Dual Roles of PARP-1 Promote Cancer Growth and Progression

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ABSTRACT

PARP-1 is an abundant nuclear enzyme that modifies substrates by poly(ADP-ribose)-ylation. PARP-1 has well-described functions in DNA damage repair and also functions as a context-specific regulator of transcription factors. With multiple models, data show that PARP-1 elicits protumorigenic effects in androgen receptor (AR)-positive prostate cancer cells, in both the presence and absence of genotoxic insult. Mechanistically, PARP-1 is recruited to sites of AR function, therein promoting AR occupancy and AR function. It was further confirmed in genetically defined systems that PARP-1 supports AR transcriptional function, and that in models of advanced prostate cancer, PARP-1 enzymatic activity is enhanced, further linking PARP-1 to AR activity and disease progression. In vivo analyses show that PARP-1 activity is required for AR function in xenograft tumors, as well as tumor cell growth in vivo and generation and maintenance of castration resistance. Finally, in a novel explant system of primary human tumors, targeting PARP-1 potently suppresses tumor cell proliferation. Collectively, these studies identify novel functions of PARP-1 in promoting disease progression, and ultimately suggest that the dual functions of PARP-1 can be targeted in human prostate cancer to suppress tumor growth and progression to castration resistance.

SIGNIFICANCE: These studies introduce a paradigm shift with regard to PARP-1 function in human malignancy, and suggest that the dual functions of PARP-1 in DNA damage repair and transcription factor regulation can be leveraged to suppress pathways critical for promalignant phenotypes in prostate cancer cells by modulation of the DNA damage response and hormone signaling pathways. The combined studies highlight the importance of dual PARP-1 function in malignancy and provide the basis for therapeutic targeting. Cancer Discov; 2(12): 1134–49. © 2012 AACR.

INTRODUCTION

Whereas the role of PARP-1 in the DNA damage response is well defined, the impact of PARP-1-mediated transcriptional regulation, especially in the context of human malignancy, is of emerging interest (1–3). PARP-1 is an abundant nuclear enzyme that catalyzes poly(ADP-ribose)-ylation (PARylation) of target proteins using NAD+ as a cofactor (1). In response to genotoxic insult, PARP-1 is recruited to sites of damage, which activates PARP-1-mediated catalytic activity (4–6). The vast majority of PARP-1 function is self-directed, and auto-modified PARP-1 recruits proteins that promote DNA repair (e.g., XRCC1), and thereby facilitates assembly and activation of the base excision repair machinery (7–9). Autodifferentiation also alters PARP-1 affinity for DNA and influences chromatin compaction at sites of PARP-1 action (8, 10–12). PARP-1 inhibitors have recently been developed as a means to sensitize to genotoxic insult, and are in clinical trials in combination with chemotherapy and/or radiation as well as for tumors with defects in DNA repair capacity (13–16).

Distinct from the role of PARP-1 in DNA repair, the transcriptional regulatory functions of PARP-1 are multifold, are not universally dependent upon enzymatic activity, and are manifest through divergent functions, including enhancer binding, association with insulators, modulation of chromatin structure, and/or direct transcription factor regulation (2, 17–19). Despite these realizations, the mechanisms and impact underlying PARP-1-mediated transcriptional control in human malignancy remain largely unexplored. Roles for PARP-1 in estrogen receptor-α (ER) transcriptional activation (20), retinoic acid receptor activation via modulation of Mediator (21), coactivation for neuron-derived orphan receptor 1 (22), and negative regulation of the retinoic acid receptor–thyroid hormone receptor heterodimer (23) suggest a potential function for PARP-1 in nuclear receptor control and possibly hormone-dependent cancers. In addition, recent studies identified a role for PARP-1 in mediating ETS activity, which harbors prooncogenic function in prostate cancer (24) and Ewing sarcoma (24, 25).

Here, a critical role for PARP-1 was identified, wherein PARP-1 regulates both tumor growth and progression through transcriptional regulatory functions. Human prostatic adenocarcinoma is dependent on androgen receptor (AR) activity for growth and survival, and is largely resistant to standard chemotherapy. As such, AR-directed therapeutics are the first-line therapeutic intervention for all patients with...
disseminated disease (26). Ablation of AR activity is initially effective; however, recurrent tumors arise wherein AR activity has been reactivated, as achieved through multiple mechanisms that have been reviewed extensively (26). As no durable cure is available for this “castrate-resistant” stage of disease, developing a means to sustainably suppress AR function and/or sensitize prostate cancer cells to cytotoxic chemotherapy is urgently needed.

