Poly(ADP-Ribose) Polymerase Inhibition Sensitizes Colorectal Cancer-Initiating Cells to Chemotherapy

AWAD JARRAR, FIORENZA LOTTI, JENNIFER DEVECCHIO, SYLVAIN FERRANDON, GERALD GANTT, ADAM MACE, GEORGIOS KARAGKOUNIS, MATTHEW ORLOFF, MONICA VENERE, MASAHIRO HITOMI, JUSTIN LATHIA, JEREMY N. RICH, MATTHEW F. KALADY

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ABSTRACT

Colorectal cancer (CRC) remains a leading killer in the U.S. with resistance to treatment as the largest hurdle to cure. Colorectal cancer-initiating cells (CICs) are a self-renewing tumor population that contribute to tumor relapse. Here, we report that patient-derived CICs display relative chemoresistance compared with differentiated progeny. In contrast, conventional cell lines failed to kill CICs, combined PARPi therapy with chemotherapy attenuated tumor growth in vivo. Clinical significance of PARPi for CRC patients was supported by elevated PARP levels in colorectal tumors compared with normal colon, with further increases in metastases. Collectively, our results suggest that PARP inhibition serves as a point of fragility for CICs by augmenting therapeutic efficacy of chemotherapy.

SIGNIFICANCE STATEMENT

Colorectal cancer is the third most common cancer in the United States. Strong evidence supports that cancer initiating cells (CICs), a self-renewing subpopulation of tumors cells, drive colorectal cancer progression and relapse. This makes CICs prime targets for therapeutic intervention. This article highlights the chemoresistant nature of CICs compared with non-CICs and commercially available colorectal cancer lines mediated by upregulation of DNA repair protein poly (ADP-ribose) polymerase (PARP). PARP Inhibitors in combination with standard of care chemotherapy-treated CIC viability, self-renewal, and DNA damage repair. Although PARPi monotherapy failed to kill CICs, combined PARPi therapy with chemotherapy attenuated tumor growth in vivo. Clinically, PARPi for CRC patients was supported by elevated PARP levels in colorectal tumors compared with normal colon, with further increases in metastases. Collectively, our results suggest that PARP inhibition serves as a point of fragility for CICs by augmenting therapeutic efficacy of chemotherapy.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and a leading cause of cancer-related deaths in the U.S. with approximately 135,430 new cases and 50,260 deaths expected in 2017 [1]. Although patients with early stage CRC generally have a good prognosis, patients with metastatic disease to lymph nodes (stage III) or distant organs (stage IV) have marked increased recurrence and mortality rates. Approximately 50% of patients present with advanced disease requiring multimodal therapy including surgery and chemotherapy [2]. Unfortunately, even with aggressive current treatment regimens approximately 50% of stage III patients develop recurrence and ultimately succumb to the disease, and long-term survival for stage IV patients may be as low as 5%. Resistance to chemotherapy remains the largest hurdle to survival in these patients. Identifying biological factors that enhance or hinder therapeutic response is crucial to improve outcomes through the development of novel therapies.

The introduction of 5-fluorouracil (5-FU)-based adjuvant chemotherapy in the late 1980s significantly improved CRC survival. The 5-year survival rate increased from 51% for cases diagnosed in the 1970s to 67% for cases diagnosed between 1999 and 2006 [1,3]. 5-FU-based chemotherapy is used as adjuvant treatment for advanced stage colon and rectal cancers. It is also used as a neoadjuvant treatment for locally advanced tumors to downstage tumors prior to surgery. Reduced toxicity and improved efficacy have been attributed to the use of continuous infusion 5-FU (5-FU-CI) as initial therapy as compared with bolus administration [4]. Despite the widespread use of 5-FU-based therapy, 30-40% of patients experience disease recurrence, with subsequent progression to failure of all available treatments [5]. Therefore, new tools are needed to improve the outcome of patients with advanced CRC.

There have been several lines of evidence that CRC arises from the transformation of a small population of cells that exhibit self-renewal and are able to give rise to tumors [6]. This subpopulation of cancer cells, referred to as cancer-initiating cells (CICs), is responsible for the self-renewal and growth of tumors [7]. CICs have been shown to have a considerable resistance to chemotherapy and radiation therapy [8]. This resistance is partially mediated by aberrant DNA repair pathways, including poly (ADP-ribose) polymerase (PARP) [9]. PARP is responsible for the repair of DNA damage caused by various mechanisms including chemotherapy and radiotherapy [10]. It catalyzes the transfer of ADP-ribose units from NAD+ onto various nuclear proteins, which results in the formation of poly(ADP-ribose) (PAR) [11]. The accumulation of PAR on specific proteins can lead to the degradation of these proteins through the ubiquitin-proteasome system [12], or the induction of cell death [13]. Inhibition of PARP prevents the degradation of PARP substrates, leading to cell death by apoptosis or autophagy [14]. This makes PARP an attractive target for cancer treatment.

