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Biology of Human Tumors

Clinical Cancer Research

Acquisition of Relative Interstrand Crosslinker Resistance and PARP Inhibitor Sensitivity in Fanconi Anemia Head and Neck Cancers

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Abstract

Purpose: Fanconi anemia is an inherited disorder associated with a constitutional defect in the Fanconi anemia DNA repair machinery that is essential for resolution of DNA interstrand crosslinks. Individuals with Fanconi anemia are predisposed to formation of head and neck squamous cell carcinomas (HNSCC) at a young age. Prognosis is poor, partly due to patient intolerance of chemotherapy and radiation requiring dose reduction, which may lead to early recurrence of disease.

Experimental Design: Using HNSCC cell lines derived from the tumors of patients with Fanconi anemia, and murine HNSCC cell lines derived from the tumors of wild-type and Fancc$^{-/-}$ mice, we sought to define Fanconi anemia–dependent chemosensitivity and DNA repair characteristics. We utilized DNA repair reporter assays to explore the preference of Fanconi anemia HNSCC cells for non-homologous end joining (NHEJ).

Results: Surprisingly, interstrand crosslinker (ICL) sensitivity was not necessarily Fanconi anemia–dependent in human or murine cell systems. Our results suggest that the increased Ku-dependent NHEJ that is expected in Fanconi anemia cells did not mediate relative ICL resistance. ICL exposure resulted in increased DNA damage sensing and repair by PARP in Fanconi anemia–deficient cells. Moreover, human and murine Fanconi anemia HNSCC cells were sensitive to PARP inhibition, and sensitivity of human cells was attenuated by Fanconi anemia gene complementation.

Conclusions: The observed reliance upon PARP-mediated mechanisms reveals a means by which Fanconi anemia HNSCCs can acquire relative resistance to the ICL-based chemotherapy that is a foundation of HNSCC treatment, as well as a potential target for overcoming chemoresistance in the chemosensitive individual. *Clin Cancer Res; 21(8); 1962–72. ©2015 AACR.*

Introduction

Fanconi anemia is a genetic disorder characterized by congenital abnormalities, progressive bone marrow failure, and cancer predisposition (1, 2). Fanconi anemia results from germ-line mutations in one of 16 genes that participate in a common DNA repair pathway, thus deregulating DNA damage responses and leading to the disorder's clinical phenotypes (3–5). It has been demonstrated that Fanconi anemia–deficient cells exhibit reduced capacity for homologous recombination (HR), whereas non-homologous end joining (NHEJ) is elevated and even functionally contributes to Fanconi anemia phenotypes under certain circumstances (6, 7). Although acute myelogenous leukemia (AML) is the most frequently occurring malignancy in Fanconi anemia, individuals with the disease also possess a strong predisposition to the development of solid tumors, particularly squamous cell carcinomas of the head and neck (HNSCC), as well as of the anogenital region (8–11). Fanconi anemia HNSCCs occur primarily in the oral cavity and in the absence of traditional risk factors for HNSCC such as tobacco and alcohol use (11, 12).

Data from the International Fanconi Anemia Registry indicate that the cumulative incidence of nonhematologic malignancies in patients with Fanconi anemia may be as high as 28% by 40 years of age (11). This dramatic risk of HNSCC is, for unclear reasons, increased further by the allogeneic hematopoietic stem cell transplantation that is the treatment of choice to correct the disorder's progressive bone marrow failure (13, 14). The hypothetical cumulative incidence of HNSCC, defined as the cumulative incidence of HNSCC development if the competing risks of death due to other causes are removed, has been estimated to approach 100% in transplanted patients versus 50% in nontransplanted patients that reach their maximal life expectancy (14). Thus, as improved transplantation and supportive care measures prolong

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Translational Relevance

Because of the sensitivity of patients with Fanconi anemia to DNA damage caused by interstrand crosslinks, current therapy for head and neck squamous cell carcinomas (HNSCC) developing in patients with Fanconi anemia requires either dose reduction or omission of the radiotherapy and chemotherapy that are mainstays of treatment for sporadically occurring HNSCCs. However, frequent early locoregional recurrence suggests a discontinuity between constitutional DNA damage sensitivity and tumor cell chemotherapy sensitivity. The surprising degree of interstrand crosslinker (ICL) resistance of Fanconi anemia HNSCC cells questions the efficacy of low-dose conventional therapies. By identifying sensitivity to PARP inhibitors, this study demonstrates that systematic testing of alternative agents will be necessary using our established murine and human Fanconi anemia HNSCC models, and that results obtained from these studies may be directly translatable into phase I/II clinical trials for treatment of Fanconi anemia HNSCC using PARP inhibition via either systemic or directed means.

survival, the risk of HNSCC will become an increasingly prominent issue for patients with Fanconi anemia.

Once HNSCCs are clinically manifest, patients with Fanconi anemia fare exceedingly poorly with 2-year overall and relapse-free survival rates of less than 50% (15). Patients tolerate surgery well, but experience significant morbidity and also mortality with the radiation and/or interstrand crosslinker (ICL)–based chemotherapy that, depending upon tumor stage at presentation, may be necessary components of treatment (12, 15, 16). Although the poor prognosis of patients with Fanconi anemia HNSCC has been attributed to intolerance of conventional clastogenic therapy due to their constitutional sensitivity to DNA damaging agents, a high rate of early locoregional recurrence (15) may suggest that the tumors are not adequately controlled by the degree of genotoxic therapy that they can tolerate.

The desire to avoid severe toxicity and the hope that Fanconi anemia HNSCCs will share in the individual's DNA damage sensitivity make the use of low-dose clastogenic treatments a possible option for therapy. However, the increased genomic instability caused by an underlying defect in error-free DNA repair by HR may facilitate Fanconi anemia tumor evolution by inducing genomic adaptations that could mitigate any inherent sensitivity to DNA damage, particularly in light of the ability of Fanconi anemia–deficient oral keratinocytes to proliferate more rapidly compared with controls, despite exhibiting increased DNA damage both *in vitro* and *in vivo* (17, 18). Thus far, the extent to which Fanconi anemia HNSCC phenotypes remain dependent on a dysfunctional Fanconi anemia pathway remains unclear, and direct and systematic examination of Fanconi anemia–dependent biologic and molecular properties of Fanconi anemia HNSCCs has been limited (19, 20), predominantly due to the paucity of available isogenic human and murine HNSCC model systems.

The PARP family of proteins contains 18 distinct proteins that catalyze the covalent attachment of ADP-ribose units from donor NAD^+ molecules onto target proteins, resulting in the attachment of monomers or linear or branched poly(ADP-ribose) (PAR) polymers that modify the receiving protein's function (21, 22). Two of these, PARP1 and PARP2, bind to sites of DNA damage and recruit and activate effector proteins that participate in numerous DNA damage repair mechanisms. PARP1 has also been shown to PARylate itself as a means of enhancing its own activity (21, 22). Although PARP proteins have been implicated in chemoresistance of several solid tumor types, including non–small cell lung cancer and sporadic head and neck cancers (23, 24), and their inhibition has been associated with synthetic lethality in tumor cells defective in BRCA1 or BRCA2 (25), they have not yet been studied in Fanconi anemia HNSCC.

To characterize the pathway-dependent cellular and molecular phenotypes of Fanconi anemia HNSCC cells, we generated isogenic cellular models of Fanconi anemia–deficient and proficient HNSCC cells, and characterize here their comparative biologic and molecular properties and DNA repair capabilities. Human patient-derived *FANCA*$^{-/-}$ and *FANCC*$^{-/-}$ HNSCC cells were transduced with either control or Fanconi anemia–complementing retroviral vectors before analysis. Surprisingly, ICL sensitivity of Fanconi anemia–deficient tumor cells was not increased compared with their Fanconi anemia–complemented cellular counterparts or to sporadic HNSCC cells. In addition, a murine HNSCC model was generated by exposing wild-type (WT) and *Fancc*$^{-/-}$ mice to the carcinogen 4-nitroquinolone 1-oxide (4-NQO). Although non-neoplastic *Fancc*$^{-/-}$ epithelial cells were hypersensitive to crosslinking agents, some *Fancc*$^{-/-}$ tumor cells lost their characteristic sensitivity, similar to the human model. To investigate potential compensatory mechanisms in DNA repair pathways of Fanconi anemia HNSCCs, we tested the degree to which PARP proteins are engaged in the repair process in both Fanconi anemia–proficient and Fanconi anemia–deficient cells. The results show that PARP activity is specifically upregulated in Fanconi anemia–deficient HNSCCs, and this increased activity is associated with a selective sensitivity to PARP inhibitors in both human and murine Fanconi anemia HNSCC cells. Taken together, the data question the expectation that Fanconi anemia HNSCCs share the individual's global DNA damage hypersensitivity, thus perhaps contributing to the high rate of early locoregional recurrence in patients treated with reduced-intensity genotoxic therapies. Importantly, we also demonstrate that this increased resistance to ICLs is caused, at least in part, through PARP activation. PARP inhibitors may thus provide new avenues for treatment of HNSCC in Fanconi anemia.