Studies herein reveal that PARP-1 is a potent modulator of both AR function and the response to DNA damage. Data show that PARP-1 regulates AR association with chromatin and controls AR function in genetically defined systems, both in vitro and in vivo. These functions of PARP-1 require enzymatic activity, and ablation of PARP-1 activity sensitizes prostate cancer cells to both genotoxic insult and androgen depletion. Strikingly, models of castration-resistant prostate cancer (CRPC) show marked upregulation of PARP-1 activity, suggesting that PARP-1 may be enhanced as a function of tumor progression. Accordingly, PARP-1 suppression enhanced the antitumor effects of castration, delayed onset to castration resistance, and was required to maintain in vivo castrate resistance. Moreover, primary human prostate tumor ex vivo cultures revealed a significant antitumor response to PARP-1 inhibition, which was correlated with diminished AR activity, thus further showing the dependence of prostate cancer on PARP-1 activity. Collectively, these studies identify a major role for PARP-1 in controlling AR signaling, and show that the dual functions of PARP-1 could be leveraged for therapeutic benefit.

RESULTS
PARP-1 Inhibition Sensitizes to Genotoxic Stress and Suppresses Cell Growth in AR-Positive Cells

To assess the impact of PARP-1 on the response to DNA damage in prostate cancer cells, a clinically relevant PARP inhibitor, ABT888, was used (27). ABT888 sensitized both androgen deprivation-sensitive [sensitive to hormone therapy (HT)] and CRPC cells to ionizing radiation (IR) and docetaxel (DCTX; Fig. 1A), the latter in a dose-dependent manner (Supplementary Fig. S1A). Unexpectedly, a reduction in cell number was observed with exposure to ABT888 alone, Fig. 1A; ref. 28). Escalating doses of ABT888 were therefore administered and cell number was assessed (Supplementary Fig. S1B); although some variation was observed, each model showed growth suppression that was induced by ABT888 as a single agent. Growth curve analyses further identified a role for PARP-1 in specifically controlling growth in AR-positive HT-sensitive cells (Fig. 1B, top). Inhibition of PARP-1 activity not only resulted in reduced growth of HT-sensitive prostate cancer cells but also cooperated with androgen deprivation to suppress cell survival (top right). Similar effects were observed in CRPC cells (Fig. 1B, middle). In contrast, no effect was seen in AR-negative prostate cancer cells (Fig. 1B, bottom) or AR-negative immortalized prostate epithelial cells (ref. 29; PrEC; Fig. 1B, bottom right). Together, these data show that PARP inhibition cooperates with genotoxic insult and elicits unexpected growth-suppressive effects that are largely confined to AR-positive prostate cancer and CRPC cells, without affecting AR-negative prostate cell growth.

PARP-1 Is a Critical Effector of AR Activity

Given the specificity of PARP inhibitors for suppressing growth of AR-positive prostate cancer and CRPC cells (but not AR-negative cells), the effect of PARP-1 on AR function was determined. Single-dose (2.5 μmol/L, below IC50) exposure of ABT888 significantly reduced expression of well-characterized, prostate-specific AR target genes of clinical relevance (KLK3/PSA, TMPRSS2, and FKBP5), compared with control (Fig. 2A, left), but did not alter mRNA levels of genes previously found to be refractory to PARP-1 knockdown in MCF7 cells (SEMA4G and PHF3; ref. 30), thus showing specificity. The clinically used AR antagonist Casodex (CSDX) served as a positive control. Observed effects on AR target-gene expression were not because of alterations in AR protein levels (Fig. 2A, middle). Importantly, chromosomal rearrangements placing the coding region of ETS genes (e.g., ERG) under control of the TMPRSS2 regulatory locus occur with high frequency in prostate cancer and therein result in AR-dependent expression of protumorigenic ETS genes (31). Therefore, the impact of PARP-1 on AR-dependent ERG gene expression was assessed in cells containing TMPRSS2:ERG fusions (VCaP cells; ref. 31). PARP inhibition significantly suppressed expression of AR-dependent target genes including ERG and the ERG target gene PLAT, while not affecting PARP-1 refractory SEMA4G or PHF3 (Fig. 2A, right). Similar effects on AR function were observed in additional prostate cancer models of HT-sensitive disease (Supplementary Fig. S2A). PARP inhibition suppressed AR-dependent activity of an integrated ARR2(probasin)-LUC reporter, thereby confirming the ability of PARP-1 to modulate AR activity (ref. 32; Supplementary Fig. S2B). Moreover, pretreatment of androgen-depleted cells with ABT888 before androgen [dihydrotestosterone (DHT)] stimulation (comparison of hormone-proficient and hormone-deficient KLK3/PSA levels, Supplementary Fig. S2C) significantly impaired androgen-dependent expression of KLK3/PSA (Fig. 2B). Thus, inhibition of PARP activity suppresses AR target-gene expression.

To determine whether the observed growth-suppressive function of PARP inhibition operates through regulation of AR, HT-sensitive cells were exposed to ABT888 alone, CSDX alone, or a combination thereof. Single-treatment regimens elicited a decrease in AR target-gene expression compared with control; however, no additive effect was observed when these agents were used in combination (Fig. 2C). In addition, combination treatment elicited no additive effect with regard to inhibition of cell growth (Supplementary Fig. S3). Taken together, these data show that PARP enzymatic activity is required for AR function in HT-sensitive prostate cancer cells and that targeting PARP activity decreases AR activity in multiple models of human disease.