In this study, we evaluated the therapeutic potential of PARP inhibitors (PARPi) in CRC cells. We used patient-derived CICs isolated from resected CRC tissues and showed that PARPi sensitized CICs to chemotherapy and reduced chemoresistance compared with differentiated progeny. In contrast, conventional cell lines failed to kill CICs, combined PARPi therapy with chemotherapy attenuated tumor growth in vivo. Collectively, our results suggest that PARP inhibition serves as a point of fragility for CICs by augmenting therapeutic efficacy of chemotherapy.

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radiotherapy sensitizer for locally advanced rectal cancers [4, 5] and neoadjuvant therapy for resectable metastatic CRC [6, 7]. Although combinations of 5-FU with newer chemotherapeutic agents such as oxaliplatin (Ox) and irinotecan have improved response rates, these remain modest for advanced CRC. Recent studies have demonstrated that even for unresectable CRC metastases, active preoperative chemotherapy was able to achieve shrinkage of liver metastases and thus qualify some patients for resection [8]. Thus, improving chemotherapy effectiveness would have significant clinical implications.

5-FU inhibits the enzyme thymidylate synthase during DNA replication [9, 10]. In contrast, Ox covalently binds DNA, forming platinum-DNA adducts that cause cell cycle arrest and growth inhibition [11]. Both compounds lead to extensive DNA damage [12, 13]. Following DNA strand breaks generated by genotoxic stress such as chemotherapy, poly(ADP-ribose) polymerase (PARP) catalyzes transfer of ADP-ribose polymers to downstream substrates that include numerous DNA repair enzymes, facilitating DNA repair [14]. In vitro, PARP inhibitors (PARPi) chemosensitize breast cancer cell lines [15]. In vivo, PARPi enhance radiation therapy in syngeneic and xenograft models of colon, lung, head and neck, and cervical cancers [11, 16–20]. Combining the standard chemotherapy agents with drugs that inhibit the DNA repair mechanism, such as PARPi, will theoretically create synthetic lethality [21]. Olaparib (AZD2281), which targets PARP, received FDA approval for BRCA1 mutated advanced ovarian cancer and is currently being studied in phase I and phase II trials for other solid tumors as a single agent or in combination with chemotherapy and/or radiotherapy [22]. A phase II study is ongoing for olaparib in patients with advanced CRC. Additional PARPi are being evaluated as single agents or in combination therapy for disseminated CRC that have failed other treatment modalities.

An emerging body of evidence indicates that tumors are composed of a hierarchy of cells with distinct characteristics [23, 24]. At the top of this hierarchy sits a subset of cancer cells that are capable of tumor initiation, and self-renewal. These molecularly and phenotypically distinct cells are referred to as tumor-initiating cells or cancer-initiating cells (CICs) [9, 25, 26]. The correlation between clinical results and experimental data obtained via commercial cancer cell lines in comparison to patient derived CICs is debatable [27, 28].

Commercial cancer cell lines have been used since the 1970s as an in vitro model for drug discovery [29]. Cell lines are relatively easy and inexpensive to use and provide rapid experimental results. However, there are several important limitations to their utilization. Cell lines represent a clonal population of tumor cells that are naturally selected to grow in culture plates and media, and thus likely differ substantially from the original tumor. Cell lines do not recapitulate the functional and genetic heterogeneity of human cancers, which is a significant factor in resistance to targeted therapies [30, 31]. This study utilizes patient CICs derived from CRC metastatic lesions to specifically mimic this clinical situation. We establish the inadequacy of commercially available CRC lines to manifest chemotherapy resistance, an observation clearly demonstrated in our patient derived CICs. No data exist regarding the efficacy of PARP inhibition on colorectal CICs. We demonstrate that PARP inhibition can synergistically reduce CIC viability, self-renewal, the ability to repair DNA damage, and in vivo tumor maintenance. A better understanding of the efficacy of PARPi in eliminating CICs can lay the scientific foundations for future clinical trials utilizing PARPi to target primary and metastatic CRC.

### Materials and Methods

#### Isolation and Culture of CD44+ (CICs) and CD44− (Non-CICs)

All human tissues were acquired from human CRC patient specimens in accordance with an approved Institutional Review Board protocol from Cleveland Clinic (IRB #4134). The current model and cell lines have been previously characterized by our group [26]. Briefly, all human tissues were acquired from primary or metastatic human CRC patient specimens according to human experimental guidelines. Tumors were dissociated using a papain dissociation system (Workington Biochemical). CD44+ cells (enriched in CICs) and CD44− (enriched for non-CICs) were enriched by flow cytometry (FACS Aria II) and grown at 37°C in an atmosphere of 5% CO2. CICs and non-CICs were cultured in serum-free media with recombinant human fibroblast growth factor-basic (bFGF, 10 ng/ml; PeproTech, Rocky Hill, NJ) and recombinant human epidermal growth factor (rhEGF, 10 ng/ml; PeproTech) as previously reported [23, 26]. Tumor specimens were maintained through subcutaneous xenografts in the flanks of NOD-SCID/IL2Rnull (NSG) mice. For cell counting prior to each experiment, a single cell suspension was achieved using TrypLE (Invitrogen, Carlsbad, CA).