Materials and Methods

Human cell cultures and vectors

Three Fanconi anemia patient–derived HNSCC cell lines used in this study were kind gifts from other institutions. VU-1131 (*FANCC*$^{-/-}$) and VU-1365 (*FANCA*$^{-/-}$) lines were obtained from Drs. Johan de Winter and Ruud Brakenhoff at VU University, Amsterdam, the Netherlands, and OHSU-974 (*FANCA*$^{-/-}$) cells were obtained from Dr. Grover Bagby at the Oregon Health and Science University (OHSU). These have been described previously as human papillomavirus (HPV)–negative head and neck cancer cells, and the respective patients were not treated with cisplatin or other ICL-causing agents before creation of the cell lines (19). Human sporadic HNSCC cell lines CAL-27, FADU, and SCC-4 were obtained from the American Type Culture Collection. Cell culture conditions are detailed in Supplementary Materials and Methods. All cell lines were authenticated regularly by their

morphologic characteristics and analysis of Fanconi anemia status and corresponding genetic and molecular markers.

The cDNAs for human *FANCA* and *FANCC* were cloned into the multicloning site of the oncoretroviral vector S91IN, which coexpresses an IRES-neomycin phosphotransferase cassette, thus conferring resistance to G418 (Invitrogen). S91IN and the two Fanconi anemia vectors, S91FAIN and S91FCIN, were transfected into ecoPhoenix cells and then supernatant generated to stable transduce PG13 cells, as previously described (26, 27). Supernatant from G418-resistant PG13 cells were collected, filtered through 0.45 μm, stored at −80°C, thawed, and then tested functionally for correction of *FANCA*- and *FANCC*-deficient reference cells with known bi-allelic mutations (data not shown). Subsequently, supernatants were utilized to transduce human HNSCC cell lines. Cultures with 0.8 mg/mL medium G418 were used for selection of transduced polyclonal HNSCC cell populations.

Murine HNSCC tumor induction

Fancc$^{-/-}$ mice were described previously (28) and were maintained and treated according to Institutional Animal Care and Use Committee guidelines at the Portland VA Medical Center. To generate murine oral HNSCCs, 2- to 4-month-old mice (22 WT and 18 *Fancc*$^{-/-}$) were treated with 20 μg/mL 4-NQO (Sigma) in water for up to 45 weeks. Mice were monitored weekly for tumor development and euthanized at the first signs of morbidity. Following euthanasia, tumor masses were preserved in formalin for histologic analyses and/or prepared for cell isolation and culture. Tumor grade and type were determined by hematoxylin and eosin (H&E) staining and analysis by a cancer pathologist at OHSU blinded to the genotype of the specimens.

Murine cell culture

Cell isolation and culture of primary tongue epithelial cells and HNSCC cells from WT and *Fancc*$^{-/-}$ mice are described in Supplementary Materials and Methods.

Western blot analysis

Trypsinized cells were washed with PBS and collected by centrifugation. For FANCA, FANCC, FANCD2, and actin immunoblots, whole-cell protein extracts were lysed using the Laemmli method (29). For DNA-PKcs and pDNA-PKcs^{S2056} immunoblots, whole-cell protein extracts were lysed using RIPA buffer (1% Triton X-100, 1% DOC, 0.1% SDS, 0.16 mol/L NaCl, 10 mmol/L Tris, pH 7.4, and 5 mmol/L EDTA) supplemented with a protease inhibitor cocktail (BD Biosciences), 10 mmol/L NaF, and 5 mmol/L NaVO3. Protein concentrations were determined using a Pierce BCA Protein Assay kit (Thermo Scientific). Lysates were resolved by SDS–PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (BioRad). Membranes were probed with the appropriate primary antibody overnight. Primary antibodies used were as follows: FANCA (Cascade), FANCC (a kind gift from the Fanconi Anemia Research Fund through OHSU), FANCD2 (Novus), actin (Seven Hills Bioresearch), DNA-PKcs (Abcam), and pDNA-PKcs^{S2056} (Abcam). Membranes were washed with TNET (10 mmol/L Tris, 2.5 mmol/L EDTA, 50 mmol/L NaCl, and 0.1% Tween 20), and secondary anti-mouse (GE) or anti-rabbit (Jackson Immunoresearch) antibodies conjugated to horseradish peroxidase were added for 30 minutes. Membranes were then exposed to chemiluminescence reagents (Thermo Scientific) for protein detection. For detection of mono-ubiquitinated FANCD2, cells were plated for 24 hours and subsequently left untreated or treated with 2 mmol/L hydroxyurea for 24 hours before collection. For detection of DNA-PKcs and pDNA-PKcs^{S2056}, cells were pretreated with DNA-PKcs inhibitors DNA-PK inhibitors NU-7026 (Tocris) or NU-7771 (Tocris) for 24 hours and subsequently with 2 μg/mL bleomycin for 20 minutes before collection.

Organotypic epithelial raft culture

Three-dimensional organotypic rafts were generated as described previously and as detailed in Supplementary Materials and Methods (18). H&E staining was performed for morphologic examination by a cancer pathologist at Cincinnati Children's Hospital Medical Center blinded to the gene complementation status of the specimens. Photographs were obtained on a Leica DM2500 microscope using Leica Application Suite software. Immunofluorescence for BrdUrd was performed as described below. The percentage of BrdUrd-positive cell population was quantified as the ratio of total BrdUrd-positive nuclei to total nuclei per 200× field. Such ratios were determined for three fields of each raft and averaged.

Immunofluorescence microscopy

Preparation of coverslips and epithelial raft sections for immunofluorescence and performance of immunofluorescence microscopy is described in Supplementary Materials and Methods.

Cell cycle analysis by flow cytometry

Assays were performed as previously described (30). Briefly, Fanconi anemia–deficient and –complemented HNSCC cells were either left untreated or treated with 0.25 μg/mL melphalan (Sigma) for 48 hours. Cells were trypsinized, washed in PBS, and fixed in 100 μL BD Cytofix/Cytoperm (BD Biosciences). Cells were prepared using the protocol for the APC BrdU Flow Kit (BD Biosciences). Cell cycle profiles were detected using 7AAD on a BD FACSCanto instrument (BD Biosciences), and these data were analyzed using FlowJo software (Tree Star).

Cellular proliferation assays

Cellular growth was measured by MTS assays as described (31) and by viable cell counts over time using dye exclusion and counted live cell assays as described in Supplementary Materials and Methods.

DNA repair assays

Flow cytometry–based DNA repair assays were performed as described (32) using constructs designed to measure the proportion of cells engaged in NHEJ. Briefly, equal numbers of Fanconi anemia–deficient and –complemented VU-1131 cells were plated in 6-well plates. Following 24 hours of growth, transfections were performed utilizing FuGENE HD transfection reagent (Promega) and Opti-MEM reduced serum media (Invitrogen). Following 24 hours, GFP expression was measured using a BD FACSCanto instrument (BD Biosciences). These data were analyzed using FlowJo software (Tree Star). At least four independent experiments were performed with each construct.

Statistical analysis

Graphs were created and statistical analyses performed using GraphPad Prism software (GraphPad). Data points and error bars indicate mean and SD, respectively, of the raw data.

