An Epigenetic Reprogramming Strategy to Resensitize Radioresistant Prostate Cancer Cells

Claudia Peitzsch1, Monica Cojoc1, Linda Hein1, Ina Kurth1, Katrin Mäbert1, Franziska Trautmann1, Barbara Klink2,3,4, Evelin Schröck2,3,4, Manfred P. Wirth5, Mechthild Krause1,3,4,6,7, Eduard A. Stakhovsky8, Gennady D. Telegeev9, Vladimir Novotny5, Marieta Toma10, Michael Muders10, Gustavo B. Baretton10, Fiona M. Frame11, Norman J. Maitland11, Michael Baumann1,3,4,6,7, and Anna Dubrovska1,3,4,7

Abstract

Radiotherapy is a mainstay of curative prostate cancer treatment, but risks of recurrence after treatment remain significant in locally advanced disease. Given that tumor relapse can be attributed to a population of cancer stem cells (CSC) that survives radiotherapy, analysis of this cell population might illuminate tactics to personalize treatment. However, this direction remains challenging given the plastic nature of prostate cancers following treatment. We show here that irradiating prostate cancer cells stimulates a durable upregulation of stem cell markers that epigenetically reprogram these cells. In both tumorigenic and radioresistant cell populations, a phenotypic switch occurred during a course of radiotherapy that was associated with stable genetic and epigenetic changes. Specifically, we found that irradiation triggered histone H3 methylation at the promoter of the CSC marker aldehyde dehydrogenase 1A1 (ALDH1A1), stimulating its gene transcription. Inhibiting this methylation event triggered apoptosis, promoting radiosensitization, and hindered tumorigenicity of radioresistant prostate cancer cells. Overall, our results suggest that epigenetic therapies may restore the cytotoxic effects of irradiation in radioresistant CSC populations.

Introduction

Prostate cancer is the most common cancer among men and the third leading cause of cancer-related deaths in men worldwide (1). A significant proportion of prostate cancer patients are diagnosed with potentially curable localized tumor that can be treated with surgery and radiotherapy alone or in combination with androgen ablation. Tumor radiosensitivity can be a limiting factor of the efficacy of radiotherapy for some patients with a high-risk prostate cancer (2, 3). Depending on the stage of disease, up to 45% of prostate cancers can relapse after radiotherapy (4–6). Tumor relapse after radiotherapy has been attributed to the population of cancer stem cells (CSC) or tumor-initiating cells (7–9). The CSC hypothesis argues that cancer cells are hierarchically organized according to their tumorigenic potential, and tumors are initiated and maintained by the populations of CSCs (10). Decades of radiobiologic research have demonstrated that the frequency of tumor cells with CSC characteristics and their intrinsic radiosensitivity varies between tumors (7, 11, 12). Radiobiologic studies provided evidence for the importance of these cells for local tumor control and suggested that efficient tumor treatment by irradiation might require eradication of the entire CSC population. The role of CSCs in tumor development and recurrence has recently motivated investigations of CSC-specific biomarkers for the analysis of CSC populations in tumor pretreatment biopsies for the prediction of clinical outcome and selection of the optimal treatment strategy (13, 14). It is also assumed that the combination of radiotherapy with agents that target or radiosensitize the CSC population might be beneficial for the treatment refinement (14). However, compelling evidence suggests a high diversity of CSCs and plasticity of their features imposed by tumor treatment, which could challenge the development of CSC-related predictive biomarkers and CSC-targeted therapy. Furthermore, CSCs, which are selected or induced following chemo- or radiotherapy, are very difficult to detect before treatment and might acquire treatment resistance, resulting in tumor relapse (15).
In this study, we show that irradiation induces genetic and epigenetic alterations that affect prostate cancer cell tumorigenicity and radioresistance. The epigenetic changes driven by irradiation are mediated by histone methylation, which induces the expression of ALDH1A1 gene regulating the maintenance of the prostate CSCs and their radioresistance. Targeting of the histone 3 methylation with DZNep, an inhibitor of lysine methyltransferase enhancer of zeste homologue 2 (EZH2), leads to the downregulation of ALDH1A1 expression and tumor cell radiosensitization.

Furthermore, irradiation causes long-term alterations in the expression of stem cell markers and induces tumor cell reprogramming. To our knowledge, this is the first study demonstrating that radioresistant cell populations within prostate cancer cells undergo a phenotypic switch during the course of irradiation. We found that in contrast to nonirradiated cells, ALDH1 activity is not indicative of a radioresistant cell subset in the cells surviving the course of fractionated irradiation. Moreover, our study demonstrates that radioresistant prostate cancer cells are more sensitive to the DZNep treatment, which induces apoptosis and abrogates tumorigenicity in combination with X-ray irradiation.

Our findings suggest that radioresistant properties of cancer cells are dynamic in nature and that a combination of radiotherapy with drugs that prevent tumor cell reprogramming may be beneficial for eradication of tumor-initiating and radioresistant cell populations. Future research must extend this work by using additional experiments on primary cultures and biopsies, and by combination of DZNep treatment with radiotherapy in xenograft mouse models of human prostate cancer.

Materials and Methods

Cell lines and culture condition

Prostate cancer cell lines DU145, PC3, and LNCaP were purchased from the ATCC and cultured according to the manufacturer's recommendations in a humidified 37°C incubator supplemented with 5% CO2. DU145 and PC3 cell lines were maintained in DMEM (Sigma-Aldrich) and LNCaP cells in RPMI1640 medium (Sigma-Aldrich) containing 10% FBS (PAA Laboratories) and 1 mmol/L-glutamine (Sigma-Aldrich). Radioresistant (RR) cell lines were established as described previously (16). The RR cell lines were kept in culture up to 6 months after the last irradiation and their radioresistance was confirmed by radiobiologic cell survival assays. The corresponding age-matched nonirradiated parental cells were used as controls for RR cell lines. Human papillomavirus 18 (HPV 18) immortalized, nontumorigenic RWPE1 cells were purchased from the ATCC and maintained in the complete keratinocyte growth medium, K-SFM (Life Technologies) supplemented with 50 µg/mL of bovine pituitary extract and 5 ng/mL of EGF. All cell lines were genotyped using microsatellite polymorphism analysis and tested for mycoplasma directly prior to experimentation.

Human prostate tumor primary cells were obtained from N.J. Maitland's laboratory at the YCR Cancer Research Unit, University of York. Primary cell culture 311/13 was established from 67-year-old prostate cancer patient with Gleason score 3+4=7, pT2c, preoperative PSA level 10.7 ng/mL. The tumor specimen PT1 was taken from a lymph node of a 69-year-old patient (pT3bN1L0V0R0G3a, Gleason score 4+3=7, preoperative PSA level 5.8 ng/mL) with newly diagnosed prostate cancer, who underwent primary surgery (bilateral lymphadenectomy and prostate vesciculectomy). The tumor specimen PT2 is a radical prostatectomy tissue taken from a 64-year-old patient (pT2c N0 M0, Gleason score 3+4=7, preoperative PSA level 19.8 ng/mL). The tumor specimen PT3 is a radical prostatectomy tissue taken from 48-year-old patient (pT3aN0L0V0P1, Gleason score 4+3=7, preoperative PSA level 10.7 ng/mL).