#### Culture of Colon Cancer Cell Lines

The commercially available colon carcinoma cell lines SW480, SW620, and HCT116 were obtained from the American Type Culture Collection. Cells were maintained in the presence of Dulbecco’s modified eagle medium containing high glucose and supplemented 10% fetal bovine serum and penicillin/streptomycin.

#### Small Molecule Inhibitors

The PARPi AZD2281 (Olaparib) and ABT888 (Veliparib; Selleck Chemicals, Houston, TX, USA) were resuspended in dimethyl sulfoxide (DMSO). DMSO served as the vehicle control for all studies at a final percentage equivalent to that of the drug suspensions.

#### Colony Formation Assay in Soft Agar

A total of 500 CICs per well were plated in triplicate in a 12-well plate and treated with vehicle (DMSO), AZD2281 alone (10 μM), or with the combination of 5-FU (50 μg/ml), Ox (10 μM), and leucovorin (1 μM). Three days later, upon visible colony/colosphere formation, cell colonies were fixed, stained with a 0.1% crystal violet in 20% methanol solution, and counted.

#### Cell Viability and Apoptosis Assays

CICs, non-CICs, and commercial lines were plated in triplicate into 96-well plates containing the appropriate growth medium. Two cell concentrations were used (500 and/or 3,500 cells per well). Vehicle or drug was then added to each well. Adenosine triphosphate (ATP) levels via a CellTiter-Glo assay (Promega, Madison, WI) were assessed on days 0, 1, 3, 5, and 7 using a
luminometer (PerkinElmer Life and Analytical Sciences, Boston, MA). Apoptosis was evaluated by measuring the activity of caspase-3 and -7 (Caspase-Glo 3/7, Promega) on day 3 using the same conditions as the ATP assay.

**Immunofluorescent Imaging**

Colospheres were suspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin and attached to slides using a cytospin (Shandon Cytospin, Thermo, Pittsburgh, PA). Cells were then post fixed in 4% paraformaldehyde (PFA), blocked (5% normal goat serum, 0.1% Triton X-100 in 1x PBS), and immunostained with anti-phospho-H2A.X (S139; H2A histone family, member X [γH2A.X], 1:500; Millipore, Billerica, MA) or antitumor protein p53 binding protein 1 ([anti-p53BP1], 1:500; Bethyl Labs, Montgomery, TX, USA; overnight at 4°C). Secondary detection was performed using Alexa Fluor-488 goat anti-mouse IgG (Invitrogen). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired using a Leica DMR upright microscope (Heidelberg, Germany), a ×100 objective, and a Retiga 2000R CCD digital camera (Q-Imaging, Burnaby, BC, Canada). Images of γH2A.X were acquired using a fluorescein isothiocyanate (FITC) cube in conjunction with an ultraviolet cube for DAPI-stained nuclei. For immunostaining analyses of tissue sections, 7-μm frozen sections were fixed, permeabilized, and stained for Ki67 (Abcam, Cambridge, U.K.) as described above. Images were taken on an upright microscope (Leica DM4000B; magnification/aperture: ×10 0.3NA – ×20 0.5NA – ×40 0.75NA; room temperature; imaging medium: air [glass coverslipped slides]; fluorochromes: 488; camera make and model: QImaging Exi aqua; acquisition software: QCapture pro6). The integrated pixel intensity of Ki67 immunofluorescence staining signals were quantified after nuclear regions were defined using the digital images of DAPI staining of the corresponding fields as previously described [32] with some modifications. For image processing, we used the same functions of ImageJ instead of Metamoph software.

**Flow Cytometric Analysis**

Flow cytometry was performed using a FACSAriva II Cell Sorter (BD). To enrich for CICs. Single cells were labeled with a phycoerythrin-conjugated monoclonal antibody against CD44 (Miltenyi Biotec, Bergisch Gladbach, Germany), and then analyzed for the expression of FITC labeled monoclonal antibody against EpCAM (R&D Systems Inc., Minneapolis, MN). Dead cells were eliminated by using the viability dye DAPI. Side scatter and forward scatter profiles were used to eliminate cell doublets. Isotype controls were used to establish proper gates. The percentage of EpCAM and CD44 expressing cells in patients’ derived specimens used in this study are summarized in Supporting Information Figure 6A.