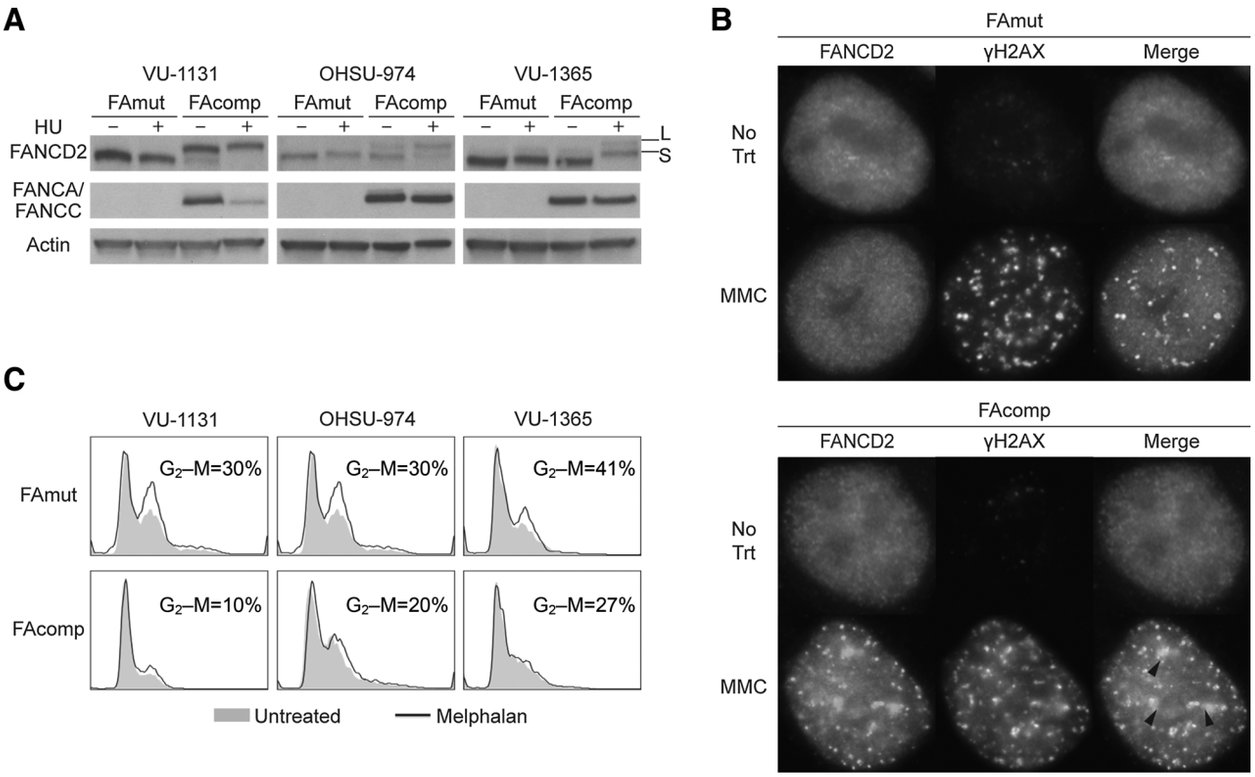

Figure 1.
Gene correction of Fanconi anemia (FA) patient–derived HNSCC cell lines. A, immunoblot of isogenic Fanconi anemia patient–derived HNSCC cell lines. Complementation of the relevant Fanconi anemia gene restores FANCD2 activity upon treatment with hydroxyurea (HU). FAmut, Fanconi anemia–deficient; FAcomp, Fanconi anemia–complemented; S, FANCD2; L, monoubiquitinated FANCD2. B, immunofluorescence for FANCD2 and γH2AX shows localization of FANCD2 to sites of DNA damage in FAcomp cells following mitomycin C (MMC) treatment. Images shown are representative of three independent experiments, each with similar results. C, FAcomp cells are rescued from cell cycle arrest in the G_2–M phase caused by melphalan treatment.

Results

Fanconi anemia complementation of patient-derived HNSCC cells reverses characteristic cellular Fanconi anemia phenotypes

The goal of this study was to determine Fanconi anemia–dependent growth and chemosensitivity properties of patient-derived HNSCC cells, with the expectation that substantial ICL sensitivity was to be observed in Fanconi anemia–deficient cells. One FANCC-deficient cell line (VU-1131) and two FANCA-deficient cell lines (OHSU-974 and VU-1365), all originally cultured from the HNSCCs of patients with Fanconi anemia, were utilized for gene correction. The cells were transduced with either control retroviral vector, *FANCC* retroviral vector for VU1131, or *FANCA* vector for OHSU-974 and VU-1365. FANCA and FANCC expression was confirmed in each case at the protein level by immunoblotting. Complementation restored pathway activation as demonstrated by FANCD2 monoubiquitination following HU treatment; thus, the mutant Fanconi anemia gene was corrected in each case (Fig. 1A). In addition, immunofluorescence experiments demonstrated that monoubiquitinated FANCD2 in complemented, but not control cells, was capable of localizing to sites of double-stranded DNA breaks following mitomycin C (MMC) treatment as shown by colocalization of FANCD2 and γH2AX foci (Fig. 1B). To verify Fanconi anemia pathway functionality, isogenic cell populations were treated with melphalan and subjected to cell cycle analysis. As predicted, Fanconi anemia complementation rescued cells from accumulation in the G_2–M phase of the cell cycle, a hallmark of Fanconi anemia pathway deficiency, following melphalan treatment (Fig. 1C; ref. 30).

Fanconi anemia complementation does not affect HNSCC proliferation in three dimensions

Our previous work utilizing HPV E6/E7-immortalized Fanconi anemia patient–derived and Fanconi anemia knockdown keratinocyte models had shown that Fanconi anemia loss confers a proliferative advantage, specifically in the environment of three-dimensional organotypic epithelial rafts, despite characteristic Fanconi anemia phenotypes and increased DNA damage (18). To examine the growth of Fanconi anemia HNSCC in the context of the epithelial milieu wherein they arise, we generated rafts utilizing the above Fanconi anemia–deficient and –complemented HNSCC cells. H&E staining revealed comparable raft thickness, as well as similar morphologic features of the constituent cells (Fig. 2A). Immunofluorescence detection of BrdUrd incorporation revealed no significant differences, indicating that Fanconi anemia correction in malignant HNSCC cells does not affect proliferation (Fig. 2B). From this, we concluded that although differentiation-associated cell cycle exit of non-malignant, HPV-positive keratinocytes is Fanconi anemia–dependent and

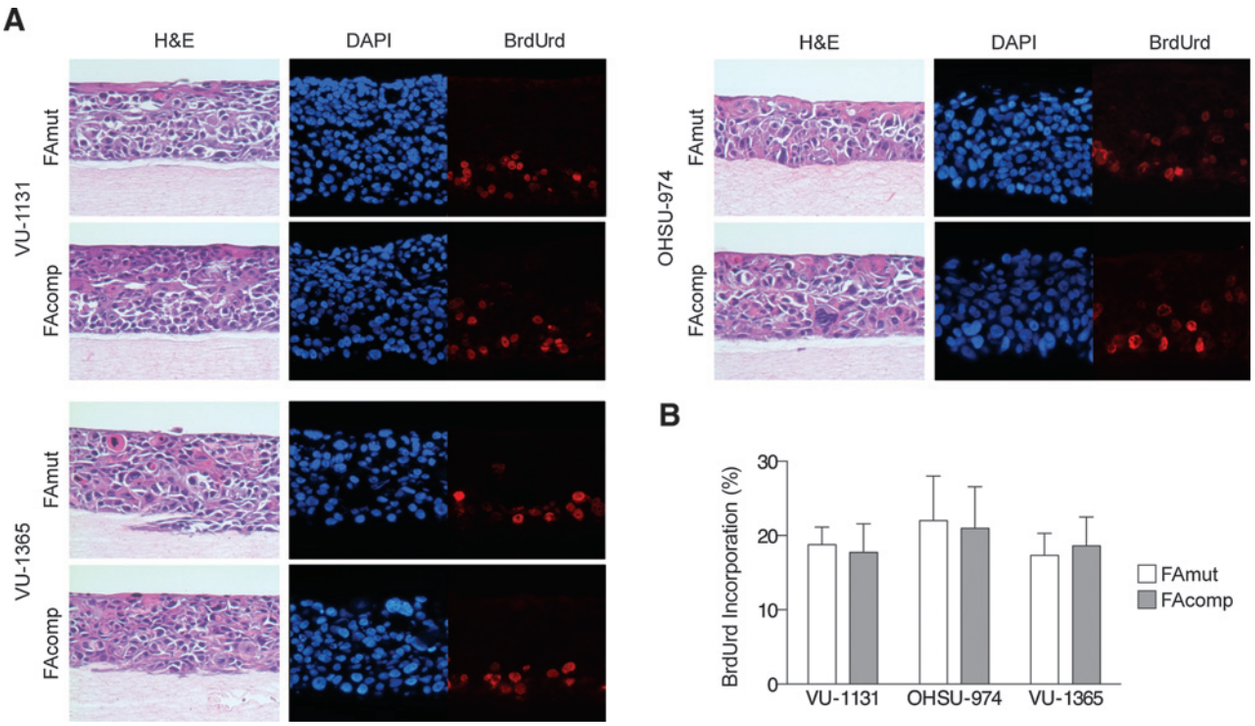

Figure 2.
Three-dimensional organotypic epithelial rafts generated from human Fanconi anemia (FA) HNSCC cells. A, H&E and immunofluorescence staining of rafts created from isogenic HNSCC cell lines. Images are representative of three independent experiments, each with similar results. H&E sections of FAmut and FAcomp rafts are equivalent in mitotic index, cellular differentiation, and stromal content. Immunofluorescence for BrdUrd incorporation indicates similar proliferative rates. B, quantification of BrdUrd incorporation of FAmut and FAcomp cells; a *t* test indicated no significant difference.