PARP-1 Modulates AR–Chromatin Interaction

As PARP enzymatic activity was shown to influence AR activity, the underlying impact on chromatin association was assessed. With the use of chromatin tethering assays,
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**Figure 1.**

A. Cell number was assessed 96 hours after exposure to indicated treatment in indicated cell models. Treatments were ABT888 (2.5 μmol/L), IR (2 Gy), ABT888 + IR (2.5 μmol/L + 2 Gy), DCTX (1 nmol/L DCTX), and ABT888 + DCTX (2.5 μmol/L + 1 nmol/L DCTX). Vehicle control is set to “100%.”

B. Model systems indicated were treated with vehicle control (solid line) or 2.5 μmol/L ABT (dashed line), and cell number was assessed at indicated time points. Cell number at the 0 hour time point is set to “1.” Data reflect averages and SD of at least 3 independent experiments, each conducted with biologic triplicates. Statistical significance was determined using Student t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. PCa, prostate cancer.
which have previously been shown to differentiate between nuclear and chromatin-associated proteins. DHT-induced AR association with chromatin, compared with cells cultured in androgen-depleted conditions, was determined (Fig. 3A; compare lanes 4 and 5). The DHT-stimulated AR occupancy of chromatin was markedly reduced in cells that were pretreated with a PARP inhibitor (compare lanes 5 and 6). To determine if AR is PARYlated in response to DHT, immunoprecipitations followed by immunoblot were conducted. AR was not PARYlated (in either the presence or absence of androgen), whereas PARP-1 autophosphorylation was suppressed by PARP inhibition (Supplementary Fig. S4A), indicating that regulation of AR by PARP-1 is not attributable to PARYlation of AR. To assess whether the functional interplay observed in the tethering assay held true at sites of known AR function, chromatin immunoprecipitation with quantitative PCR (ChIP-qPCR) was used to assess recruitment of AR and PARP-1 to the regulatory loci of AR target genes. As expected, DHT stimulation induced AR occupancy at the KLK3/PSA enhancer (Fig. 3B, top left), the KLK3/PSA promoter (top right), and a critical androgen responsive element that controls expression of TMPRSS2 (bottom left). Strikingly, PARP-1 was associated with each region as a function of AR occupancy. In contrast, DHT-induced occupancy of both AR and PARP-1 was markedly suppressed in cells pretreated with ABT888 (Fig. 3B), thus suggesting that PARP-1 function may be critical for AR and PARP-1 residence at key sites of AR function. To determine if AR and PARP-1 cooccupy sites of chromatin, ChIP-reChIP was conducted. reChIP signals of AR/PARP-1 were not discernible from IgG controls (Supplementary Fig. S4B), indicating that although AR and PARP-1 occupy the same AR target-gene loci, they do so in separate protein complexes. To eliminate the possibility that these observations represent random binding of either AR or PARP-1, ChIP assays were done to assess the residency of these proteins in a genomic region devoid of coding genes. Neither PARP-1 nor AR binding was detectable (Fig. 3B, bottom right). Previous studies showed that PARP-1–dependent transcriptional regulation can be manifest through the

Figure 2. A, left: LNCaP cells were cultured in media containing complete serum, treated for 24 hours with either ethanol control (0.01%), ABT888 (2.5 μmol/L), or CSDX (1 μmol/L). Cells were harvested, and quantitative PCR (qPCR) analyses for indicated target genes were conducted. Data reflect averages and SE of at least 3 independent experiments, each conducted with technical triplicates. Results were normalized to 18S and control is set to “1.” Middle: LNCaP cells were cultured, treated, and harvested as in A, then lysed; and total protein was separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF), and immunoblotted for indicated proteins. Representative image of at least 3 independent experiments is shown. Right, same as left, except cell model is VCaP. B, LNCaP cells were cultured in media containing complete serum, treated for 24 hours with either ethanol control (0.01%), ABT888 (2.5 μmol/L), or a combination of ABT888 (1.75 μmol/L) and CSDX (0.5 μmol/L). Cells were harvested, and qPCR analyses for indicated target genes were conducted. Data reflect averages and SE of at least 3 independent experiments, each conducted with technical triplicates. Results were normalized to 18S and control is set to “1.” Statistical significance was determined using Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 3. A, LNCaP cells were steroid deprived for 72 hours and then pretreated for 30 minutes with either vehicle or ABT888 (2.5 μmol/L), stimulated with DHT (1 nmol/L, 1 hour) or ethanol control. Cells were then harvested, lysed, and differentially centrifuged as described in the Materials and Methods section of the Supplementary Data, resulting in a soluble fraction (glyceraldehyde-3-phosphate dehydrogenase serves as control) or a chromatin-tethered fraction (histone H4 serves as control). Immunoblots were conducted for the indicated proteins. A representative image of at least 3 independent experiments is shown. B–E, LNCaP cells were steroid deprived for 72 hours, pretreated for 30 minutes with vehicle or ABT888 (2.5 μmol/L), and then stimulated with DHT (10 nmol/L, 1 hour) or ethanol control. Cells were fixed and lysed, and ChIP for AR and PARP-1 (% input) was conducted. DNA was purified from immunoprecipitates (IP) and used in qPCR reactions for amplicons spanning 400 bp on either side of the transcriptional start site (TSS) of KLK3/PSA. Data are representative of at least 3 independent experiments, each conducted with technical triplicates, and depicted as the averages of MNase sensitive:protected DNA and SE. Statistical significance was determined using Student’s t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001. G, LNCaP treated as in A. Micrococcal nuclease (MNase) protection assays were conducted (as described in Methods) from which DNA was purified and used in qPCR reactions for amplicons spanning 400 bp on either side of the transcriptional start site (TSS) of KLK3/PSA. Data are representative of at least 3 independent experiments conducted in technical triplicate, depicted as the averages of MNase sensitive unprotected DNA and SD. Gray box indicates amplicons that are statistically significant between DHT-treated samples and ABT-pretreated-DHT-treated samples by Student t test.
Regulates Castration-Resistant AR Activity