**Limiting-Dilution Assay**

CD44+ CICs were sorted as described using flow cytometry into 96-well plates at decreasing final cell numbers of 50, 10, and 1 cell per well. Colosphere formation was evaluated at 14 days after sorting. Wells were scored positive or negative for the presence of at least one colosphere. The estimated cancer initiating cell frequency was calculated using extreme limiting dilution analysis software [33].

**Real-Time Quantitative Polymerase Chain Reaction**

Total cellular mRNA was isolated with the RNaseasy kit (Qiagen, Hilden, Germany) and reverse-transcribed into cDNA using the SuperScript III Reverse Transcriptase kit (Invitrogen). Quantitative real-time polymerase chain reaction (RT-qPCR) was performed on an Applied Biosystems 7900HT cycler using SYBR Green Master mix (SA Biosciences, Heidelberg, Germany) and gene-specific primers with the following sequences: β-actin forward 5′-AGAAA ATCTGGCACCACACC-3′; β-actin reverse 5′-AGAGGCGTACAGGAT AGCA-3′; CD44 forward 5′-GCCCTTTGATGCACTTAATCC-3′; CD44 reverse 5′-CCTTGGTGTCCTCCAGAAGC-3′; OCT4 forward 5′-TCTCC CATGCAATTCAAACTGAG-3′; OCT4 reverse 5′-CCCTTGTGTCCCAAT TCCTTC-3′. Fold changes in mRNA expression levels were calculated using the delta/delta threshold cycle method. Each sample was run in triplicate, and all human derived cancer specimens were normalized to normal colonic epithelium.

**Animals and in vivo Studies**

All animal studies described were approved by the Cleveland Clinic Institutional Animal Care and Use Committee and conducted in accordance with the national institutes of health guide for the care and use of animals. To investigate the effect of PARPi on tumor maintenance, a subcutaneous xenograft flank model was used. 1 × 105 viable CICs derived from a primary CRC patient specimen were injected into the flanks of 6-week-old NSG mice obtained from Jackson Labs. Once the tumor volume reached a size of 200 mm3, treatment was initiated. Four treatment arms with five mice per arm were used: (a) vehicle (nontreated); (b) chemotherapy alone (5-FU and Ox); (c) PARPi alone (AZD2281); and (d) PARPi and chemotherapy. All agents were administered via intraperitoneal injection over a 22-day time period. Chemotherapeutic agents were injected in the lower left abdominal quadrant. In the chemotherapy groups, 5-FU was administered at 15 mg/kg per day, 5 days/week for the first 2 weeks only and Ox was administered at 0.25 mg/kg once a week for 4 weeks. In the PARPi groups, AZD2281 was administered at a concentration of 50 mg/kg daily for 22 days. When coadministration of AZD2281 and chemotherapeutic agents was required, AZD2281 was given 30 minutes before chemotherapy. The maximum volume injected per mouse was 200 μl. Tumor growth rate was calculated as the ratio of tumor volume at the end of the treatment cycle compared with the tumor volume at the beginning of treatment (200 mm3). Mice were euthanized at day 22 and the xenografts were excised.

**Patient Database Bioinformatics**

Gene expression data was obtained from the Oncomine database (www.oncomine.com). mRNA expression levels of PARPi were compared between CRCs and normal colonic epithelium. mRNA expression levels of PARPi were compared in primary CRC specimens stratified according to development of distant recurrence during 5-year follow-up after surgical resection. PARPi mRNA levels were also compared between CRC specimens showing high level microsatellite instability (MSI-H) and microsatellite stable (MSI-S) CRCs.

**Immunoblotting**

CICs, non-CICs or tissue lysates were harvested and immunoblotted. The membrane was probed for 53BP1 (Bethyl Labs, and anti-β-actin (Sigma–Aldrich, St. Louis, MO). Secondary
detection was achieved using HRP conjugated secondary antibodies.

**Mutational Analysis**

KRAS, BRAF, and P53 mutations were evaluated by PCR sequencing analysis. Genomic DNA was amplified at 2 KRAS exons (exons 2 and 3), 2 BRAF exons (exons 11 and 15) and exons 4–9 of P53 gene by standard PCR as previously reported [34, 35]. All PCR products were purified using the Qiagen QiaQuick Kit. Sequences were analyzed for mutations using FinchTV version 1.4.0 (Geospiza, Seattle, WA). The characteristics of patients’ derived specimens used in this study are summarized in Supporting Information Figure 6B.

**Statistical Analysis**

Statistical significance was calculated with GraphPad Prism utilizing a one-way or two-way analysis of variance (ANOVA) with a Bonferroni’s post-test, Student’s t test, or log-rank (Mantel–Cox) test where appropriate (GraphPad Software Inc., La Jolla, CA, USA). Data are represented as the mean ± SD.