reversible upon complementation, the Fanconi anemia pathway is unable to exert any such antiproliferative influence following tumorigenesis.

Fanconi anemia HNSCC cells acquire relative resistance to ICLs

The expectation that Fanconi anemia–deficient HNSCC cells possess the same hypersensitivity to ICLs as nonmalignant cells from patients with Fanconi anemia has not previously been tested in murine or human systems. We therefore sought to develop a murine model of nonmalignant oral keratinocytes and HNSCCs using *Fancc*$^{-/-}$ and WT mice. Oral keratinocytes were harvested from either WT (W-NR) or *Fancc*$^{-/-}$ (M-NR) mice, SV40-transduced for immortalization, and analyzed in survival assays to test for relative sensitivities to MMC and cisplatin. As expected, SV40-immortalized *Fancc*$^{-/-}$ oral keratinocytes exhibited significantly increased sensitivity to MMC and cisplatin when compared with their WT counterparts (Fig. 3A). Specifically, *Fancc*$^{-/-}$ cells displayed an approximately 5-fold average decrease in half maximal effective concentration (EC_{50}) compared with WT cells (Fig. 3B; Supplementary Table S1).

For HNSCC induction, we utilized a well-known carcinogen, 4-NQO, which has been shown to cause the development of murine HNSCCs that closely mimic human tumors histopathologically (33, 34). WT and *Fancc*$^{-/-}$ mice were treated with 4-NQO in water for up to 45 weeks. Mice were monitored weekly for visible tumor development and euthanized at the first signs of morbidity. Survival (time to morbidity that necessitated sacrifice) and tumor incidence were similar for WT and *Fancc*$^{-/-}$ mice (Supplementary Fig. S1A and S1B). Median survival for both cohorts of mice was 40 weeks. Greater than 80% of mice of both genotypes developed tumors that were located mainly on the tongue, with a subset developing on or in the lip, buccal mucosa, and esophagus (Supplementary Fig. S1C). All tumors were well-differentiated HNSCCs, ranging from low- to high-grade (Supplementary Fig. S1D and Supplementary Table S1). We did not detect metastases in either genotype, analogous to previous studies (33, 34), perhaps due to the necessity of early euthanasia after tumor development. Tumors were harvested for generation of WT (W-SCC) or *Fancc*$^{-/-}$ (M-SCC) cell lines. These were subsequently tested in survival assays for relative sensitivities to MMC and cisplatin. Interestingly, *Fancc*$^{-/-}$ mutant compared with WT HNSCC cells did not differ significantly in their sensitivity to MMC or cisplatin (Fig. 3C). In fact, three of six WT lines displayed an MMC EC_{50} of 10 to 20 nmol/L, similar to an EC_{50} of 5 to 20 mmol/L in *Fancc*$^{-/-}$ lines, whereas one other WT line displayed an only slightly higher EC_{50} of 29 nmol/L (Fig. 3D; Supplementary Table S1). The lack of uniform ICL sensitivity in *Fancc*$^{-/-}$ versus WT cell lines does not appear to be due to increased chromosomal instability in WT cells during malignant transformation, as *Fancc*$^{-/-}$ cell lines showed more complex karyotypes and had greater levels of MMC-induced chromosomal breakage (Supplementary Table S1).

To compare the murine with human Fanconi anemia HNSCC cell models, we also subjected uncorrected Fanconi anemia patient–derived cell lines and cell lines derived from sporadically occurring HNSCCs to MMC treatment and performed viable cell counts after 5 days of exposure. These experiments revealed results similar to those obtained with murine HNSCC cells; overlap of survival curves of Fanconi anemia and sporadic cell lines was

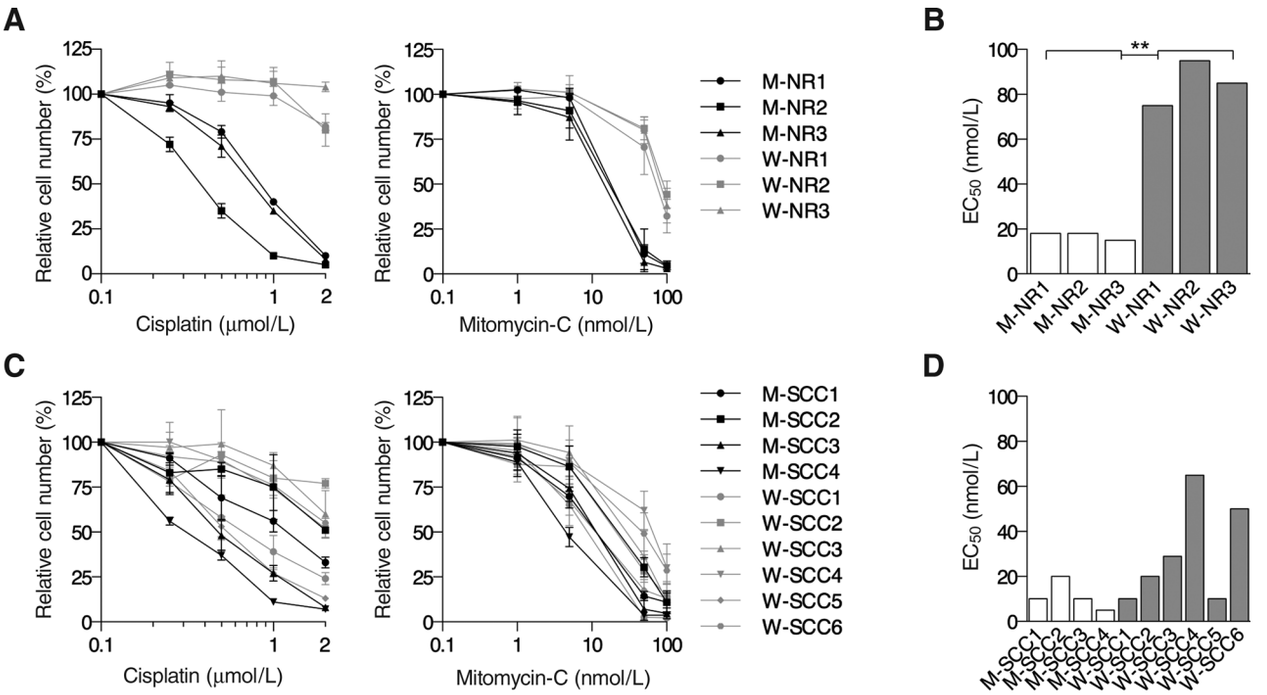

Figure 3.
ICL sensitivity of murine Fanconi anemia HNSCC. A, cisplatin (left) and MMC (right) cellular growth assays of immortalized, nonmalignant oral epithelial cells of Fancc$^{-/-}$ (M-NR) and WT (W-NR) mice show significantly increased sensitivity of Fancc$^{-/-}$ cell lines. B, MMC EC_{50}s of immortalized, nonmalignant murine Fancc$^{-/-}$ and WT oral epithelial cells. **, $P < 0.01$ (t test). C, cisplatin (left) and MMC (right) cellular growth assays of murine Fancc$^{-/-}$ (M-SCC) and WT (W-SCC) mice indicate overlap of sensitivities. D, MMC EC_{50}s of Fancc$^{-/-}$ and WT HNSCC cell lines following 5 days of exposure; a t test revealed no significant difference.

observed, and two of three Fanconi anemia and two of three sporadic lines had an EC_{50} of 9 to 17 nmol/L (Fig. 4A and B). Taken together, we concluded that Fanconi anemia–deficient HNSCC cells can largely overcome Fanconi anemia–dependent sensitivity to chemical crosslinkers.

