uniquely sensitive to IR after PARP inhibition. (A) FA depletion (FANCD2 or FANCG) has no significant effect on cell survival following irradiation. (B) FA depletion (FANCD2 or FANCG) confers sensitivity to irradiation after PARP inhibition (veliparib, 40uM). (C) sensitization of FA-depleted cells to irradiation following PARP inhibition, relative to NT. Error bars represent S.E.M of at least 3 independent experiments. n.s. not significant, * $P < 0.05$ determined by student's t-test.

Figure 5.

FA depleted/deficient cell sensitivity to MMC is rescued by inhibition of DNA-PKcs. **(A)** FA-depleted (FANCD2 or FANCG) U2OS cells are sensitive to MMC exposure. Left; non-target siRNA-treated cells do not show additional sensitivity or rescue from MMC exposure after DNA-PKcs inhibition (PKi; NU7441 1 μ M), with FANCD2/G shown for comparison. Middle and right; FANCD2- and FANCG-depleted cells, respectively, demonstrate a partial rescue of sensitivity to MMC following DNA-PKcs inhibition (PKi; NU7441, 1 μ M). **(B)** Rescue of FA-depleted cells by DNA-PKcs inhibition after MMC exposure, relative to DMSO control. **(C)** Cell viability assay demonstrating FANCG-deficient cells rescue of sensitivity of MMC exposure by DNA-PKcs inhibition, in comparison to the wild-type complemented cell line.

Figure 6.

FA proteins regulate recruitment of DSB repair proteins to sites of replication-associated DNA damage in U2OS cells. (A) Representative immunofluorescence images of BRCA1 (green), Mre11 (green), phosphorylated DNA-PKcs (pDNA-PKcs; red), and 53BP1 (red) foci in response to aphidicolin treatment (25 μ g/ml, 4 hours) after depletion of FANCD2 or FANCG. (B–E) quantification of immunofluorescence images demonstrating protein response of BRCA1, MRE11, pDNA-PKcs, and 53BP1 to indicated treatments. Error bars represent S.E.M. of at least 3 independent experiments, at least 250 cells counted per experiment. n.s. not significant, * $P < 0.05$, ** $P < 0.01$, determined by student's t-test.