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**RESULTS**

**Colorectal CICs Are Resistant to Chemotherapy**

To interrogate the efficacy of the commonly used 5-FU-based chemotherapy, human-derived CICs from primary and metastatic CRCs were sorted for CD44+ cells and expanded in serum-free medium. We previously demonstrated the ability to isolate and expand a subpopulation of tumor cells, designated CICs, based on the preferential ability of these cells to propagate secondary tumors that phenocopy the original tumor [26]. CICs and the corresponding isolated non-CICs were treated with a combination of 5-FU (50 μg/ml), oxaliplatin (10 μM), and leucovorin (1 μM). Cell viability was measured on days 0, 1, 3, 5, and 7 after treatment using an ATP-based cell viability assay. The data for each time point were normalized to the DMSO-treated controls. Data are presented as mean ± SD (n = 3); *, p < .05; ***, p < .001.

**Figure 1.** Colorectal cancer-initiating cells (CICs) are more resistant to 5-fluorouracil (FU)-based therapy than non-CICs and conventional cell lines. CICs and non-CICs derived from freshly dissociated human specimens (RL199 and CW656) and commercially available colon cancer lines (HCT116 and SW480) were treated with vehicle (dimethyl sulfoxide [DMSO]) or a combination of 5-FU (50 μg/ml), oxaliplatin (10 μM), and leucovorin (1 μM). Cell viability was measured on days 0, 1, 3, 5, and 7 after treatment using an ATP-based cell viability assay. The data for each time point were normalized to the DMSO-treated controls. Data are presented as mean ± SD (n = 3); *, p < .05; ***, p < .001.
proliferation rate and the sensitivity of chemotherapy, we repeated the experiment with different cell concentrations per well which yielded similar results for both cell lines, CICs, and non-CICs (Supporting Information Fig. 1B, 1C). CICs were significantly more viable than both non-CICs and commercially available cell lines despite the seeding density. These results support using patient-derived CICs as a model to interrogate chemoresistance.

PARP Inhibition Increases the Chemosensitivity of Colorectal CICs

We next sought to assess the effect of PARP inhibition on CIC survival. CICs derived from metastatic and primary CRCs were treated with AZD2281, 5-FU/Ox/leucovorin, or a combination of both. Cell viability was monitored over a 7-day time course. AZD2281 monotherapy modestly reduced CIC viability (Fig. 2A, 2D and Supporting Information Fig. 2A; blue line). Chemotherapy

Figure 2. Poly(ADP-ribose) polymerase inhibition increases the chemosensitivity of colorectal cancer-initiating cells (CICs). CICs from the specimens RL199 (A), CW656 (B), E80 (C), and 0064 (D) were plated at 500 cells per well in a 96-well plate. Cells were treated with vehicle (dimethyl sulfoxide [DMSO]), AZD2281 (10 μM), or 5-fluorouracil (FU; 50 μg/ml), oxaliplatin (10 μM), and leucovorin (1 μM) alone or in combination with AZD2281 (10 μM). Cell viability was measured on days 0, 1, 3, 5, and 7 using an ATP-based cell viability assay. The data for each time point were normalized to the DMSO-treated controls. Data are presented as mean ± SD (n = 3); *, p < .05, ***, p < .001. A total of 500 CICs per well from the specimens RL199 (E) and CW656 (F) were plated in a 12-well plate and treated with vehicle control (DMSO), AZD2281 alone (10 μM), or the combination of 5-FU (50 μg/ml), oxaliplatin (10 μM), and leucovorin (1 μM). Three days later, upon visible colony/colosphere formation, cell colonies were fixed, stained with a 0.1% crystal violet in 20% methanol solution, and counted. Data are presented as mean ± SD; ***, p < .001.
alone, as expected, did not affect proliferation and all lines had significant expansion post-treatment (Fig. 2A, 2D and Supporting Information Fig. 2A; red line). However, the addition of AZD2281 to chemotherapy completely blocked CIC proliferation (Fig. 2A, 2D and Supporting Information Fig. 2A; pink line). This combinational effect was demonstrated at 1 μM (Fig. 2A, 2B; pink line) and 10 μM (Supporting Information Fig. 2B, 2C; pink line) of AZD2281 with chemotherapy. Comparable results were obtained using another PARP inhibitor currently in clinical trials (ABT888; Supporting Information Fig. 2D), thus supporting observations with AZD2281.

To confirm the efficacy of AZD2281 alone or in combination with 5-FU/Ox/leucovorin against CICs, we used a soft agar colony-formation assay (Fig. 2E, 2F). Following 3 days of treatment, ADZ2281 monotherapy had a modest effect on colony formation, which was consistent with the previous result on viability obtained after 3 days of treatment (Fig. 2A, 2B). The combination of chemotherapy with AZD2281 significantly decreased CIC colony formation (Fig. 2E, 2F).