Fanconi anemia HNSCC cells engage in increased NHEJ at baseline, but do not require Ku-dependent NHEJ for repair of cisplatin-induced DNA damage

Given the reported stimulation of NHEJ that is regulated by Fanconi anemia in other cellular models (6, 7), we next sought to define Fanconi anemia–dependent NHEJ DNA repair properties of Fanconi anemia HNSCC using established reporter constructs (Fig. 5A). Isogenic VU-1131 cell lines were cotransfected with I-*Sce*I endonuclease plus NHEJ-GFP reporter plasmids as described in mammary epithelial cell lines (32). Flow cytometry was then used to detect the percentage of cells with the corresponding repair events. As expected, Fanconi anemia HNSCC cells had significantly increased occurrences of NHEJ in comparison with their complemented counterparts (Fig. 5B).

NHEJ has been identified as encompassing two distinct and competing pathways (35). Classical NHEJ is dependent upon recruitment of the Ku70/80 heterodimer to DNA double-strand breaks (DSB) and subsequent activation by phosphorylation of DNA-PKcs (36); alternative NHEJ is suppressed by the binding of Ku70/80 to DSBs and is initiated by binding of PARP1 to DSB ends (37, 38). The performance of Ku-dependent NHEJ has been implicated in the increased defective DNA repair that occurs in Fanconi anemia–deficient cells (6, 7). To determine whether Fanconi anemia HNSCC cells relied upon increased performance of Ku-dependent NHEJ in response to ICLs, we next investigated the effect of its inhibition using the DNA-PKcs inhibitors NU-7026 and NU-7441 on cisplatin sensitivity of human Fanconi anemia–deficient and –complemented cell populations. We hypothesized that, if Ku-dependent NHEJ were the necessary DNA repair pathway used by Fanconi anemia HNSCC cells following ICL exposure, then inhibition would produce an early decrease in survival in deficient versus complemented cells. To understand baseline behavior, isogenic cell lines were first treated with cisplatin alone for two days, following which growth was quantified by MTS assays. Fanconi anemia–deficient and corrected cells for each donor possessed similar sensitivities (Supplementary Fig. S2A). Viable cell counts following 2 days of MMC treatment of VU-1131 and OHSU-974 cell lines also revealed comparable survival (Supplementary Fig. S2B). Sensitivity to other chemotherapeutic agents that are used clinically for the treatment of head and neck cancer was also evaluated, including paclitaxel, 5-fluorouracil, and rapamycin. No differences in the response to these drugs were observed between Fanconi anemia–deficient versus proficient cells (Supplementary Fig. S2C and S2D). Reduced DNA-PKcs phosphorylation in the presence of NU-7026 or NU-7441 was confirmed via immunoblotting (Fig. 5C; Supplementary Fig. S3A). Next, the cells were exposed to cisplatin, and treated versus untreated cells were subjected to cellular growth assays. Interestingly, DNA-PKcs inhibition did not differentially affect the cisplatin sensitivity of Fanconi anemia–deficient and –complemented human HNSCC cells (Fig. 5D; Supplementary Fig. S3B), suggesting that Ku-dependent NHEJ was not specifically upregulated by Fanconi anemia HNSCC cells following ICL exposure.

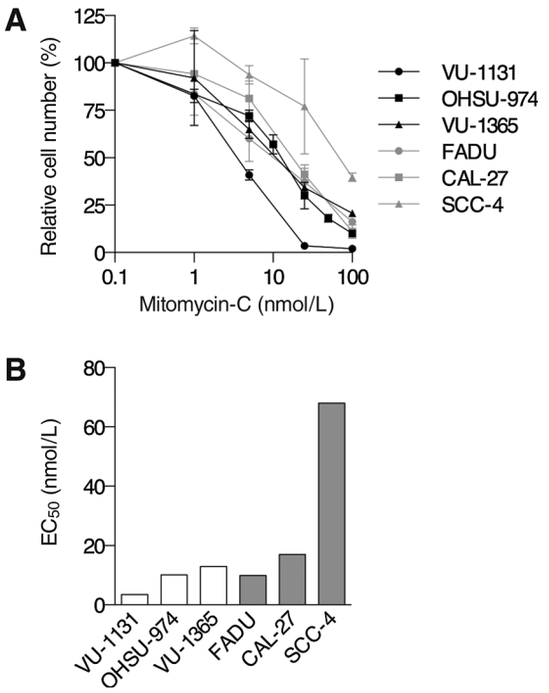

Figure 4.
ICL sensitivity of human Fanconi anemia HNSCC. A, MMC cellular growth assays of Fanconi anemia patient–derived (black) and sporadic (gray) HNSCC cell lines indicate overlap of sensitivities following 5 days of treatment. B, MMC EC_{50}s of Fanconi anemia patient–derived and sporadic HNSCC cell lines following 5 days of exposure; a *t* test revealed no significant difference.

PARP activity is required by Fanconi anemia HNSCC, and tumor cells are sensitive to PARP inhibitors

PARP inhibitors were initially developed as chemotherapeutic agents for BRCA-deficient cancers following the identification of synthetic lethality of PARP inhibition in BRCA1-mutated cells (39). In light of the intrinsic relationship between the Fanconi anemia and BRCA pathways, we sought to determine the effect of PARP inhibition on the growth of Fanconi anemia HNSCC cells. Viable cell counts were taken over time in the presence of the combined PARP1/PARP2 inhibitor olaparib and the PARP1 inhibitor PJ-34. The results indicated profound sensitivity of human Fanconi anemia HNSCC cells to olaparib that was significantly decreased by complementation (Fig. 6A). A similar result was observed in VU-1131 cells treated with PJ-34 (Supplementary Fig. S4A). Intranuclear PAR foci, but not cytoplasmic signal, are an indicator of PARP-mediated DNA damage sensing and repair activity (40). Thus, we next quantified PAR polymer foci following MMC treatment, and detected increased formation of intranuclear PAR foci in Fanconi anemia–deficient cells (Fig. 6B and C). To test olaparib sensitivity in the above malignant murine tumor cell system, we quantified viable cell counts using WT and *Fancc*$^{-/-}$ cell lines. PARP inhibitor sensitivity was present uniformly in the *Fancc*$^{-/-}$ cell lines (Fig. 6D; Supplementary Fig. S4B). Taken together, activation of PARP-mediated DNA damage responses provides a mechanism upon which Fanconi anemia HNSCC cells can rely for response to both endogenous and exogenous DNA damage.

Discussion

The lack of knowledge about the natural behavior and response to therapy of HNSCCs arising in patients with Fanconi anemia is a major hindrance to their successful treatment. Therapy for HNSCC includes surgery and possibly radiotherapy or chemotherapy, depending upon disease stage. In light of the established sensitivity of patients with Fanconi anemia to genotoxic agents, their poor survival has traditionally been attributed to intolerance of therapy. However, long-term follow-up of patients who survive initial therapy and obtain a complete response indicates a very high rate of recurrence of 50% by age 40 (15). Most of these recurrences are at the original site of disease, suggesting incomplete disease control rather than origination of a metachronous tumor. Although the rate of second or multiple primary tumor formation in patients with Fanconi anemia HNSCC had been reported to be over 60% (15), the majority of these are in the anogenital regions, further underscoring that tumors arising in the head and neck area after a first occurrence of HNSCC are likely to be recurrent tumor. Given that current therapy provided for these tumors may be insufficient to provide lasting progression-free survival, and that treatment of Fanconi anemia patients with HNSCC could benefit from an in-depth understanding of tumor biology and response to therapy, we considered whether patients' poor prognosis extends beyond their constitutional susceptibility to DNA damage. To address this lack of understanding in human and murine models, we utilized a panel of HNSCC cell lines derived from the tumors of patients with Fanconi anemia or mice and their Fanconi anemia–proficient counterparts.