PARP-1 Activity Is Increased in CRPC and Regulates Castration-Resistant AR Activity

The lethal stage of prostate cancer, CRPC, arises because of resurgent AR activity despite maintenance of AR-directed therapeutics (26). Remarkably, analyses of PARP-1 activity in cell-line models of CRPC revealed that PARP-1 is highly PARylated in CRPC cells, as compared with HT-sensitive prostate cancer (Fig. 4A; compare lanes 1–3 with lanes 4 and 5). Noting that autodigestion accounts for much of PARP-1 activity (40), these findings suggested that PARP-1 function may be enhanced in CRPC. This postulate was confirmed by ELISA, which showed that global PAR levels are significantly elevated in CRPC cells (Fig. 4B) compared with HT-sensitive LNCaP cells. Given the ability of AR to remain active after androgen deprivation in CRPC cells, the impact of enhanced PARP-1 activity on AR function was determined under conditions of hormone depletion. Whereas CSDX was incompletely effective at suppressing AR activity in CRPC cells (Supplementary Fig. S6), suppression of PARP activity attenuated AR-target gene expression (Fig. 4C, top). Importantly, PARP inhibition suppressed expression of UBE2C, a key regulator of cell-cycle progression in CRPC cells (38). Consistent with observations in HT-sensitive cells, no changes in AR protein were apparent (Fig. 4C, bottom). Although the present findings identify PARP-1 activity as a major effector of AR–chromatin occupancy, AR function, and AR-dependent cell growth in prostate cancer, PARP inhibitors show modest impact on other PARP family members, especially PARP-2 (27). Therefore, additional means were used to address the specificity of PARP-1 for modulation of AR function. Validated short hairpin RNAs (shRNA) directed against PARP-1 (or control; ref. 30) were introduced into CRPC cells, and AR-target gene expression was assessed. Moderate PARP-1 knockdown (Supplementary Fig. S7A, middle top) resulted in reduced PARP-1 activity, as determined by immunoblotting for total PAR (right); in addition, decreased AR-target gene expression was observed in CRPC cells deprived of androgen (left) without altering AR protein levels (middle bottom). Moreover, combining AR knockdown with pharmacologic PARP inhibition did not result in a cooperative effect with respect to inhibition of cell growth (Supplementary Fig. S7B), suggesting that the growth inhibitory effects of PARP inhibition are likely attributed to suppressed AR function in CRPC cells. Moreover, ChIP analyses showed that AR is recruited to loci regulating KLK3/PSA and TMPRSS2 expression in the absence of hormone and that PARP-1 cooccupied these regions (Fig. 4D). Within each region, suppression of PARP activity resulted in depletion of both AR and PARP-1 on chromatin. In combination, these observations show that PARP-1 activity is upregulated in models of CRPC and indicate that CRPC-associated, ligand-independent AR function and chromatin occupancy are dependent on PARP-1 enzymatic activity.

PARP-1 Is Required for AR Function in Genetically Defined Systems and in Vivo Models

To assess PARP-1–regulated AR function in defined genetic systems, mouse embryonic fibroblasts (MEFs) isolated from wild-type and Parp−/− (41) mice were transfected with plasmids encoding β-galactosidase (to normalize for transfection efficiency), AR, and the ARR2-LUC reporter (for determination of AR activity). Both basal and ligand-induced AR reporter activity was reduced in Parp−/− MEFs when compared with wild-type MEFs (Fig. 5A, left). To ensure specificity, a reporter specific for E2F1 function was also assessed. There was no discernible difference of reporter activity in wild-type versus Parp−/− MEFs transfected with a 3xE2F reporter construct (Supplementary Fig. S8). Parallel studies were conducted wherein Parp−/− MEFs were transfected with plasmid encoding either a wild-type or a catalytically inactive allele of PARP-1 (E988A; ref. 42). Expression of wild-type PARP-1 in Parp−/− MEFs restored AR activity, whereas the catalytic mutant failed to support AR function (Fig. 5A, middle). Expression of transfected PARP-1 alleles was...
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**A**

![Image](image1)

**B**

![Image](image2)

**C**

![Image](image3)