**PARP Inhibition Leads to a Delayed DNA Damage Repair and Increased Apoptosis**

Based on the effect of AZD2281 on CIC viability, we interrogated the mechanism of cell death caused by PARP inhibition. We first established the extent of DNA damage caused by the various treatments. CICs derived from CRC metastasis (CW656) and primary CRC (E80) were treated with vehicle control, AZD2281, 5-FU/Ox/leucovorin, or with a combination of both, and the levels of DNA damage were measured using the DNA damage marker γH2AX and tumor suppressor p53-binding protein 1 (53BP1). After treatment with chemotherapy, CICs were able to resolve damaged DNA, whereas cells treated with both chemotherapy and AZD2281 demonstrated compromised ability to repair damage, as demonstrated by persistent γH2AX and 53BP1 signals (Fig. 3A, 3C). These data highlight a central role for PARP in both primary and metastatic CICs response to DNA damage. We subsequently sought to examine the role of PARP inhibition in apoptotic cell death. CICs from CRC liver metastasis (RL199) and brain metastasis (CW656) were treated with AZD2281 with or without 5-FU/Ox/leucovorin, and apoptosis was measured via caspase-3/7 activity after 3 days. In all cells, AZD2281 potently increased chemotherapy-induced apoptosis (Supporting Information Fig. 3).

**Targeting PARP Compromises the Stem Cell Phenotype of CICs In Vitro**

A key defining characteristic of CICs is the ability to form and grow spheres (i.e., colospheres) initiated from a single cell as a putative functional assay of self-renewal capacity. The impact of a treatment on the colosphere-forming potential of CICs can be evaluated through a limiting dilution assay, which permits a quantitative estimation of stem-like cells. To examine the impact of AZD2281 in combination with 5-FU/Ox/leucovorin on sphere formation, we plated single cell suspensions of CICs in the presence of increasing concentrations of AZD2281 and evaluated sphere formation 14 days later. Consistent with its anti-proliferative effects, AZD2281 decreased self-renewal by 5- to 20-fold at the higher concentration. Importantly, self-renewal was almost ablated with the addition of chemotherapy (Fig. 4A, 4D). Although cell death likely contributed to this result, the lower concentration of AZD2281 plus chemotherapy also decreased the cancer initiating cell frequency (Fig. 4A, 4B). To rule out the effect of cell death on the functional assay, CICs derived from the RL199 and CW656 (Supporting Information Fig. 4A, 4B) were pretreated with the same conditions as above for duration of 3 days. One, 10, and 50 viable CICs were then plated in a 96-well plate as previously described. The same pattern was observed at 14 days (Supporting Information Fig. 4A, 4B) suggesting a compromise of self-renewal in the presence of AZD2281 plus chemotherapy.

**Targeting PARP Compromises the Stem Cell Phenotype of CICs In Vivo**

As in vivo tumor maintenance is an important aspect of CIC function, we interrogated the ability of AZD2281 treatment to inhibit tumor growth in NSG mice. Each NSG mouse bearing a 200 mm³ xenograft was treated with DMSO, AZD2281, 5-FU/Ox/leucovorin chemotherapy, or AZD2281 plus chemotherapy for 22 days. Tumor growth rate was monitored for the duration of the treatment. Indeed, the combination of AZD2281 and chemotherapy treatment compromised tumor growth in comparison to the other arms of the study (Fig. 5A, 5B). Explanted xenografts were cut, mounted onto slides, and stained for the proliferation marker Ki67. Xenografts harvested from those mice receiving the combination of AZD2281 and chemotherapy showed the least proliferation (Fig. 5C). As reported previously by our group [26], the percentage of CD44+ cells is enriched after chemotherapy treatment. To expand these findings to the current in vivo model, explanted xenografts were evaluated for CD44 expression along with expression of a stem cell regulatory gene (OCT4) using RT-qPCR (Fig. 5D, 5E). In the explanted xenograft tumors that were harvested from mice treated with the combination of PARPi and chemotherapy, there was a decrease in the expression levels of both CD44 and OCT4 compared with PARPi or chemotherapy alone (Fig. 5D, 5E). Xenografts treated with combination chemotherapy and PARPi demonstrated increased levels of DNA damage as evidenced by increased 53BP1 (Supporting Information Fig. 5).

These data support the use of PARP inhibition against CICs in vivo, offering preclinical insight into targeting the stem cell-like population and possibly improving the response to commonly used chemotherapeutic agents.

**PARP1 Is Highly Expressed in CIC Compared with Non-CICs, in CRC Compared with Normal Colon, and in CRC Metastases Compared with Primary Cancers**

The abundance of PARP1 expression in CRC compared with normal colonic epithelium may dictate the specificity of PARPi to target cancer tissue. The mRNA expression profile of PARP1 was determined in two patient datasets available through Oncomine (www.oncomine.com). The mean expression levels of PARP1 (Fig. 6A) were higher in CRC compared with normal colonic tissues, suggesting a role in CRC biology and a possible target. Since the main focus of the current study was the role of PARPi in recurrent CRCs, we evaluated PARP expression in primary CRC specimens that subsequently developed distal recurrence upon 5-year follow-up. PARP1 levels were significantly higher in the primary tumors of patients who developed CRC recurrence (Fig. 6B). Furthermore, we evaluated differences in PARP1 expression between matched primary tumors
and metastatic lesions in the same patient from the Cleveland Clinic CRC biobank. PARP1 levels were significantly elevated in the metastases compared with the primary cancers (Fig. 6C).