A significant body of research has provided insight into the behavior of Fanconi anemia hematopoietic cells. Bone marrow transplantation for Fanconi anemia patients with severe bone marrow failure, AML, or myelodysplastic syndrome can be successfully performed with low rates of toxicity-related morbidity using T-cell–depleted grafts and reduced-intensity preparative regimens (41). Unfortunately, the hope that Fanconi anemia HNSCC could also be treated both effectively and safely with low-dose clastogenic therapies may be incorrect. Published research suggests that the extrahematopoietic compartments of these patients possess a distinct set of characteristics; for instance, in both *in vitro* and *in vivo* models of the epidermal compartment, Fanconi anemia deficiency leads to unique and unexpected gains in keratinocyte proliferation despite increased DNA damage (17, 18).

Thorough understanding of Fanconi anemia HNSCC has been impaired by the need of a comprehensive model. We used an isogenic human Fanconi anemia HNSCC model that allowed for observations of tumor cell characteristics that were strictly Fanconi anemia–dependent. Three-dimensional organotypic tumor rafts utilized here provide a view of Fanconi anemia HNSCC as a carcinoma *in situ*, and allow for quantifiable examination of tumor cell proliferation in a physiologic but controlled environment. However, although available human Fanconi anemia HNSCC cell lines are well characterized (19), they are few in number. The difficulty in faithfully recapitulating the Fanconi anemia epithelial compartment is underscored by the fact that Fanconi anemia mice do not spontaneously form HNSCCs (42). We thus used 4-NQO to induce HNSCCs in WT and *Fancc*$^{-/-}$ mice. The cell lines isolated from these and nonmalignant oral keratinocytes of WT and *Fancc*$^{-/-}$ mice reveal data similar to that obtained in the human Fanconi anemia HNSCC cell system.

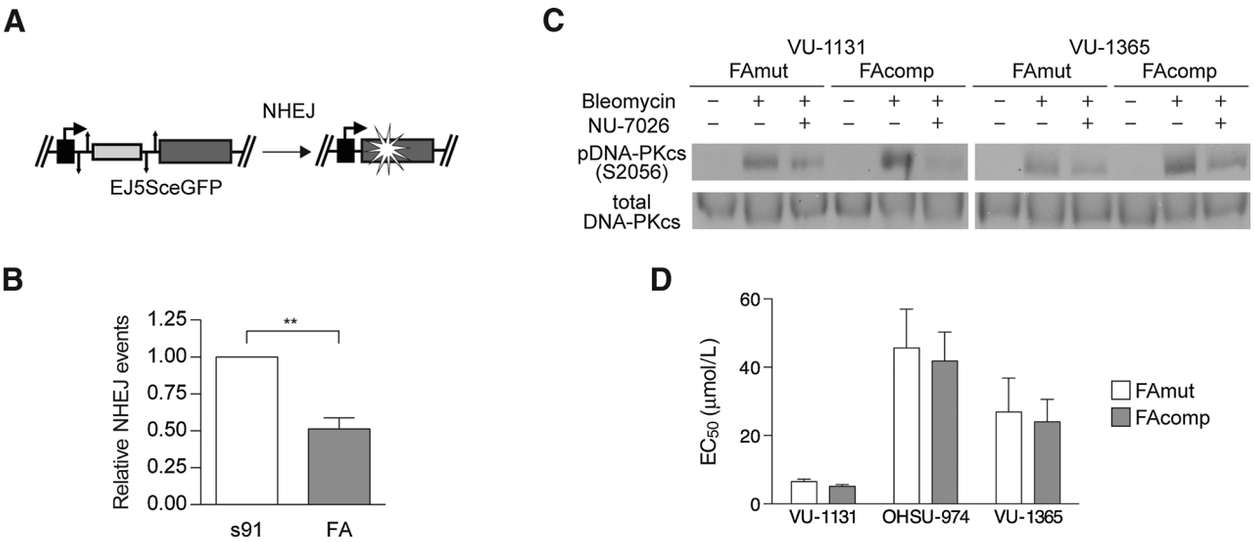

Figure 5.

Preference for NHEJ of mutant and complemented Fanconi anemia HNSCC cells. A, schematic of DNA repair reporter assay constructs for NHEJ. B, reporter assays performed on isogenic VU-1131 cells indicate that Fanconi anemia gene correction decreases baseline preference for NHEJ. **, $P < 0.01$ (*t* test). C, immunoblot of total DNA-PKcs and pDNA-PKcsS2056 in FAmut and FAcomp cell lines treated with 2 μg/mL bleomycin for 20 minutes in the presence and absence of 24 hours of pretreatment with the DNA-PKcs inhibitor NU-7026 (2 μmol/L). Pretreatment with NU-7026 decreases phosphorylation of DNA-PKcs caused by bleomycin treatment. D, chemical inhibition of DNA-PKcs does not decrease the cisplatin EC_{50} of FAmut HNSCC cells relative to FAcomp cells following 2 days of exposure.

We find that the growth characteristics between Fanconi anemia–deficient and Fanconi anemia–complemented HNSCC cells are similar. In contrast, Fanconi anemia complementation of patient-derived nonmalignant keratinocytes decreases hyperplasia (17, 18). In light of the chromosomal instability induced by Fanconi anemia deficiency, loss of the suppressive effect of the Fanconi anemia pathway on proliferation of the premalignant epithelium could conceivably contribute to the increased risk of HNSCC in patients with Fanconi anemia. However, the loss of growth suppression seen in Fanconi anemia–complemented HNSCC cells suggests that, following malignant transformation, cellular machineries become less dependent upon Fanconi anemia deficiency.

Previous work utilized colony assays to explore the chemosensitivity of Fanconi anemia compared with sporadic HNSCC cells, and found a lack of MMC sensitivity in the FANCA-deficient OHSU-974 cell line (20). Importantly, the present study confirms this result. In contrast, ICL sensitivity has been observed in Fanconi anemia fibroblasts (5, 20, 43, 44). We postulated that, in the background of Fanconi anemia deficiency, tumorigenesis and the resulting genomically unstable environment, as illustrated by the complex karyotypes of *Fancc*$^{-/-}$ HNSCCs (Supplementary Table S1), could lead to adaptations in cellular processes that may confer relative chemoresistance. Such adaptation is in line with comparisons between murine keratinocytes versus HNSCC-derived cell lines; early passage–immortalized oral keratinocytes are consistently hypersensitive to ICLs, whereas HNSCC cell populations are not (Fig. 3A–D). Alterations in DNA repair mechanisms are one of a variety of means for tumor cells to become chemoresistant, and would be especially advantageous to a cancer arising in a patient with intrinsic DNA damage sensitivity. It thus stands to reason that Fanconi anemia HNSCCs would, in the process of tumor generation and development, and in response to the increased cellular stress during transformation, be preferentially selected for cells that have enhanced DNA repair mechanisms.

Increased performance of NHEJ at the expense of HR is an expected result of Fanconi anemia pathway loss and so is a natural first choice for examination of the impact of DNA repair on chemosensitivity of Fanconi anemia HNSCCs. However, the extent to which NHEJ participates in the survival of Fanconi anemia HNSCC has not previously been explored, nor has DNA repair by NHEJ been directly measured in Fanconi anemia HNSCC cells. Using DNA repair reporter assays, we show that, as expected, Fanconi anemia–deficient VU-1131 cells exhibit increased NHEJ (Fig. 5B). We found that DNA-PKcs inhibition does not decrease the cisplatin EC_{50} of the human Fanconi anemia–deficient HNSCC cell lines (Fig. 5D), while all are uniformly sensitive to PARP inhibition. The lack of enhanced cisplatin sensitivity of Fanconi anemia–deficient HNSCC cells following DNA-PKcs inhibition suggests that Ku-dependent NHEJ is not the DNA repair mechanism required by Fanconi anemia HNSCC cells for repair of damage caused by ICLs.