**D**

![Image](image4)

**Figure 4.** A, indicated model systems were cultured in media containing complete serum, harvested, and lysed; total protein was separated by SDS-PAGE, transferred to PVDF, and immunoblotted for indicated proteins. Representative image of at least 3 independent experiments is shown. B, Poly(ADP-ribose) (PAR) ELISA was conducted on whole-cell lysates of indicated model systems. Data reflect averages and SD of at least 3 independent experiments, each conducted with technical and biologic triplicates. C, top, indicated model systems were steroid deprived for 72 hours, then treated for 30 minutes with Poly(ADP-ribose) vehicle or ABT888 (2.5 μmol/L). Cells were harvested and qPCR analyses were conducted for indicated target genes. Data reflect averages and SE of at least 3 independent experiments, each conducted with biologic triplicates. Results were normalized to 18S and control is set to “1.” Bottom, indicated model systems were treated as above, then harvested, and lysed; total protein was separated by SDS-PAGE, transferred to PVDF, and immunoblotted for indicated proteins. Representative image of at least 3 independent experiments is shown. D, C4-2 cells were steroid deprived for 72 hours, then treated for 30 minutes with vehicle or ABT888 (2.5 μmol/L). Cells were fixed and lysed, and ChIP for AR and PARP-1 was conducted. DNA was isolated from IPs and used in qPCR reactions for indicated genomic loci. Data are representative of at least 3 independent experiments, each conducted with technical triplicates, and depicted as the average of immunoprecipitated signal to input signal and SE. Statistical significance was determined using Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
confirmed by immunoblot (Fig. 5A, right). Together, these data show that PARP-1 promotes basal and ligand-induced AR activity and further establish the requirement of PARP-1 catalytic activity for AR function. To determine whether the observed regulation of AR by PARP-1 can be targeted in vivo by another PARP inhibitor (olaparib), short-term (4 hours) treatments were initially conducted using human tumor xenografts. Upon tumor development, mice were treated with olaparib, CSDX, or control for 4 hours before sacrifice, and gene expression was analyzed (Fig. 5B, top). Tumors exposed to PARP inhibition showed decreased expression of KLK3/PSA, TMPRSS2, and FKBP5, as compared with control, whereas responses to CSDX were varied and target gene dependent (Fig. 5B, bottom), findings consistent with the observations in Supplementary Fig. S3, in which there was no additive effect in vitro when PARP inhibition was combined with CSDX. To further these observations, parallel xenograft studies were conducted wherein host animals were treated with ABT888 instead of olaparib. PARP inhibition resulted in significant decreases in AR target-gene expression (Fig. 5C) and, importantly, strongly suppressed prostate cancer tumor growth in vivo (Fig. 5D; tumor volumes depicted in Supplementary Fig. S9). PARP inhibition, compared with control, significantly suppressed tumor doubling to a degree strikingly similar to castration alone. When castration and PARP-1 inhibition were combined, the duration of tumor doubling significantly lengthened, compared with castration alone (50%). This observation is significant, as these findings indicate that PARP-1 inhibition not only cooperates with castration to limit tumor doubling in vivo but also suppresses the transition to CRPC. In addition, in castrated animals treated with the PARP inhibitor, 20% of the tumors never achieved a full doubling through the duration of the study (60 days). To examine the in vivo effect of PARP inhibition on CRPC cell tumor growth, C4-2 xenografts were established. Castration plus PARP inhibition, compared with castration alone, significantly decreased tumor volume (Fig. 5E, top), and this decreased tumor volume...
was associated with attenuated AR target-gene expression (Fig. 5E, bottom). Together, these data show that suppressing or silencing PARP-1 results in decreased AR-dependent target-gene expression, AR function, and ability of AR to promote tumor progression both in vitro and in vivo. These observations predict that PARP-1 could serve as a viable therapeutic target in AR-positive prostate cancer, as PARP inhibitors not only suppressed tumor growth in cooperation with castration but also delayed the onset of castration resistance.