A subset of CRCs arises due to a DNA mismatch repair (MMR) deficiency [36]. The resultant tumors are termed MSI-H and are characterized by genetic hypermutability resulting from impaired DNA MMR. MMR proteins correct errors that spontaneously occur during DNA replication, such as single base mismatches or short insertions and deletions. Since PARP plays a role in DNA repair, we evaluated the relationship of DNA hypermutability (MSI-H CRC) with PARP expression via publically available MSI-H and microsatellite stable CRC datasets through Oncomine database (www.oncomine.com). As we expected, MSI-H tumors had significantly higher PARP1 expression levels compared with microsatellite stable counterparts (Fig. 6D).

In light of the previous results, we then hypothesized that CICs might differentially express PARP1 compared with non-CICs. Indeed, CICs showed a higher protein abundance of PARP1 as well as its enzymatic product, poly(ADP-ribose; PAR), suggesting a more abundant and active PARP1 and in CICs versus non-CIC (Fig. 6E).

Taken together, these data demonstrate that PARP is increased in CRC and also is related to advancing stages of disease. In addition, its enhanced expression in hypermutated CRC suggests PARP is a relevant potential target particularly in metastatic and MSI-H CRC.

Figure 3. Poly(ADP-ribose) polymerase inhibition delays DNA damage repair in colorectal cancer-initiating cells (CICs). Immunostaining analysis of CW656 (A) and E80 (B, C) CICs grown as colospheres and treated with vehicle (dimethyl sulfoxide), AZD2281 (10 μM), or 5-fluorouracil (50 μg/ml), oxaliplatin (10 μM), and leucovorin (1 μM) alone or in combination with AZD2281 (10 μM) for 24 hours. Colospheres were attached to a slide via cytospin, fixed, and stained for the DNA damage marker γH2A.X (A, B) and 53BP1 (C). High-magnification (×100) image fields were acquired across the entire slide section. The number of γH2A.X and 53BP1 nuclear foci per cell was quantified for a total of 100 cells. Data are presented as mean ± SD (n = 2); *** p < .001.
Patients diagnosed with advanced stage CRC (stages III and IV) have higher recurrence rates and worse prognosis despite the utilization of 5-FU and platinum-based chemotherapy, with minimal progress over the last few decades [36, 37]. Our group and others have shown an enrichment of colorectal CICs following chemotherapy, making them an appealing target for future chemosensitizers [26, 38, 39]. In the current study, we investigated the reliance of colorectal CICs on PARPs as a possible resistance mechanism. We showed that targeting metastatic CICs with PARPi in the context of 5-FU-based chemotherapy compromised the ability of CICs to respond to DNA damage, forcing the cells to undergo apoptosis. This phenomenon could lead to the elimination of CIC expansion following chemotherapy. Given the important role CICs play in tumor metastasis and dissemination [40], our results suggest that PARP inhibition with chemotherapy impairs the ability of CICs to drive CRC maintenance and future recurrence. The potential role for PARP inhibition in the treatment of metastatic disease is further supported by the fact that PARP expression was significantly higher in more advanced CRCs and in metastases compared with matched primary cancers.
Numerous agents proposed to chemosensitize cancers have shown promise in preclinical trials but have not produced substantial benefit in patients during clinical trials. This may partially due to the observation that commercial colon cancer lines as often used in preclinical work do not adequately represent the primary tumor and the newly identified highly resistant subset of CICs. Indeed, our results show a dramatic difference in the response to chemotherapy between metastatic and primary CRC CICs and commercial CRC lines. The translational impact of such differences should be taken into consideration in designing future preclinical studies.

Sensitivity to chemotherapy is in part determined by the integrity of DNA repair ability. For example, cisplatin sensitivity is enhanced in breast and ovarian tumors that arise secondary to inherited mutations in DNA repair genes, \textit{BRCA1} and \textit{BRCA2} [41]. \textit{BRCA} mutant tumors are defective in DNA repair via homologous recombination (HR), which contributes to processing of DNA interstrand crosslinks induced by platinum agents such as cisplatin. Upon DNA damage, PARP1 is rapidly recruited to DNA nicks where it induces the synthesis of protein-conjugated polymers of ADP-ribose (PAR). PARP1 itself is a prime target of PARylation and the resulting PAR