In contrast with the NHEJ machinery, PARP appears to be a more promising target in Fanconi anemia HNSCCs. We show increased activation of PARP in Fanconi anemia–deficient HNSCC cells by greater formation of intranuclear PAR foci following MMC treatment (Fig. 6B and C). In addition, rescue of PARP inhibitor sensitivity of human Fanconi anemia HNSCC cells occurred by gene complementation (Fig. 6A; Supplementary Fig. S4A), and uniform PARP inhibitor sensitivity was additionally observed in murine Fanconi anemia HNSCC cells (Fig. 6D; Supplementary Fig. S4B). PARP inhibitor sensitivity has previously been examined in MMC-sensitive fibroblasts derived from Fanconi anemia mice as well as patients with Fanconi anemia, with conflicting results (5, 44); the present work adds to this not only by showing PARP sensitivity in Fanconi anemia HNSCC cells but also by linking PARP activity to cellular response to ICLs and

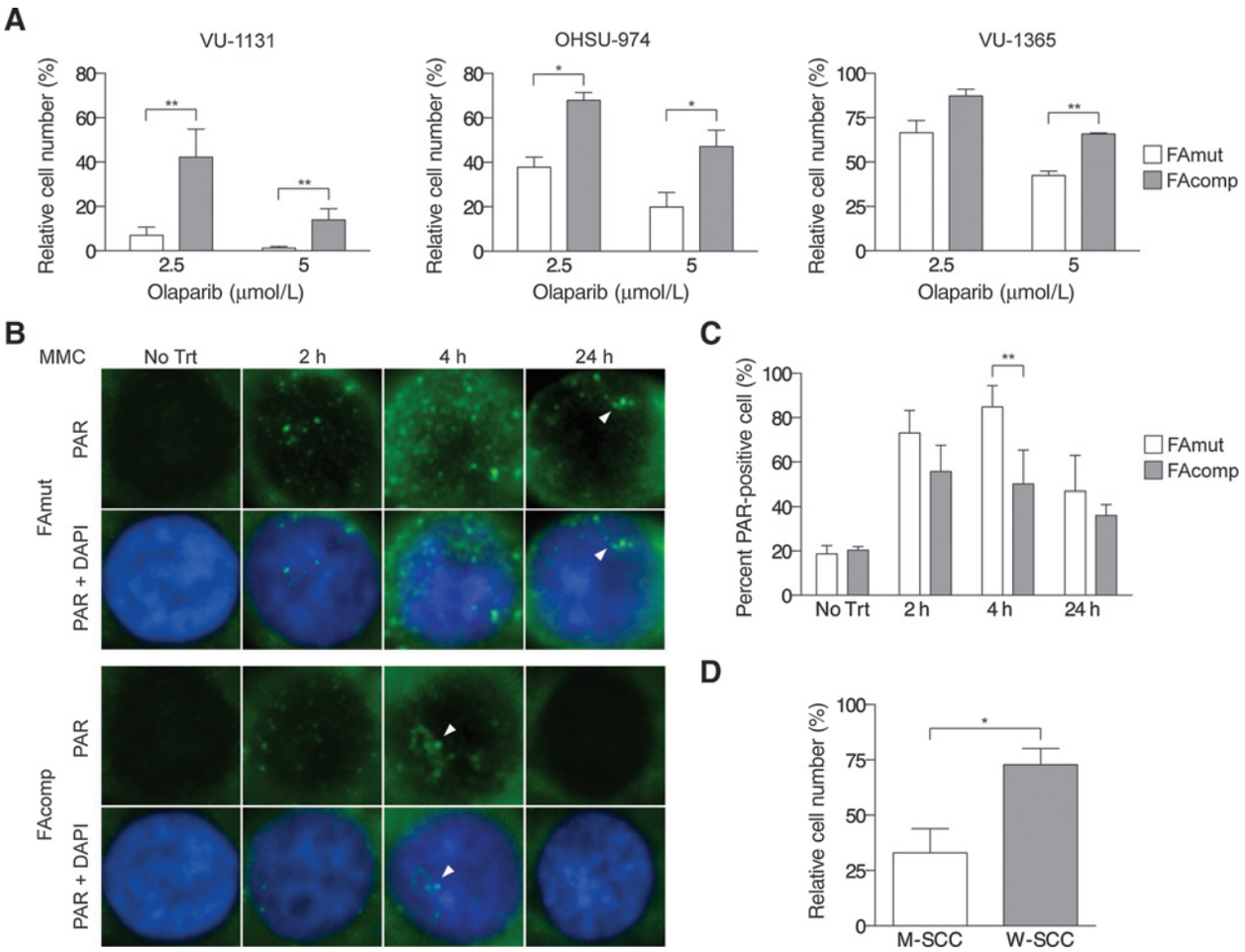

Figure 6.
PARP inhibitor sensitivity of human and murine Fanconi anemia HNSCC cells. A, cellular growth assays on isogenic human HNSCC cells exposed to the PARP1/PARP2 inhibitor olaparib show uniform sensitivity of FAmut cell lines. *, $P < 0.05$; **, $P < 0.01$ (*t* test). B, immunofluorescence for PAR foci in VU-1131 cells shows increased PAR foci formation in FAmut cells over a 24-hour course of MMC treatment. Results shown are representative of two (no treatment) or three (2, 4, and 24 hours) independent experiments of each time point, each with similar results. C, quantification of intranuclear PAR foci in VU-1131 cells over a 24-hour course of MMC treatment reveals a significantly increased number of PAR foci in FAmut cells at 4 hours of exposure. **, $P < 0.01$ (*t* test). D, cellular growth assays performed on murine Fancc$^{-/-}$ (M-SCC) and WT (W-SCC) HNSCC cell lines treated with olaparib show significantly increased sensitivity of Fancc$^{-/-}$ cell lines. *, $P < 0.05$.

subsequent relative resistance. We thus postulate that PARP hyperactivation is a mechanism frequently acquired during malignant transformation whereby Fanconi anemia HNSCC overcome constitutional DNA damage sensitivity.

PARP activation could conceivably overcome Fanconi anemia pathway deficiency by multiple mechanisms. PARP1, which comprises approximately 90% of intranuclear PARP, engages numerous modes of DNA repair, including single-strand break repair (45), base excision repair (45), nucleotide excision repair (46), Ku-independent NHEJ (37), and HR (47). PARP1 has also been implicated in Chk1 signaling at stalled replication forks (40), plays a role in control of transcription by maintaining chromatin in a transcriptionally active state (48), and may promote survival by functioning as a cofactor for NF-κB–dependent transcription (49). PARP2 has been associated with the later steps of single-strand break repair and base excision repair (50). It remains to be seen what aspects of PARP protein function are most critical for Fanconi anemia HNSCC cell adaptation.

The relative ICL resistance of Fanconi anemia HNSCC cells highlights the delicate balance between providing effective therapy and avoiding excessive toxicity in cancer treatment. The difficulty in achieving this balance becomes especially profound in patients with Fanconi anemia HNSCC, as the therapy de-escalation that may be necessary to avoid overwhelming toxicity-related morbidity may simultaneously undertreat their malignancy. In this light, it is essential to identify new therapies that will enhance survival of this fragile patient population. Identification of PARP-mediated DNA repair as a key survival mechanism employed by Fanconi anemia HNSCCs provides a promising new potential avenue of treatment. PARP inhibitor therapy could enhance efficacy of low-dose clastogenic treatments via synergistic effects. PARP inhibition could greatly benefit patients that have undergone bone marrow transplantation that are at the highest risk for HNSCC development, as the presence of a hematopoietic compartment unaffected by Fanconi anemia could prevent excessive myelotoxicity in a patient group with an otherwise grim

prognosis. Further studies targeting PARP will hopefully allow for forward progress in improvement of outcomes of Fanconi anemia patients with HNSCC.

Disclosure of Potential Conflicts of Interest

L. Wiesmüller is an inventor of a patent on a test system for determining genotoxicities. No potential conflicts of interest were disclosed by the other authors.