PARP-1 Promotes Tumor Cell Proliferation in Primary Human Disease

As the above studies identified PARP-1 as a potent mediator of AR activity and prostate cancer growth and progression, the impact of PARP inhibition on primary human tumor growth was assessed using a novel ex vivo culture system. Tumor tissue was obtained immediately upon resection at radical prostatectomy and placed into the explant system as described (43, 44). Explant specimens retain all salient features of the original tumor, including AR expression, tumor and normal tissue histoarchitecture, stromal/tumor cell interaction, and proliferative rate, and can be used to monitor response to treatment(s). As depicted in Fig. 6A, ex vivo culture of primary tumor tissue obtained at radical prostatectomy was subdivided, cultured on a matrix, and subjected to targeted therapy. To assess efficacy of suppressing PARP activity, immunohistochemistry for total PAR was conducted after ABT888 treatment. Global PAR levels in ABT888-treated tumor cells were significantly decreased, compared with control (Fig. 6A, bottom). Although no observable alteration in histoarchitecture was observed after ABT888 exposure, the Ki67 index of the tumor-cell population was markedly decreased (Fig. 6B and 6C). This observed decrease in proliferative index was associated with diminished AR target-gene expression (Fig. 6D). In combination, these data show that PARP-1 can be targeted in human prostate cancer and that suppressing PARP activity results in decreased primary tumor cell proliferation.
Figure 6. A, top: schematic of explant assay is depicted as described in Methods. Bottom, representative images of explant tissues that were treated with either control or 2.5 μmol/L ABT888, then were formalin fixed and paraffin embedded. Tissue from these paraffin blocks was cut with a microtome and placed on microscope slides. Slides were then used to determine relative levels of PAR in these explant tissues by immunohistochemistry using standard techniques. B, same as in A, but tissue slides were either stained with hematoxylin & eosin (top) or stained for Ki67 via immunohistochemistry using standard techniques. C, quantification of Ki-67 (as a percentage of tumor cells that are nuclear positive) as depicted in B from 3 explants harvested at indicated time points treated with either control or 2.5 μmol/L ABT888. Data are presented as mean ± SD of 3 explant assays from 3 distinct prostatectomy specimens. D, explants were conducted as in A, tissue was homogenized in TRizol, cDNA was generated, and qPCR for the indicated mRNA was conducted. Data are presented as mean ± SD of at least 3 distinct explant tissue per treatment group. Statistical significance was determined using Student’s t test. ***, P < 0.001.
DISCUSSION

Although the role of PARP-1 in the DNA damage response is well understood, the impact of PARP-1–mediated transcriptional effects on tumor cell behavior remains poorly elucidated. The present study illuminates the dual roles of PARP-1 in supporting AR activity and resistance to genotoxic insult in prostate cancer. Key findings show that (i) PARP-1 inhibition sensitizes human prostate cancer cells to genotoxic insult, independently yields cytostatic effects in AR-positive cells, and cooperates with androgen depletion to suppress cell proliferation; (ii) PARP-1 is recruited to sites of AR action and PARP-1 enzymatic activity is critical for AR occupancy on chromatin and for AR activity in both HT-sensitive and CRPC cancer cells; (iii) PARP-1 activity is enhanced and required in CRPC, wherein AR activity is sustained in castrate conditions; (iv) ablation of PARP-1 activity in vivo is sufficient to suppress AR function, decrease tumor growth and delay the onset to castration resistance, and is required to maintain castrate resistance, and (v) PARP-1 inhibition suppresses proliferation of primary human tumor cells. Together, these findings identify the dual functions of PARP-1 as critical in the DNA damage response, as well as supporting AR signaling, protumorigenic phenotype, and tumor progression.

The observed recruitment of PARP-1 to sites of AR function as well as the ability of PARP-1 inhibitors to reduce AR occupancy on chromatin is consistent with the observation that PARP-1 can interact with histones and alter chromatin accessibility (11, 19, 45–50). It is enticing to speculate that upregulation of PARP-1 activity in CRPC cells may assist in the development of inappropriate AR activity and concomitant transition to CRPC; this postulate is strongly supported by data herein, which show that PARP inhibitors are highly effective in CRPC models. Recent findings in both prostate cancer and Ewing sarcoma indicate that similar effects are observed upon activation of ERG (24, 25). This function of the PARP-1/ERG interaction is both a transcriptional regulatory and a DNA damage effect, similar to the functions of PARP-1 described herein. These observations provide the first evidence that PARP-1 function is a requisite for AR-chromatin association, AR activity, and AR-dependent cellular proliferation, and unveil a critical new understanding of AR regulation in prostate cancer cells.

Chemo- and radiosensitization by PARP inhibition suggests that PARP-1 may be important for promoting resistance to these modalities. This idea is of potentially significant clinical relevance, as prostate cancer is largely resistant to genotoxic chemotherapies, and as of yet no means exist to sensitize prostate cancer to the combination of irradiation and AR-directed therapy, which is the standard of care for management of locally advanced disease (26, 51). That PARP-1 inhibition can sensitize prostate cancer to DCTX could be of clinical significance, as DCTX is currently used after the disease has transitioned to the CRPC stage, in which treatment options are limited. As it has been shown that DCTX affords only a modest (~2–3 month) survival advantage (52, 53), using PARP-1 inhibitors as an adjuvant to DCTX may help increase the therapeutic window in advanced disease.

The ability of PARP-1 to promote aberrant ligand-independent AR activity was remarkable, as the vast majority of lethal, late-stage prostate cancers remain dependent on AR for growth and survival (26, 54). PARP-1 inhibitors proved effective at enhancing the effect of androgen depletion on cell survival, suggesting the provocative hypothesis that PARP-1 inhibitors could be assessed clinically as a means to sensitize prostate cancer to AR-directed therapeutics. The PARP-1 inhibitors used in this study are currently being evaluated in clinical trials for multiple tumor types, with favorable toxicity profiles (55–58), and show preclinical and clinical efficacy (14, 55, 59). The data herein bring forth important new understanding for PARP-1 inhibitors and show that the transcriptional regulatory functions of PARP-1 may significantly promote clinically important transcriptional events in prostate cancer. Currently, endeavors to design clinical trials combining ABT888 and AR-directed therapy (abiraterone; NCI 9012, also designated as NCT01576172), as well as ABT888 and IRR, are under way.