Human tumor tissue ex vivo culture

Tumor tissue samples were cut into small pieces of approximately 3 mm × 3 mm and transferred to 12-well ultra-low attachment plates (Corning) in MEM (Lonza) supplemented with 4 µg/mL insulin (Sigma-Aldrich), B27 (Invitrogen), 20 ng/mL EGF (Peprotech), 20 ng/mL basic fibroblast growth factor (FGF, Peprotech). Penicillin-streptomycin solution was added to 100 µL of cell culture media for a final concentration of 50 to 100 IU/mL penicillin and 50 to 100 (µg/mL) streptomycin. The tissues were treated with 2 µmol/L of DZNep for 24 hours, consequently irradiated with 4 Gy of X-ray and fixed 24 hours after irradiation. Tissue fragments incubated under the same conditions but treated with DMSO and sham irradiation were used a control. The presence of tumor cells in the tissue pieces was analyzed by hematoxylin and eosin staining, by fluorescent immunostaining with PSMA and CKS/14 antibody, and was confirmed by a pathologist.

Animals and in vivo tumorigenicity assay

The animal facilities and the experiments were approved according to the institutional guidelines and the German animal welfare regulations [protocol number 24(D)-9168.1TVV2014/30]. The experiments were performed using 8- to 12-week-old male NMRI (nu/nu) mice that were bred in-house (Experimental Centre, Faculty of Medicine, Technische Universität Dresden). To immunosuppress the nude mice further, the mice underwent total body irradiation 1–3 days before tumor transplantation with 4 Gy...
(200 kV X-rays, 0.5 mm Cu filter, 1 Gy/minute). Mice were examined once a week and the relative tumor volumes (mm³) based on caliper measurements were calculated as length × width × height/2. At a tumor volume of 100 mm³, mice were scored as tumor bearing to calculate tumor uptake. Kaplan–Meier plots were analyzed using the log-rank test (GraphPad Prism 5). Analysis of the tumor-initiating cell numbers was performed using the web-based ELDA (Extreme Limiting Dilution Analysis) statistical software at http://bioinf.wehi.edu.au/software/elda/index.html, which uses the frequency of tumor positive and negative animals at each transplant dose to determine the number of tumor-initiating cells within the injected cell populations.

Microarrays analysis of the prostate cancer cell lines
Gene expression profiling of the DU145, DU145-RR, ALDH⁺ DU145, ALDH⁺ DU145, ALDH⁻ DU145, ALDH⁺ DU145-RR, ALDH⁻ DU145-RR cells was performed using SurePrint G3 Human Gene Expression 8 × 60 K v2 Microarray Kit (Design ID 039494, Agilent Technologies) according to the manufacturer's recommendations. Total RNA was isolated from cell pellets using the RNeasy kit (Qiagen). Sample preparation for analysis was carried out according to the protocol detailed by Agilent Technologies (Santa Clara). Briefly, first and second cDNA strands were synthesized, double-stranded cDNA was in vitro transcribed using the Low Input Quick Amp Labeling Kit, and the resulting cRNA was purified and hybridized to oligonucleotide arrays representing about 60,000 features, including 27,958 Entrez Gene RNAs and 7,419 lincRNAs. Arrays were processed using standard Agilent protocols. Probe values from image files were obtained using Agilent Feature Extraction Software. The dataset was normalized using GeneSpring software, and the list of differentially regulated genes with fold change > 2 and P < 0.05 was further analyzed using the web-based Panther Pathway Analysis tool (http://www.pantherdb.org/tools/index.jsp).

Data deposition. All data are MIAME compliant and the raw data have been deposited in the Gene Expression Omnibus (GEO) database, accession no GSE53902 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE53902).

Immunofluorescence microscopy
Cells were plated onto Millicell EZ SLIDE 8-well glass slides (Millipore) in a regular culture medium containing 10% serum. After 18 hours, the cells were fixed for 30 minutes with 3.7% formaldehyde at room temperature and permeabilized with 0.125% Triton X-100 for 10 minutes followed by washing with PBS, and blocked by incubation with 10% BSA in PBS. The cells were then incubated for 12 hours at 4°C with primary antibody against phospho-histone H2AX (S139; Millipore), β-catenin (#D10A8, Cell Signaling Technology), ALDH1A1 (clone H-4, Santa Cruz Biotechnology), cleaved PARP (Asp214; #5625, Cell Signaling Technology), or ALDH1A3 (HIPA046271, Sigma-Aldrich) diluted in 3% BSA in PBS as 1/100 and washed ten times with PBS. Cells were then incubated for 0.5 hour with a secondary antibody conjugated with Alexa Fluor 488 or 555 (Invitrogen) diluted 1/500 in 3% BSA in PBS. After appropriate washes with PBS, cells were stained with DAPI and examined by fluorescent microscopy. For quantification, 200 to 1,000 cells per condition in at least three randomly selected fields were counted. Nuclear intensity of γH2AX and cleaved PARP signal was evaluated using ImageJ software.

Clonogenic cell survival assay
Cells were plated at a density of 500–2,000 cells/well depending on the cell line in 6-well plates and irradiated with doses of 2, 4, 6, and 8 Gy of 200 kV X-rays (Yxlon YTFU 320; dose rate 1.3 Gy/minute at 20 mA) filtered with 0.5 mm Cu. The absorbed dose was measured using a Duplex dosimeter (PTW). After 10 days, the colonies were fixed with 10% formaldehyde (VWR) and stained with 0.05% crystal violet (Sigma-Aldrich). Colonies containing >50 cells were counted using a stereo microscope (Zeiss). The plating efficiency (PE) was determined as the ratio between the generated colonies and the number of cells plated. The surviving fraction (SF) was calculated as the PE from the irradiated cells divided by the PE from the nonirradiated control. For three-dimensional (3D) colony formation analysis, a cell suspension (500 cells/ml) in 0.2% low-melting SeaPlaque GTG agarose (Cambrex Bio Science Rockland, Inc.) with MEM medium (Lonza) supplemented with 4 μg/ml insulin (Sigma-Aldrich), B27 (Invitrogen), 20 ng/ml EGF (Peprotech), and 20 ng/ml FGF (Peprotech) was overlaid into 24-well low attachment plates (Coming). All samples were plated in triplicates. The plates were incubated at 37°C, 5% CO₂ in a humidified incubator for 14 days.

Sphere formation assay
To evaluate the self-renewal potential, cells were grown as nonadherent multicellular cell aggregates (spheres). Cells were plated at a density of 1,000 cells/2 mL/well in 6-well ultra-low attachment plates (Coming) in MEM medium (Lonza) supplemented with 4 μg/ml insulin (Sigma-Aldrich), B27 (Invitrogen), 20 ng/ml EGF (Peprotech), and 20 ng/ml FGF (Peprotech). Media containing supplements were refreshed once a week and spheres with a size > 100 μm were assayed after 14 days using Axiovert 25 microscope (Zeiss) or were automatically scanned using the Celigo S Imaging Cell Cytometer (Brooks). Sphere number and size were calculated using ImageJ software.

Cell proliferation and cytotoxic activity
Cell proliferation and cytotoxicity was analyzed using the MTT assay (Sigma-Aldrich). A total of 3 × 10⁴ cells/well were seeded into 96-well plates and quantified after 1–4 days at 560 nm absorbance, with reference at a wavelength of 690 nm using a microplate reader (Tecan).