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**Figure 5.** Poly(ADP-ribose) polymerase inhibition compromises the stem cell phenotype in vivo. The effect of colorectal cancer-initiating cell (CIC) treatment with AZD2281 or chemotherapy on tumor growth was evaluated in vivo. Four arms were included: control, AZD2281, 5-fluorouracil + oxaliplatin, and AZD2281 + 5-fluorouracil + oxaliplatin. CICs derived from a primary colorectal cancer patient specimen were implanted into the flanks of 20 (5 mice per condition) 6-weeks-old female NSG mice. Once subcutaneous xenografts reached 200 mm$^3$ treatment was initiated. The tumor growth was monitored. Mice were sacrificed at 22 days after injection. (A): Relative tumor volume of various arms. (B): Representative images of explanted tumors are presented. Scale bar, 1 cm. Data are presented as mean ± SD (n = 5); *, p < .05; ***, p < .001. ANOVA, followed by Bonferroni’s post hoc test was used to assess the significance. Explanted tumors were sectioned and stained for Ki67 proliferation marker. (C): Quantification and representation of staining intensity in relation to DAPI nuclear staining is presented. Data are presented as mean ± SD (n = 4); *, p < .05. Quantitative real time polymerase chain reaction analysis of CIC markers CD44 (D) and OCT4 (E) was performed on performed on explanted xenografts. Data are presented as mean ± SD (n = 3); *, p < .05; ***, p < .001.

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chains serve as a platform for the recruitment of downstream repair factors. PARylation is a transient and reversible modification, as it is counteracted by the activity of PAR glycohydrolase (PARG), which degrades PAR. Inhibition of PARP1, leading to the accumulation of unresolved single-strand breaks (SSBs). Loss of PARG has been recently reported as a major resistance mechanism to PARPi [42]. Although cancer cells are equipped with redundant DNA repair mechanisms, the loss of a second crucial mechanism as accomplished by inhibition of the PARP pathway leads to cell death. BRCA mutant cancer cells cannot compensate for the disruption of both SSB repair (involving PARP) and HR (involving BRCA1/2) and thus synthetic lethality occurs through a combination of pharmacologic inhibition and cellular mutation [43]. The occurrence of sporadic BRCA mutations in CRCs is a low-frequency event [44]. However, approximately 20% of CRCs have DNA MMR deficiency (MSI-H tumors), a subset of cancers that are characterized by hypermutation [44, 45]. As MSI-H tumors are relatively resistant to 5-FU-based chemotherapy [46], PARP inhibition in this subset of patients may offer a novel approach to personalized care.

The current study does not aim to interrogate the mechanism behind the differential role PARP inhibition plays in CIC chemoresistance. However, our observation of increased PARP1 and PAR levels in CICs versus non-CICs suggests a contributing role. Multiple mechanisms could underlie the advantageous PARP-mediated DNA repair mechanism in CICs. Over-activation of PARG is one potential hypothesis. Another possibility that

Figure 6. Poly(ADP-ribose) polymerase 1 (PARP1) mRNA mean expression level is increased in colorectal cancer. The mean mRNA expression levels of PARP1 (A) were compared in patient-derived colorectal cancers and normal colonic epithelial specimens. Analysis was done using the Oncomine database (www.oncomine.org); *** p < .001. (B): The mean expression levels of PARP1 were compared in primary colorectal cancer specimens stratified according to development of distal recurrence during 5-year follow-up. Analysis was done using the Oncomine database (www.oncomine.org); *** p < .001. (C): PARP1 mRNA levels were compared in colorectal cancer liver metastases compared with matched primary colorectal cancer specimens using quantitative real time polymerase chain reaction. Data are presented as mean ± SD (n = 3); *, p < .05. (D): The mean mRNA expression levels of PARP1 were compared in patients derived colorectal cancer specimens stratified according to microsatellite instability status. Analysis was done using the Oncomine database (www.oncomine.org); *** p < .001. The protein levels of PARP1 and its activity surrogate poly(ADP-ribose) polymer, were assessed using Western blotting in CICs and non-CICs derived from CW656 (E).
warrants further exploration is the reliance of CICs on the NAD+ salvage pathway. Catastrophic DNA damage-mediated PARP activation leads to cytosolic NAD+ depletion and the release of mitochondrial apoptosis-inducing factor (AIF). This is an established mechanism behind PARP1-driven apoptosis in cells with extensive, unreparable DNA damage [46]. Recent reports suggest an active role for the NAD+ salvage pathway in modulating cancer cell viability via the replenishing of the NAD+ reservoir [47]. Such a pathway, if proven to be preferentially present in CICs, could further explain the active role of PARP. Future studies are required to explore these hypotheses.

CONCLUSION

We demonstrated that PARP inhibition reduces metastatic CIC viability, self-renewal, and the ability to repair DNA damage. This work opens the door for future exploitation of this CIC vulnerability in the context of preclinical and clinical trials with the goal of improving CRC survival.

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