The cooperation between castration and PARP-1 inhibition in vivo holds significance. Mimicking human disease, xenografts used reactivated AR despite continued therapeutic targeting of AR. In vivo data show that combining PARP-1 inhibition and HT may result in slowing evolution of the tumor to the lethal CRPC stage and may therefore have an advantage over AR-directed strategies alone. The combination of castration and PARP-1 inhibition appears to target not only ligand-dependent AR activity but also aberrant, ligand-independent, CRPC-associated AR activity, as was determined in model systems of CRPC under castrate conditions. This finding is potentially of considerable clinical significance, in that the vast majority of mortality associated with prostate cancer occurs after the disease has undergone transition to castration resistance, as the data suggest that PARP-1 inhibition may provide benefit for tumors that have already undergone the transition to CRPC.

Together, the data support a new paradigm in regard to PARP-1 function in human malignancy, whereby the dual functions of PARP-1 in DNA damage repair and AR regulation can be leveraged to suppress pathways critical for prostate cancer cell survival and progression. Mechanistically, PARP-1 enzymatic activity was determined to be required for AR transcriptional function. This finding is important, given the well-understood role of AR at all stages of prostate cancer. On the basis of the data, PARP-1 inhibition may afford therapeutic benefit in several contexts: (i) as a means to radiosensitize in primary disease, as well as in salvage and palliative radiotherapy; (ii) as a single agent in primary disease; (iii) in combination with existing AR-directed therapeutics, potentially increasing the time to development of CRPC; and (iv) in combination with DCTX (or other DNA damage–inducing therapeutics) in late-stage, metastatic CRPC that is, by definition, refractory to other AR-directed strategies. These data provide evidence that PARP-1 is potentially a novel therapeutic target in prostate cancer, based on its role in DNA damage repair and its heretofore unknown role in regulating the function of AR.

METHODS

Cell Culture and Treatments

LNCaP, C4-2, and VCaP cells were maintained in improved minimum essential media (IMEM) supplemented with 5% FBS.
(heat-inactivated FBS). LNCaP cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with 10% FBS, PC3, DU145, Parp-1+/− MEFs, and Parp-1−/− MEFs were maintained in Dulbecco’s modified Eagle’s media supplemented with 10% FBS. The 22Rv1 cells were maintained in RPMI supplemented with 10% FBS. LNCAP-abl cells were maintained in phenol red-free media supplemented with 5% charcoal dextran-treated serum (CDT). PreEC cells were maintained in PreEBM Prostate Epithelial Cell Basal Medium, supplemented with PreEGM SingleQuots (Clonetics). All media were supplemented with 2 mmol/L of l-glutamine and 100 units/mL penicillin-streptomycin. ABT888 was obtained from Alexis Biochemicals and dissolved in H2O and used at indicated concentrations. For steroid-depleted conditions, cells were plated in appropriate phenol red-free media supplemented with 5% to 10% CDT. CSIDX and DHT were dissolved in ethanol. Cell lines were not cultured longer than 6 months after receipt from the original source or American Type Culture Collection.

**Cell Growth Assays**

Cells were seeded at equal densities, treated as indicated, and harvested at indicated time points. At the time of harvest, cell number was determined using trypsin blue exclusion and a hemocytometer.

**Gene Expression Analysis**

Cells were seeded at equal density in steroid-proficient (FBS) or -depleted (CDT-treated) FBS conditions as indicated and were treated as specified; RNA was isolated using TRIzol and cDNA generated using SuperScript III (Invitrogen). Quantitative PCR was conducted with primers described in Supplementary Table S1 and with an ABI StepOne machine and PowerSybr in accordance with the manufacturer’s specifications.

**ChIP and reChIP Analysis**

Cells were cultured in media containing CDT for 72 hours and treated as indicated. ChIP analyses and qPCR were conducted as previously described (60), using primers described in Supplementary Table S1. ReChIP was conducted as ChIP, except after the first IP, samples were eluted at 37°C for 30 minutes and the supernatant was collected, subjected to the second IP, and then processed as ChIP samples.

**MNase Protection Assay**

These assays were conducted as previously described (45). Briefly, cells were cross-linked with formaldehyde, nuclei were collected, and chromatin was isolated. Chromatin was divided into 2 aliquots, one receiving MNase treatment. Chromatin was incubated overnight at 65°C to reverse cross-links, then treated with proteinase K and RNase H. DNA was extracted and precipitated and used in qPCR reactions with primers described in Supplementary Table S1 and with an ABI StepOne machine and PowerSybr in accordance with the manufacturer’s specifications.

**Antibody Immunoprecipitation and Immunoblotting**

Protein isolation, immunoprecipitation, and immunoblotting were conducted as previously described (61), using antisera described in Supplementary Materials and Methods.