Transwell migration assay
Prostate cancer cells were starved for 24 hours under serum-free conditions. A total of 10⁴ cells were plated on top of the membrane with 8 μm pore size (Boyden chamber, BD Biosciences) in a 24-well plate. After 24 hours, membranes were fixed with 10% paraformaldehyde and stained with 0.04% crystal violet. Absolute migration was evaluated by counting migrating cells on the membrane using Axioview25 microscope (Zeiss).

Chemical inhibitors and Western blot analysis
XAV939, BIX01294, and DZNep inhibitors were purchased from Biomol GmbH. DMSO was used at concentration ≤ 1% v/v DMSO as a drug solvent, and corresponding concentrations of DMSO were used as controls in all the experiments, which included cell treatment. Cells were lysed in RIPA buffer (Santa Cruz Biotechnology) and protein concentrations were determined using the bicinchoninic acid assay according to manufacturer's recommendations (Pierce). All antibodies were used at concentrations recommended by the manufacturer followed by
the incubation with a 1:5,000 dilution of appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology). The signal was visualized using the enhanced chemiluminescence detection reagent (GE Healthcare). Semi-quantitative analysis of protein expression relative to the loading control β-actin or GAPDH was performed with ImageJ software.

Flow cytometry analysis

For single cell analysis and multi-color staining, cells were dissociated with Accutase (PAA) and stained at 4°C in PBS buffer with 1 mmol/L ethyleneediaminetetraacetic acid (EDTA) and 5% FBS according to the manufacturer’s protocol. Aldehyde dehydrogenase activity was analyzed using Aldeflour assay (Stem Cell Technologies) according to manufacturer’s protocol. Samples were analyzed with the BD LSRII flow cytometer (Beckton Dickinson). A minimum of 100,000 viable cell events were collected per sample. Data were analyzed using FlowJo software (version 7.6.2) and gates were set according to the individual isotype controls. To purify ALDH+h cell populations, cells were stained using the Aldeflour assay (Stem Cell Technologies). Cells incubated with the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) served as negative control. Dead cells were excluded by 1 μg/mL DAPI staining and doublets were excluded using the FSC-W&H and SSC-W&H function of the BD FACSDiva 6.0 software. ALDH+h and ALDH−h populations were sorted on a BD FACS Aria III (Beckton Dickinson) using 100 μm nozzle with an average purity of >90%.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed using the ChIP Assay Kit (17-295, Millipore) according to the manufacturer’s instructions. DNA shearing was performed using Covaris S2 ultrasonicator under the following conditions: intensity 4, duty cycle 10%, cycles per burst 200, treatment time 55 seconds. The following antibodies were used to immunoprecipitate DNA: anti-H3 (D2B12, Cell Signaling Technology), anti-H3K36me3 (D5A7, Cell Signaling Technology), and normal rabbit IgG (Cell Signaling Technology).

Luciferase reporter assay

ALDHH1A1-promoter luciferase reporter was purchased from Switchgear genomics. M50 Super 8x TOPFlash and M51 Super 8x FOPFlash (TOPFlash mutant) were a gift from Randall Moon (Addgene plasmid # 12456 and # 12457; Supplementary ref. 1). Transient transfection of plasmid DNA in DU145 and LNCaP cells was performed using Lipofectamine 2000 (Life Technologies GmbH) according to the manufacturer’s instructions. Luciferase activity was normalized to FOP-Flash reporter. Luciferase assay was conducted using LightSwitch Assay (Switchgear genomics) or Bright-Glo luciferase assay system (Promega).

Histology

Human tumor specimens were fixed by immersion in 4% paraformaldehyde, cryoprotected in 30% sucrose, and embedded in TissueTek O.C.T. compound (Sakura Finetek). The 10-μm thick tissue sections (Microm HM 560, Cryo-Star Cryostat) were collected on Superfrost plus slides (Thermo Fisher Scientific). Slides were blocked for 1 hour in PBS buffer containing 10% of horse serum and 0.4% TritonX100 and then incubated overnight at 4°C with the primary antibodies against EpCAM (sc-21792, Santa Cruz Biotechnology), γH2AX (05-656, Millipore), and cleaved PARP (Asp214; #5625, Cell Signaling Technology). Staining with antibody against CK5/14 (#C1857/C01, DCS Innovative Diagnostik-Systeme, clone XM26/SFI-3) was used to distinguish benign prostate glands (CK5/14 positive) from cancer glands (CK5/CK14 negative). Bound antibodies were detected with appropriate secondary antibodies conjugated with AlexaFluor 488 or 555 (Molecular Probes) at room temperature for 1 hour. Slides were analyzed using the Meta Confocal Microscope (LSM510, Zeiss). Images were analyzed using ImageJ software. For quantification, two independently treated tissue pieces validated for the presence of tumor cells were analyzed for each condition.

Determination of glutathione and reactive oxygen species levels

To analyze glutathione (GSH) level, cells were stained with 40 μmol/L monochlorobimane (mBCl; Life Technologies) for 30 min at 37°C, washed with PBS at the end of culture and analyzed by Celigo cytometer. To measure the level of reactive oxygen species (ROS), cells were incubated with 5 μmol/L 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (CMH2DCFDA, Invitrogen Molecular Probes) for 15 minutes at 37°C. Samples were analyzed with the BD LSRII flow cytometer (Beckton Dickinson). A minimum of 100,000 viable cell events were collected per sample. Data were analyzed using FlowJo software (version 7.6.2) and gates were set according to the individual controls.

Array comparative genome hybridization

Array comparative genome hybridization was performed on Agilent’s SurePrint G3 Human CGH + SNP Microarray Kit 2 × 400 K (Design ID 028081, Agilent) according to the manufacturer’s instructions with the exception that the labeling of reference and test DNA was reversed. Arrays were scanned using an Agilent microarray scanner. Agilent’s CytoGenomics Editions 2.7 and 2.9 were used for extraction and processing of Raw data (using the integrated Feature Extraction software) and to determine deleted and amplified regions based on the draft of the reference human genome (GRCh37/hg19) using theDefault Analysis Method - CGH v2. All results were additionally checked by eye and were evaluated by a board-certified medical geneticist (Barbara Klink). CNVs that were commonly found in the Database of Genomic Variants (http://dgv.tcag.ca/) and therefore can be considered as polymorphisms that are most likely germline were excluded from further analysis.

Statistical analysis

The results of colony formation assays, microscopy image analysis, gene reporter assay, ChIP assay, cell migration, flow cytometry analysis, cell proliferation assays, gene expression, and siRNA-mediated gene silencing were analyzed by paired t test. Tumor uptake rate was calculated using log-rank (Mantel–Cox) test. Multiple comparison analysis for the data depicted on Fig. 2D, Supplementary Fig. S9B and S9C was performed using one-way ANOVA analysis by GraphPad Prism software. The differences between cell survival curves depicted on Figs. 3A and 4C, and Supplementary Fig. S7C were analyzed using the statistical package for the social sciences (SPSS) v23 software as described by Franken and colleagues (18) by fitting the data into the linear-quadratic formula S(D)/S(0) = exp(aD + bD2) using stratified linear regression. A P value of <0.05 was regarded as statistically significant. IC50 values were calculated...
Results

Irradiation causes long-term alterations in the expression of stem cell markers

In our previous studies, we have shown that radioresistant sublines obtained by multiple irradiation of prostate cancer cell lines with 4 Gy of X-ray in vitro are characterized by an increased expression of stem cell markers and enhanced tumorigenicity in mice (16). Here, we analyzed the time-dependent effect of irradiation on the expression of different CSC markers and activation of prosurvival pathways in parental (P) and radioresistant (RR) DU145 and LNCaP cells (Supplementary Fig. S1A). Irradiation of DU145 and LNCaP cells with a single X-ray dose of 4 Gy led to long-term and time-dependent changes in the cell phenotype, such as enhanced ALDH activity, increased expression of stem cell markers including OCT4, NANOG, BMI1, ABCG2, activation of PI3K/AKT signaling pathway, and gain of epithelial–mesenchymal transition (EMT) signatures such as upregulation of the β-catenin and vimentin expression (Fig. 1A and B and Supplementary Fig. S1B and S1C). The expression level of these stem cell markers and EMT signatures continuously increased during 3 to 4 weeks after irradiation and was correlated with an increase in clonogenic potential of DU145 and DU145-RR cells (Fig. 1C).