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References

1. Auerbach AD. Fanconi anemia and its diagnosis. Mutat Res 2009;668:4–10.
2. Kottemann MC, Smogorzewska A. Fanconi anaemia and the repair of Watson and Crick DNA crosslinks. Nature 2013;493:356–63.
3. Kennedy RD, D'Andrea AD. The Fanconi anemia/BRCA pathway: new faces in the crowd. Genes Dev 2005:2925–40.
4. Kee Y, D'Andrea AD. Molecular pathogenesis and clinical management of Fanconi anemia. J Clin Invest 2012;122:3799–806.
5. Kim Y, Spitz GS, Veturi U, Lach FP, Auerbach AD, Smogorzewska A. Regulation of multiple DNA repair pathways by the Fanconi anemia protein SLX4. Blood 2013;121:54–63.
6. Adamo A, Collis SJ, Adelman CA, Silva N, Horejsi Z, Ward JD, et al. Preventing nonhomologous end joining suppresses DNA repair defects of Fanconi anemia. Mol Cell 2010;39:25–35.
7. Pace P, Mosedale G, Hodskinson MR, Rosado IV, Sivasubramaniam M, Patel KJ. Ku70 corrupts DNA repair in the absence of the Fanconi anemia pathway. Science 2010;329:219–23.
8. Alter BP. Fanconi's anemia and malignancies. Am J Hematol 1996;53:99–110.
9. Rosenberg PS, Alter BP, Ebell W. Cancer risks in Fanconi anemia: findings from the German Fanconi Anemia Registry. Haematologica 2008;93:511–7.
10. Rosenberg PS, Greene MH, Alter BP. Cancer incidence in persons with Fanconi anemia. Blood 2003;101:822–6.
11. Kutler DI, Singh B, Satagopan J, Batish SD, Berwick M, Giampietro PF, et al. A 20-year perspective on the International Fanconi Anemia Registry (IFAR). Blood 2003;101:1249–56.
12. Birkeland AC, Auerbach AD, Sanborn E, Parashar B, Kuhel WI, Chandrasekharappa SC, et al. Postoperative clinical radiosensitivity in patients with fanconi anemia and head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2011;137:930–4.
13. Masserot C, Peffault de Latour R, Rocha V, Leblanc T, Rigolet A, Pascal F, et al. Head and neck squamous cell carcinoma in 13 patients with Fanconi anemia after hematopoietic stem cell transplantation. Cancer 2008;113:3315–22.
14. Rosenberg PS, Socie G, Alter BP, Gluckman E. Risk of head and neck squamous cell cancer and death in patients with Fanconi anemia who did and did not receive transplants. Blood 2005;105:67–73.
15. Kutler DI, Auerbach AD, Satagopan J, Giampietro PF, Batish SD, Huvos AG, et al. High incidence of head and neck squamous cell carcinoma in patients with Fanconi anemia. Arch Otolaryngol Head Neck Surg 2003;129:106–12.
16. Marcou Y, D'Andrea A, Jeggo PA, Plowman PN. Normal cellular radiosensitivity in an adult Fanconi anaemia patient with marked clinical radiosensitivity. Radiother Oncol 2001;60:75–9.
17. Park JW, Pitot HC, Strati K, Spardy N, Duensing S, Grompe M, et al. Deficiencies in the Fanconi anemia DNA damage response pathway increase sensitivity to HPV-associated head and neck cancer. Cancer Res 2010;70:9959–68.
18. Hoskins EE, Morris TA, Higginbotham JM, Spardy N, Cha E, Kelly P, et al. Fanconi anemia deficiency stimulates HPV-associated hyperplastic growth in organotypic epithelial raft culture. Oncogene 2009;28:674–85.
19. van Zeeburg HJ, Snijders PJ, Pals G, Hermsen MA, Rooimans MA, Bagby G, et al. Generation and molecular characterization of head and neck squamous cell lines of Fanconi anemia patients. Cancer Res 2005;65:1271–6.
20. Kachnic LA, Li L, Fournier L, Willers H. Fanconi anemia pathway heterogeneity revealed by cisplatin and oxaliplatin treatments. Cancer Lett 2010;292:73–9.
21. Schreiber V, Dantzer F, Ame JC, de Murcia G. Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol 2006;7:517–28.
22. Kim MY, Zhang T, Kraus WL. Poly(ADP-ribosyl)ation by PARP-1: `PAR-laying' NAD +into a nuclear signal. Genes Dev 2005;19:1951–67.
23. Michels J, Vitale I, Galluzzi L, Adam J, Olaussen KA, Kepp O, et al. Cisplatin resistance associated with PARP hyperactivation. Cancer Res 2013;73:2271–80.
24. Forster M, Mendes R, Fedele S. Synthetic lethality and PARP-inhibitors in oral and head & neck cancer. Curr Pharm Des 2012;18:5431–41.
25. Helleday T. The underlying mechanism for the PARP and BRCA synthetic lethality: clearing up the misunderstandings. Mol Oncol 2011;5:387–93.
26. Hanenberg H, Batish SD, Pollok KE, Vieten L, Verlander PC, Leurs C, et al. Phenotypic correction of primary Fanconi anemia T cells with retroviral vectors as a diagnostic tool. Exp Hematol 2002;30:410–20.
27. Meindl A, Hellebrand H, Wiek C, Erven V, Wappenschmidt B, Niederacher D, et al. Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. Nat Genet 2010;42:410–4.
28. Chen M, Tomkins DJ, Auerbach W, McKerlie C, Youssoufian H, Liu L, et al. Inactivation of Fac in mice produces inducible chromosomal instability and reduced fertility reminiscent of Fanconi anaemia. Nat Genet 1996;12:448–51.

29. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
30. Chandra S, Levran O, Jurickova I, Maas C, Kapur R, Schindler D, et al. A rapid method for retrovirus-mediated identification of complementation groups in Fanconi anemia patients. Mol Ther 2005;12:976–84.
31. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
32. Keimling M, Wiesmuller L. DNA double-strand break repair activities in mammary epithelial cells–influence of endogenous p53 variants. Carcinogenesis 2009;30:1260–8.
33. Steidler NE, Reade PC. Experimental induction of oral squamous cell carcinomas in mice with 4-nitroquinolone-1-oxide. Oral Surg Oral Med Oral Pathol 1984;57:524–31.
34. Kanojia D, Vaidya MM. 4-nitroquinoline-1-oxide induced experimental oral carcinogenesis. Oral Oncol 2006;42:655–67.
35. Bennardo N, Cheng A, Huang N, Stark JM. Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair. PLoS Genet 2008;4:e1000110.
36. Lieber MR. The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. Annu Rev Biochem 2010; 79:181–211.
37. Wang M, Wu W, Wu W, Rosidi B, Zhang L, Wang H, et al. PARP-1 and Ku compete for repair of DNA double strand breaks by distinct NHEJ pathways. Nucleic Acids Res 2006;34:6170–82.
38. Cheng Q, Barboule N, Frit P, Gomez D, Bombarde O, Couderc B, et al. Ku counteracts mobilization of PARP1 and MRN in chromatin damaged with DNA double-strand breaks. Nucleic Acids Res 2011;39:9605–19.
39. Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 2005;434:917–21.
40. Min W, Bruhn C, Grigaravicius P, Zhou ZW, Li F, Kruger A, et al. Poly(ADP-ribose) binding to Chk1 at stalled replication forks is required for S-phase checkpoint activation. Nat Commun 2013;4:2993.
41. Chaudhury S, Auerbach AD, Kernan NA, Small TN, Prockop SE, Scaradavou A, et al. Fludarabine-based cytoreductive regimen and T-cell-depleted grafts from alternative donors for the treatment of high-risk patients with Fanconi anaemia. Br J Haematol 2008;140: 644–55.
42. Parmar K, D'Andrea A, Niedernhofer LJ. Mouse models of Fanconi anemia. Mutat Res 2009;668:133–40.
43. Jakobs PM, Sahaayaruban P, Saito H, Reifsteck C, Olson S, Joenje H, et al. Immortalization of four new Fanconi anemia fibroblast cell lines by an improved procedure. Somat Cell Mol Genet 1996;22: 151–7.
44. McCabe N, Turner NC, Lord CJ, Kluzek K, Bialkowska A, Swift S, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. Cancer Res 2006;66:8109–15.
45. Hegde ML, Hazra TK, Mitra S. Early steps in the DNA base excision/single-strand interruption repair pathway in mammalian cells. Cell Res 2008; 18:27–47.
46. Robu M, Shah RG, Petitclerc N, Brind'Amour J, Kandan-Kulangara F, Shah GM. Role of poly(ADP-ribose) polymerase-1 in the removal of UV-induced DNA lesions by nucleotide excision repair. Proc Natl Acad Sci U S A 2013;110:1658–63.
47. Li M, Yu X. Function of BRCA1 in the DNA damage response is mediated by ADP-ribosylation. Cancer Cell 2013;23:693–704.
48. Kim MY, Mauro S, Gevry N, Lis JT, Kraus WL. NAD+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. Cell 2004;119:803–14.
49. Hassa PO, Haenni SS, Buerki C, Meier NI, Lane WS, Owen H, et al. Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription. J Biol Chem 2005;280:40450–64.
50. Yelamos J, Farres J, Llacuna L, Ampurdanes C, Martin-Caballero J. PARP-1 and PARP-2: New players in tumour development. Am J Cancer Res 2011; 1:328–46.