**PAR ELISA**

Total cell lysates were analyzed for PAR using the HT Colorimetric PARP/Apoptosis Assay (Trevigen) per the manufacturer’s instructions.

**Reporter Assays**

Parp-1+/− and Parp-1−/− MEFs were transfected in serum-free media, as described in Supplementary Materials and Methods, and treated with DHT or control for approximately 36 hours posttransfection. Relative luciferase activity was assessed using the Promega Luciferase Assay Kit and Galacto-Star reagent (Applied Biosystems) was used to detect β-galactosidase activity.

**Chromatin Tethering**

LNCaP cells were steroid deprived for 72 hours and treated as indicated. At harvest, plates were harvested and processed as previously described (62). Supernatant was removed and SDS sample buffer was added. The insoluble, chromatin-tethered fraction was resuspended in SDS sample buffer and sonicated briefly; then both the supernatant and insoluble fraction were denatured at 95°C for 5 minutes. After denaturation, samples were separated by SDS-PAGE, transferred to PVDF, and immunoblotted as usual.

**Expression Vectors and shRNA Retroviruses**

Retroviral shRNA constructs were generously provided by W. Lee Kraus (UT Southwestern, Dallas, Texas) and were previously described (30). Plasmid constructs used in reporter assays were described earlier (63). Wild-type and catalytic-deficient Parp-1 expression plasmid constructs were also previously described (46).

**Xenograft Analysis**

Four-week-old male BALB/c nu/nu mice were purchased from Charles River, Inc. VCaP (2 × 106 cells) were resuspended in 100 μL of saline with 50% Matrigel (BD Biosciences) and were implanted subcutaneously into the flank of the mice. All tumors were staged for 4 weeks before starting the drug treatment. For assessment of in vivo AR target-gene expression, tumors from mice were treated with a single dose of olaparib (100 mg/kg via intraperitoneal injection) or ABT888 (100 mg/kg via oral gavage) and harvested 4 or 72 hours after treatment, respectively. Total RNA was extracted by TRIzol. For long-term xenograft growth studies, treatment was initiated when tumors were approximately 150 mm3, with tumor volumes balanced between all treatment groups. Mice allocated to the castration group were surgically castrated on the first day of treatment initiation (day 0). Mice allocated to the ABT888 group were given 100 mg/kg of ABT888 via oral gavage twice per day (with treatments separated by 10–12 hours) for 5 consecutive days each week, starting on the first day of treatment initiation until tumor harvest (i.e., 0–4, 7–11, 14–18, and so on). Tumor volume (via caliper measurement) and mouse weight were assessed 3 times weekly. No mice lost more than 5% of their initial body weight. Mice were sacrificed once their tumors reached a size of approximately 800 mm3. All procedures involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan and conformed to their relevant regulatory standards. Changes in tumor volume within each treatment group were depicted using cumulative incidence plots per tumor volume doubling, with statistical differences among treatment groups assessed with the log-rank (Mantel–Cox) analysis.

**Human Prostate Tumor Ex Vivo Explant Cultures**

Human prostate ex vivo explant cultures were conducted as previously described (43). Briefly, fresh tissue was obtained from a pathologist immediately following radical prostatectomy. The deidentified specimen was processed under a laminar flow hood, using sterile technique, and transported to the lab in RPMI on ice. The Thomas Jefferson University Institutional Review Board has reviewed this procurement protocol and determined this research to be in compliance with federal regulations governing research on deidentified specimens and/or clinical data [45 CFR 46.102(d)]. The following procedures were conducted under sterile tissue-culture conditions. Veterinary dental sponges (Novaris Cat. #96002) were placed in...
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12-well plates and soaked in 500 μL media (IMEM supplemented with 5% heat-inactivated FBS, hydrocortisone, insulin from bovine pancreas, and 100 units/mL penicillin-streptomycin) and appropriate treatment (either vehicle control or 2.5 μmol/L ABT888) for 5 to 10 minutes at 37°C. Tissue was placed into the lid of a 10-cm plate and dissected into 1-mm³ pieces with a scalpel. Three pieces of tissue were placed on each sponge, using sterile tweezers or forceps. Plates were placed in an incubator at 37°C and 5% CO₂. Media were replaced every day with appropriate treatment. Tissue was harvested at indicated time points and fixed in 4% formalin. Formalin-fixed, paraffin-embedded blocks were cut with a microtome, and slides underwent standard hematoxylin and eosin staining, as well as standard Ki67 immunohistochemistry, and were scored by a clinical pathologist (Dr. P. McCue, Thomas Jefferson University, Philadelphia, PA).

Disclosure of Potential Conflicts of Interest

The University of Michigan has filed a patent linking ETS fusions in Ewing sarcoma and prostate cancer as markers of sensitivity to PARP inhibitors and DNA-PKcs inhibitors. A.M. Chinnaiany and J.C. Brenner are named as inventors. G.V. Raj has an honorarium from the speakers' bureau of Amgen and is a consultant/advisory board member of Bristol-Myers Squibb, Janssen, and C-Diagnostics. No potential conflicts of interest were disclosed by the other authors.

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