Fractionated irradiation with more than 56 Gy (14 × 4 Gy) of X-rays resulted in the establishment of cell sublines with a stable radioresistant phenotype, which has upregulated CSC marker expression, increased migratory potential, and high tumorigenicity in vivo (Supplementary Fig. S1D; ref. 16). We and others have shown that prostate cancer cells with a high ALDH activity (ALDH+) have functional characteristics of tumor-initiating cells (16, 19). We found that irradiation might cause ALDH+ cell enrichment in both, ALDH+ and ALDH− cell populations isolated by fluorescence-activated cell sorting (FACS) from DU145 prostate cancer cells and cultured in serum-containing medium for two weeks (Fig. 1D). Notably, the ALDH+ cell population did not show an increased proliferation in response to irradiation, as compared with ALDH− cells (Supplementary Fig. S1E). These results suggest that irradiation can not only select but also induces a prostate tumor cell population with CSC features. Taken together, these findings have shown that irradiation might influence cell clonogenicity and radioresistance, and has a long-term effect on the expression of stem cells markers and cell phenotype.

Irradiation induces epigenetic and genetic alterations in prostate cancer cells

Our previous studies demonstrated that regrowth of DU145 xenograft tumors after chemotherapy can be attributed to certain genetic aberrations, which lead to an increase in tumor-initiating cells (20). Microsatellite polymorphism analysis revealed genetic alterations in DU145-RR cells as compared with the parental DU145 cells, suggesting that X-ray treatment might induce genomic instability in prostate cancer cells (Supplementary Fig. S2A).
To identify genomic aberrations in the irradiated prostate cancer cells more comprehensively, we analyzed DNA from DU145 and DU145-RR cells using comparative genome hybridization (CGH) microarray. CGH array revealed a high number of various copy number variations (CNV) in parental DU145 cells, including partial chromosome deletions and duplications (e.g., partial gains and losses on chromosome 4). There were also marked differences between the parental and radioresistant cells (Supplementary Fig. S2B and S2C). Noteworthy, comparative analysis of the gene expression profiling and CGH data revealed that 53.5% (711/1,328) differentially regulated genes (>2 fold, \( P < 0.05 \)) for the parental and radioresistant DU145 cells did not map within the deleted or amplified chromosomal regions. This suggests that expression of these genes is mediated by genetic alterations in other genes or by epigenetic mechanisms (Supplementary Table S1). Gene expression profiling of the parental and radioresistant prostate cancer cells and FACS-isolated ALDH\(^+\) and ALDH\(^-\) cell populations revealed changes in the expression level of a number of genes involved in the epigenetic regulation of protein expression through DNA and histone modifications as well as methyltransferases involved in the various metabolic processes. 

Figure 2.
Epigenetic alterations in the irradiated prostate cancer cells. A, gene expression profiling of the DU145 parental and DU145-RR cells and FACS-isolated ALDH\(^+\) and ALDH\(^-\) cell populations revealed a number of genes involved in the epigenetic regulation of protein expression through DNA and histone modifications as well as methyltransferases involved in the various metabolic processes. B, GSEA enrichment analysis for the genes upregulated in DU145-RR as compared with DU145 cells. The "Hallmark Epithelial Mesenchymal Transition" gene set includes genes defining epithelial–mesenchymal transition, as in wound healing, fibrosis, and metastasis. The "Mikkelsen MEF LCP with H3K4me3" gene set includes genes with low-CpG-density promoters (LCP) bearing a trimethylation mark at H3K4 (H3K4me5) in MEF cells (embryonic fibroblasts). C, irradiation induces long-lasting changes in H3K4, H3K36, and H3K27 trimethylation. DU145 and LNCaP cells were irradiated with 4 Gy of X-ray, and the lysates of cells were prepared at the indicated time points during 6 weeks after irradiation and analyzed by Western blotting. NI, nonirradiated cells. D, analysis of H3K4 and H3K36 methylation in response to irradiation. Densitometric analysis of the protein immunoblots is shown in the accompanying graph. The level of H3K4 and H3K36 methylation was normalized to the total H3 level. Error bars, SEM; \( n = 3 \). P, parental cells; RR, radioresistant cells. E, the results of ChIP analysis showed that irradiation induces a H3K36me3 transcription activation mark on ALDH1A1 promoter within two-core β-catenin/TCF binding sites (i) and as described earlier (ii, ref. 16). Error bars, SEM; \( n = 3 \). *P < 0.05. F, the results of the luciferase reporter assay where luciferase gene expression is regulated by an endogenous ALDH1A1 gene promoter showed that ALDH1A1 transcription is significantly increased in DU145-RR and LNCaP-RR cells as compared with the parental cell lines. Error bars, SEM; \( n = 3 \). *P < 0.05; P, parental cells; RR, radioresistant cells.
We next investigated whether irradiation induces global chromatin modifications. Methylation of lysine residues K4 and K36 of histone H3 correlates with transcriptional activation, whereas trimethylation of histone H3 on lysine 27 enforces gene silencing (21). We found that irradiation induced long-term changes in H3K4, H3K36, and H3K27 tri-methylation in prostate cancer cells, which can be observed at least 6 weeks after treatment (Fig. 2C and Supplementary Fig. S3A). In accordance with this observation, Western blot analysis revealed long-term and time-dependent changes in the expression of the histone methyltransferases involved in H3 methylation on residues K27 (EZH2) or K36 (SETD2; Supplementary Fig. S3A and S3B). In addition, Ingenuity Pathway Analysis (IPA) revealed histone methyltransferase EZH2 as one of the most significantly upregulated transcriptional regulators in DU145-RR cells as compared with parental cells (P < 0.05) (Supplementary Fig. S3C). Interestingly, H3K4 and H3K36 tri-methylation increased during TGF-β-induced EMT and cell reprogramming (21) and was significantly upregulated in the highly migratory DU145-RR and LNCaP-RR cells as compared with their nonirradiated parental counterparts, as well as in parental DU145 cells after irradiation (Fig. 2D and Supplementary Fig. S3D). In contrast, radiosensitive cells showed no significant change in H3K4 and H3K36 methylation in response to irradiation (Fig. 2D). In line with this observation, we found that DU145-RR and LNCaP-RR cells display an increased expression of EMT and stem cell markers, which was altered after irradiation to a lesser extent than in parental cell lines (Fig. 1A and B and Supplementary Fig. S4A). Moreover, irradiation induced an ALDH positive cell population to a lesser degree in both DU145-RR ALDH+ and DU145-RR ALDH− cell populations, as compared with the ALDH+ and ALDH− cells isolated from the parental cells (Supplementary Fig. S4B).

To determine whether histone H3 methylation can regulate ALDH1A1 gene transcription after X-ray irradiation, a chromatin immunoprecipitation assay (ChIP) was performed. The cross-linked DNA–protein complexes from X-ray irradiated or nonirradiated parental and DU145-RR cells were immunoprecipitated with either anti-H3K36me3 or with anti-H3 and control IgG antibodies. PCR amplification was performed using primers flanking two regions of the ALDH1A1 promoter containing binding sites for β-catenin/TCF transcription factor and named ALDH1A1(i) and ALDH1A1(ii) with sizes of 508 bp and 518 bp, respectively (Fig. 2E; refs. 16, 22). The results of the ChIP analysis showed that the H3K36 methylation mark at ALDH1A1 promoter increased after irradiation with 4 Gy of X-ray (Fig. 2E and Supplementary Fig. 4C). To perform a direct analysis of the transcriptional regulation of ALDH1A1, we used a reporter system where luciferase gene expression is regulated by the endogenous ALDH1A1 gene promoter. The results of the luciferase reporter assay confirmed that ALDH1A1 transcription is highly activated in the RR prostate cancer cell lines and irradiation of parental cells led to a significant increase of ALDH1A1 transcription as well as to upregulation of β-catenin/TCF reporter gene expression (Fig. 2F and Supplementary Fig. S5A). These results suggest an epigenetic regulation of ALDH1A1 transcription by irradiation, which is mediated by histone H3 methylation and is consistent with flow cytometry data for the ALDH enzymatic activation in response to irradiation (Fig. 1B). Taken together, these findings suggest that genetic alterations and changes in the histone methylation that have occurred during treatment contribute to the emergence of cell radiosensitivity and stem cell reprogramming.

Characterization of the properties of ALDH+ and ALDH− cell populations in radioresistant prostate cancer cells

Our previous study demonstrated that ALDH activity is indicative of prostate tumor—initiating cells (23). We have recently reported that ALDH+ cell population among the nonirradiated prostate cancer cells has a lower level of DSBs and more efficient DNA repair after irradiation as compared with the ALDH− cell population (1/961, at 95% confidence interval: 1/2,061 to 1/448 cells vs. 1/4,637, at 95% confidence interval: 1/10,558 to 1/2,037 cells, respectively; P < 0.01; Supplementary Fig. S5F). Moreover, radiosensitive subsets isolated from the parental and DU145-RR cell lines showed an increase in sphere-forming potential over DU145-RR ALDH− cells (Supplementary Fig. S5E). To analyze the tumorigenic potential of DU145-RR ALDH+ and DU145-RR ALDH− populations, the FACS-purified cells were injected subcutaneously into athymic immunodeficient NMRI nu/nu mice at varying cell numbers (100, 1,000, and 3,000). The DU145-RR ALDH+ population showed a significantly higher frequency of tumor-initiating cells than the DU145-RR ALDH− cell population (1/361, at 95% confidence interval: 1/2,061 to 1/448 cells vs. 1/4,637, at 95% confidence interval: 1/10,558 to 1/2,037 cells, respectively; P < 0.01; Supplementary Fig. S5F). We demonstrated that ALDH activity and cell radioresistance not only correlate but are causally linked. Taking into account the genetic, phenotypical, and functional differences between the parental and radiosensitive prostate cancer cell lines, we hypothesized that irradiation can induce plasticity of the tumor stem cell phenotype. To test this assumption, we performed a comparative analysis of the spherogenic, tumorigenic, and radioresistance properties of ALDH+ and ALDH− cell subsets isolated from the parental and DU145-RR cell lines. Similarly to the DU145 ALDH+ cells, DU145-RR ALDH− cells showed an increase in sphere-forming potential over DU145-RR ALDH+ cells (Supplementary Fig. S5E). To analyze the tumorigenic potential of DU145-RR ALDH− and DU145-RR ALDH+ populations, the FACS-purified cells were observed in either the 2D or the 3D survival assays (Fig. 3A and Supplementary Fig. S5G).

The phosphorylation of histone H2AX at Ser 139 by ATM or ATR protein kinases on the sites of DNA damage is a marker of DNA double-strand break (DSB) response. Residual nuclear foci of the phosphorylated H2AX (γ-H2AX), measured 24 hours after irradiation, are associated with DNA lesions, which cannot be repaired and can lead to cell death (23, 24). We have recently reported that ALDH+ cell population among the nonirradiated prostate cancer cells has a lower level of DSBs and more efficient DNA repair after irradiation as compared with the ALDH− cells. We found that, in contrast to the parental DU145 cells, the number of residual γ-H2AX foci 24 hours after irradiation was not increased in DU145-RR ALDH+ cells compared with DU145-RR ALDH− cells (Supplementary Fig. S5H). Various ALDH isoforms contribute to the high ALDH activity in prostate cancer cells including ALDH1A1 and ALDH1A3, which show abundant expression in prostate cancer tissues (25). Both of these isoforms are expressed at high levels in the radioresistant prostate cancer cell lines, and in the ALDH+ cell populations (Supplementary Fig. S6A–S6C; ref. 16). Microscopy analysis of the residual γ-H2AX foci in the ALDH1A3-positive DU145 and DU145-RR cells confirmed that, in contrast to the parental cells, ALDH1A3 expression in DU145-RR cells is not indicative of more efficient DNA DSB repair (Fig. 3B). Taken together, these data suggest that, in contrast to the parental prostate cancer cells, ALDH activity is not a marker of more radioresistant cell population in cells with...
acquired radioresistance. These findings suggest that radioresistant prostate cancer cell populations can change their phenotype during the course of irradiation.

Comparative gene expression profiles of ALDH<sup>+</sup> and ALDH<sup>−</sup> cell populations in parental and radioresistant prostate cancer cells

To better understand the biological mechanisms that may underlie the lack of difference in radiosensitivity between ALDH<sup>+</sup> and ALDH<sup>−</sup> cell populations in cells with acquired radioresistance, we employed comparative gene expression profiling of ALDH<sup>+</sup> and ALDH<sup>−</sup> cells isolated from the parental DU145 cell line and from its radioresistant derivative DU145-RR cell line. Whereas the differences between DU145 ALDH<sup>+</sup> and DU145 ALDH<sup>−</sup> cell populations are largely based on the deregulation of the WNT and cadherin signaling pathway, the distinct characteristics of the DU145-RR ALDH<sup>+</sup> and DU145-RR ALDH<sup>−</sup> cells depend on the differential regulation of DNA replication, which might be reflective of a high rate of DU145-RR ALDH<sup>−</sup> cell proliferation (Fig. 3C and Supplementary Fig. S6D). Moreover, as indicated by the number of commonly regulated genes, the set of genes that is differentially expressed between DU145 ALDH<sup>+</sup> and DU145 ALDH<sup>−</sup> cells is more similar to the gene set that is differentially regulated between parental DU145 and DU145-RR cells, as compared with the genes that are differently expressed between DU145-RR ALDH<sup>+</sup> and DU145-RR ALDH<sup>−</sup> cells (Fig. 3C).

Analysis of the genes that are involved in the WNT signaling pathway and differentially regulated between DU145 ALDH<sup>+</sup> and ALDH<sup>−</sup> population and between DU145 and DU145-RR cells revealed that expression of these genes does not differ between DU145-RR ALDH<sup>+</sup> and DU145-RR ALDH<sup>−</sup> cells except WNT9A.

Figure 3.
Irradiation-induced plasticity of the mechanisms of radioresistance in prostate cancer cells. A, clonogenic cell survival assay showing that, in contrast to ALDH<sup>+</sup> and ALDH<sup>−</sup> cell populations isolated from parental DU145 cells, DU145-RR ALDH<sup>+</sup> and DU145-RR ALDH<sup>−</sup> cells do not differ in their radioresistance properties. Error bars, SEM; n ≥ 5. B, immunofluorescence analysis of the residual γ-H2AX foci in ALDH<sup>+</sup> and ALDH<sup>−</sup> cells 24 hours after irradiation with 4 Gy of X-ray. Error bars, SEM; n = 5; *P < 0.05. C, comparative gene expression profiling of ALDH<sup>+</sup> and ALDH<sup>−</sup> cell populations in the parental DU145 and DU145-RR cells. Venn diagrams show an overlap in the sets of genes differently regulated between DU145 and DU145-RR cells in comparison with the DU145 ALDH<sup>−</sup> and DU145 ALDH<sup>+</sup> cells or to DU145-RR ALDH<sup>−</sup> and DU145-RR ALDH<sup>+</sup> cells. PANTHER analysis was used to identify the pathways overrepresented among the genes that are differentially regulated in DU145 ALDH<sup>−</sup> and DU145 ALDH<sup>+</sup> cells and DU145-RR ALDH<sup>−</sup> and DU145-RR ALDH<sup>+</sup> cells. D, hierarchical clustering analysis of the WNT signaling pathway genes differentially regulated between DU145 and DU145-RR, DU145 ALDH<sup>−</sup> and DU145 ALDH<sup>+</sup>, as well as DU145-RR ALDH<sup>−</sup> and DU145-RR ALDH<sup>+</sup>. E, immunofluorescence analysis of the intracellular distribution of β-catenin in DU145 RR ALDH<sup>+</sup> and DU145 RR ALDH<sup>−</sup> cell subsets. The data for DU145 ALDH<sup>−</sup> and DU145 ALDH<sup>+</sup> are included for comparison (16). Error bars, SEM; n = 2. F, the inhibition of WNT signaling pathway with XAV939. n.s., inhibitor does not affect radiosensitivity of LNCaP-RR and DU145-RR cells. The data for LNCaP and DU145 cells are included for comparison (16). The cells were serum starved in DMEM with 3% FBS for 24 hours followed by treatment with XAV939 antagonist at concentration 1 μmol/L for 72 hours. Error bars, SEM; n ≥ 3; *, P < 0.01.
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Figure 4.
Inhibition of the histone methylation activity decreases prostate cancer cell viability and radioresistance. A, DZNep, a global histone methylation inhibitor, is more potent for the inhibition of viability of LNCaP-RR and DU145-RR cells as compared with their parental counterparts. IC_{50} values were determined after 24 hours (n > 3) and 72 hours (n ≥ 2) of treatment with the drugs. B, plating efficacy of the LNCaP, LNCaP-RR, DU145, and DU145-RR cells pretreated with DZNep or BIX-01294 at the different concentrations for 72 hours. Error bars, SEM; Ctrl., n = 2. P, 0.063, n = 7. C, cells were pretreated with DZNep or BIX-01294 at the different concentrations in DMEM with 3% FBS for 72 hours and their susceptibility to X-ray irradiation was evaluated by clonogenic cell survival assay. Error bars, SEM; Ctrl., n = 2, P < 0.0005. P, parental cells; RR, radioresistant cells.

(wingless-type MMTV integration site family, member 9A), which is significantly downregulated in DU145-RR ALDH^+ cells as compared with the DU145-RR ALDH^- cell population (Supplementary Tables S3–S5). Consistent with this observation, hierarchical clustering analysis of the WNT signaling pathway genes that were significantly up- or downregulated by ≥1.5 fold (P < 0.05) in DU145-RR as compared with DU145 cells and in ALDH^- versus ALDH^+ cells demonstrated that DU145-RR ALDH^- and DU145-RR ALDH^+ cell clusters together. In contrast, DU145 ALDH^- cells formed a cluster separated from DU145 ALDH^+ cells (Fig. 3D).

Activation of the canonical WNT signaling pathway leads to the stabilization and nuclear accumulation of β-catenin, which interacts with T-cell factor/lymphoid enhancer factor-1 (TCF/LEF) proteins to regulate gene transcription. We found that ALDH^+ cells have a higher level of nuclear β-catenin, which is consistent with an activation of the WNT signaling pathway in this cell population (16). In contrast to the parental DU145 cells, ALDH^- and ALDH^+ cell populations isolated from DU145-RR cells both showed a high nuclear accumulation of β-catenin and no differences in its intracellular distribution (Fig. 3E). WNT signaling, which is overrepresented in both radioresistant prostate cancer cells and ALDH^+ tumor-initiating cell population is a potent inducer of EMT and cell migration (26). We next analyzed the migratory properties of ALDH^- and ALDH^+ cell populations isolated from the parental and radioresistant DU145 and LNCaP cell lines. The results of the Boyden chamber based cell migration analysis demonstrated that in contrast to ALDH^- and ALDH^+ cell subsets isolated from the radioresistant cell lines, ALDH^- cells isolated from the parental cell lines have a higher migratory potential as compared with ALDH^+ cell population (Supplementary Fig. S6E). Inhibition of the WNT signaling by tankyrase inhibitor XAV939, which stimulates β-catenin degradation, led to the inhibition of cell clonogenicity and increases in cell radiosensitivity, with a more pronounced effect for the parental cell lines LNCaP and DU145, compared with their radioresistant counterparts (Fig. 3F and Supplementary Fig. S6F). Recent studies have demonstrated that overexpression of LEF1 might lead to cell resistance to tankyrase inhibition (27). Noteworthy, DU145-RR cells express a significantly higher level of LEF1 as compared with the parental DU145 cells, which contribute to XAV939 resistance (Supplementary Fig. S6G). Therefore changes in the phenotype of the radioresistant cell population during treatment are associated...
with deregulation of multiple intracellular signaling pathways, which can be attributed to the epigenetic and genetic alterations acquired by cells after irradiation.

Alteration of histone methylation affects cell radioresistance and tumorigenicity

Next, we determined whether epigenetic regulation of gene expression controlled by histone lysine methylation plays a role in the regulation of prostate cancer cell radioresistance. Our current studies suggest that irradiation induces activation of a number of proteins involved in the regulation of epigenetic modification of DNA and histones. As shown above, IPA-based interrogation of the gene expression data revealed histone H3 lysine methyltransferase EZH2 as one of the most significantly upregulated transcriptional regulators in DU145-RR cells, compared with their parental counterparts (Supplementary Fig. S3C). To determine whether modulation of histone H3 methylation contributes to prostate cancer cell radioresistance, we examined the effect of inhibitors of lysine methyltransferases EZH2 and G9a, DZNep, and BIX01294, respectively, on the regulation of the viability and radiosensitivity of the parental and radioresistant DU145 cells. Noteworthy, DZNep is a broad-spectrum methyltransferase inhibitor that affects both inhibitory and activating histone methylation marks, thus allowing a more comprehensive epigenetic resetting. Treatment with DZNep was significantly more potent for the inhibition of the viability of radioresistant LNCaP as compared with parental LNCaP cells and radioresistant DU145 cells as compared with parental DU145 cells (Fig. 4A). In contrast, BIX01294 showed no significantly different effect in the radioresistant and parental LNCaP and DU145 cells (Supplementary Fig. S7A). Moreover, treatment of prostate cancer cells with DZNep decreased clonogenicity and radiosensitivity of the parental and radioresistant LNCaP and DU145 cells, with more pronounced effects on the radioresistant cell lines (Fig. 4B and C and Supplementary Fig. S7B). In contrast to DZNep, BIX01294 had no significant inhibitory effects on the cell clonogenicity and radioresistance of the radioresistant and parental cell lines at the same range of concentrations (Fig. 4B and C and Supplementary Fig. S7C). Analysis of the residual γ-H2AX foci, 24 hours after irradiation, showed that pretreatment of cells with DZNep significantly increased the number of unrepaired DSBs compared with the untreated cells. This effect was greater in the radioresistant cell lines than in the parental cells. Noteworthy, treatment of the cells with DZNep alone at a concentration of 1 μmol/L for 72 hours also led to significant accumulation of DNA DSBs as indicated by γ-H2AX analysis (Fig. 5A).

To understand the molecular mechanism of the more pronounced inhibitory effect of DZNep treatment on DU145-RR cells in comparison with their parental counterparts, DZNep was applied alone or in combination with X-irradiation, and the cells were analyzed by Western blotting for the activation of the apoptotic markers. Interestingly, DU145-RR cells had a higher basal expression level of PARP, which is involved in DNA repair, genomic instability, and transcription regulation, including prevention of the H3K4me3 demethylation (28). Recent evidence points to the potential role of PARP in prostate cancer progression and therapy resistance, inferring that targeting of PARP could serve as important therapeutic modality in hormone-refractory prostate cancer (28). In contrast to the parental DU145 cells, treatment of DU145-RR cells with DZNep induced caspase cascade and cleavage of PARP at Asp214, which mediates the apoptotic program (Supplementary Fig. S8A and S8B). Induction of the PARP cleavage in the DU145-RR and LNCaP-RR cells after treatment with DZNep was confirmed by fluorescent microscopy (Supplementary Fig. S8C).

An accumulation of DNA DSBs and the sensitivity of the cells to apoptosis can be attributed to disruption of intracellular redox balance. To determine whether treatment with DZNep does disrupt redox balance in prostate cancer cells, GSH and ROS levels were assessed by imaging cytommetry or flow cytometry after incubation with mBCI or 2′,7′-dichlorofluorescein diacetate, respectively. Treatment with DZNep resulted in a decrease in GSH levels, and upregulation of ROS (Fig. 5B and C). The GSH level in DU145-RR cells was significantly more affected by the DZNep treatment as compared with the parental DU145 cells (Fig. 5C). These results suggest that protective, ROS-scavenging mechanisms of the radioresistant cells might rely on epigenetic regulation of genes involved in DNA repair and apoptosis.

Next we analyzed whether DZNep treatment can induce DNA DSBs and apoptotic signaling in prostate tumor specimens and primary prostate tumor cells. A freshly isolated lymph node biopsy of human hormone refractory prostate cancer (PT1) and a radical prostatectomy specimen (PT2) were dissected into pieces of approximately 3 × 3 mm, treated with 2 μmol/L of DZNep for 24 hours, then irradiated with 4 Gy of X-ray and fixed 24 hours after irradiation. Tissue culture was performed in serum-free, sphere-forming cell medium. The presence of tumor cells in the tissue pieces was confirmed by staining with hematoxylin and eosin and by fluorescent immunostaining with anti-prostate-specific membrane antigen (PSMA) and CK5/14 antibody (Fig. 6A and Supplementary Fig. S9A). Microscopy analysis of the nuclear intensity of γ-H2AX and cleaved PARP 24 hours after irradiation confirmed that inhibition of H3 methylation by DZNep led to an increase in DNA DSBs and induced activation of tumor cell apoptosis after ex vivo irradiation (Fig. 6A). Primary cell cultures 312/13 and 311/13 were established from targeted needle biopsies of Gleason grade 7 tumors from the prostate of two patients who underwent radical prostatectomy at Castle Hill Hospital, Cottingham, United Kingdom (kindly provided by Prof. Norman Maitland, University of York). For these experimental models, only combination of DZNep treatment with 4 Gy irradiation led to significant increase in the nuclear γ-H2AX intensity 24 hours after irradiation compared with untreated control cells, whereas no significant difference was seen between control cells and irradiated cells without DZNep treatment (Supplementary Fig. S9B).

In comparison, treatment of the immortalized, nontransformed human cell line RWPE1 with DZNep did not lead to a significant increase in residual DNA damage as compared with the treatment by irradiation alone, suggesting that radiosensitizing effect of DZNep can be more pronounced in malignant cells as compared with normal cells (Supplementary Fig. S9C).

Next we addressed the question of whether DZNep inhibition directly affects expression of stem cell markers such as ALDH1A1. Western blot analysis revealed that treatment of prostate cancer cells with DZNep led to an inhibition of EZH2 and ALDH1A1 and to a decrease in tri-methylation of lysine residue K36 in histone H3 (Fig. 6B and Supplementary Fig. S9D). Inhibition of ALDH1A1 expression was also observed in DU145 cells pretreated with DZNep and subsequently irradiated with 4 Gy of X-ray. These findings suggest that this inhibitor can be used to prevent tumor
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Figure 5. Mechanisms of DZNep-mediated prostate cancer cell radiosensitization. A, analysis of γH2A.X foci in cells treated with DZNep at a concentration of 1 μmol/L for 72 hours alone or in combination with 4 Gy of X-ray irradiation 48 hours after start of DZNep treatment. Residual γH2A.X foci were analyzed 24 hours after irradiation. Error bars, SEM; n = 3; *, P < 0.05; **, P < 0.01. B, analysis of ROS level after treatment of cells with DZNep at a concentration of 1 μmol/L for 72 hours. Cells treated with DMSO were used as control. Error bars, SEM; n = 3; *, P < 0.05. C, measurement of the GSH levels in the cells pretreated with DMSO or DZNep at a concentration of 1 μmol/L for 72 hours by incubation with monochlorobimane (mBCl) and Celigo cytometry. Error bars, SEM; n = 8; *, P < 0.05. P, parental cells; RR, radioresistant cells.

In accordance with the results of gene expression analysis and our previous findings (16), siRNA-mediated inhibition of β-catenin expression decreases expression of EZH2 and ALDH1A1 in prostate cancer cells, and DZNep treatment has an additive effect on β-catenin knockdown with regard to EZH2 and ALDH1A1 expression (Supplementary Fig. S10A). These results suggest a link between the activation of WNT/β-catenin signaling pathway after cell irradiation and irradiation-induced epigenetic changes. Strikingly, ALDH1A1 and EZH2 genes are frequently co-expressed with CTNNB1 (β-catenin) in prostate tumor specimens (Supplementary Fig. S10B). Moreover, a gene signature, which includes expression of ALDH1A1 and EZH2, correlated with reduced disease-free survival in patients with prostate cancer, as suggested by the analysis of the provisional The Cancer Genome Atlas dataset extracted from cBioPortal for Cancer Genomics (Supplementary Fig. S10C).

To analyze whether inhibition of the H3 methylation by DZNep can affect cell tumorigenicity after irradiation, we compared the relative in vivo growth of DU145 and DU145-RR cells after in vitro pretreatment with DZNep alone or in combination with irradiation. The cells were treated with 1 μmol/L of DZNep or with DMSO as a control for 72 hours, irradiated with 6 Gy of X-ray or left nonirradiated, embedded in Matrigel, and injected subcutaneously into NMRI nu/nu mice. Animals alive and tumor-free at the end of the observation period (77 days) were defined as long-term survivors. In contrast to the parental cells, DU145-RR cells pretreated with DZNep had a significantly lower relative tumor growth rate and tumor take than control cells, and the inhibitory effect of the DZNep treatment on DU145-RR cell tumorigenicity was significantly increased after irradiation, leading to complete inhibition of DU145-RR cell tumorigenicity (Fig. 6C and D).

Discussion

Radiotherapy is one of the most effective treatments for cancer and is a potentially curative treatment option for patients with clinically localized prostate carcinoma (29). Recent discoveries have provided compelling evidence that CSC population within each individual tumor is a key contributor of radiotherapy failure, regardless of whether this population is transient or stable (30). Therefore, tumors can only be cured if all CSCs are targeted, or...
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Regulation of histone methylation impacts prostate cancer cell tumorigenicity. A, analysis of apoptosis induction and γH2AX increase in the specimens of node metastasis of prostate tumor (PT1) and radical prostatectomy (PT2 and PT3), which were treated with 4 Gy of X-ray alone or in combination with 2 μmol/L of DZNep for 48 hours. The tissues were fixed 24 hours after irradiation. Error bars, SEM. H&E, hematoxylin and eosin. B, Western blot analysis of the parental and radioresistant LNCaP and DU145 cell lines treated with DZNep or BIX-01294 at a concentration of 1 μmol/L for 72 hours. Densitometric analysis of the protein immunoblots is shown in the accompanying graph. Error bars, SEM. C, tumor uptake rates in mice injected with 5 × 10⁶ DU145 or DU145-RR cells. The cells were pretreated with DZNep before injection at a concentration 1 μmol/L for 72 hours alone or in combination with 6 Gy of X-ray irradiation given 48 hours after the start of DZNep treatment. Cells treated with DMSO and sham-irradiation were used as a control; the P value was calculated using log-rank (Mantel–Cox) test. D, relative tumor growth of DU145 and DU145-RR cells treated as indicated. Error bars, SEM, *P < 0.05, P, parental cells; RR, radioresistant cells.

Figure 6.

when the host is able to inactivate those CSC surviving anticancer treatment (7). However, development of therapeutic agents eradicating CSCs remains challenging due to the high diversity and plasticity of CSC phenotypes. Recent advances in DNA sequencing allowing global analysis of genetic landscape in individual tumor cells revealed a high heterogeneity of different subclones within the same tumor specimen and has provided an insight into the subclonal evolution, suggesting that CSCs and clonal evolution models are not mutually exclusive (15, 31). Furthermore, evolved CSCs that survive after chemo- or radiotherapy might confer treatment resistance. By using CGH analysis, we revealed multiple genomic alterations and pronounced changes in the global gene expression profiling in prostate cancer cells that have been irradiated with multiple doses of X-rays. Therefore, we hypothesized that irradiation-induced genomic instability might lead to the evolution of the tumor-initiating populations that change their phenotypic features during the course of irradiation.

In our previous study, we found that prostate tumor-initiating cells with high aldehyde dehydrogenase activity (ALDH⁺ cells) were more radioresistant than ALDH⁻ cells, and inhibition of ALDH activity radiosensitizes prostate cancer cells (16).

In this study, we addressed the question whether tumor-initiating cells with acquired radioresistance can also be defined by a high ALDH activity. Surprisingly, although RR ALDH⁺ population possesses higher clonogenic and tumorigenic potential compared with RR ALDH⁻ cells, these two populations cannot be distinguished by either their radiosensitivity properties or by the activation of WNT/b-catenin signaling pathway and migratory potential. These results indicate that the radioresistant tumor-initiating cell populations undergo a phenotypic switching during the course of radiotherapy by analogy with a lineage switch in leukemia patients after chemotherapy (32–34), and functional changes in ovarian and prostate CSCs following paclitaxel or docetaxel treatment (20, 35). Furthermore, recent data suggest that ALDH1A1 expression is predictive of poor prognosis in hormone-naive prostate cancer patients. In contrast, in patients with castration-resistant disease, ALDH1A1 expression does not correlate with clinical outcome (25).

The previously published studies suggested that phenotypic plasticity of tumor-initiating cells might be associated not only with certain genetic but also with epigenetic changes, which can be
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Epigenetic changes: Cancer stem cell plasticity
Cell reprogramming
Emergence of radioresistance

- WNT/β-catenin signaling
- DZNep
- Apoptosis
- Radiosensitization

Cancer stem cell population
Radiosensitive cancer stem cell population
The course of irradiation

Figure 7.
Proposed model of regulation of prostate cancer cell radiosensitivity by epigenetic mechanisms. Irradiation-mediated activation of the WNT/β-catenin signaling pathway leads to cell reprogramming and CSC plasticity. Inhibition of the global histone methylation by DZNep results in impaired DNA repair, induction of apoptosis, and increase of cell radiosensitivity.

essential for cell tumorigenicity and therapy resistance (15, 36). Indeed, accumulating experimental evidence suggests that tumor cell dedifferentiation, based on the induced reprogramming of cancer cells is analogous to the reprogramming of the differentiated somatic cells into pluripotent cells (37). Therefore, efficient anticancer treatment must eradicate both CSCs and the tumor bulk to prevent tumor cell dedifferentiation. The populations of tumor cells with salient features of CSCs can be induced in vivo in response to the different microenvironmental stimuli, which include hypoxia, genetic alterations that trigger EMT, as well as cancer therapies (26, 37, 38). In agreement with this, recently published data showed that irradiation may induce reprogramming of differentiated breast and hepatocellular carcinoma cells (39–41). Consistent with these findings, we have previously shown that fractions of the cells positive for CSC markers, for example, ABCC2, CD133, ALDH1 activity were significantly enriched after fractionated irradiation that can be potentially attributed to cell selection as well as to cell induction mechanisms including both, accelerated proliferation and reprogramming (16, 39, 40).

Notably, our data revealed that a single 4 Gy irradiation induces a prolonged effect on the clonogenic potential and expression of both CSC and EMT markers, which lasts for more than 6 weeks. The dynamics of protein expression after cell irradiation can reflect a continuous process, resulting in consequent changes of cell phenotype and functional properties, and resembling the reprogramming process of somatic cells. Indeed we have shown that irradiation induces methylation of H3K4 and H3K36, which is a hallmark of the reactivated transcription of the epigenetically silenced target genes. In support of the hypothesis that irradiation can induce prostate tumor cell populations with CSC features, our study shows that irradiation of ALDH+ prostate carcinoma cells leads to an induction of an ALDH+ cell subset within this population. This mechanism can potentially contribute to the enrichment of the cells with CSC phenotype after irradiation.

Heritable epigenetic regulation of gene expression, which includes changes in DNA methylation and histone posttranslational modification, plays an important role in the regulation of tumor response to the treatment. Histone modification patterns have been correlated to tumor recurrence in patients with prostate cancer (42). In support of the hypothesis that irradiation-induced epigenetic changes can regulate tumor response to therapy, we found that expression of the ALDH1A1 gene, which is indicative of prostate cancer cells with increased tumorigenicity and radioresistance, is regulated by the irradiation-induced epigenetic changes. Our previous studies demonstrated that ALDH1A1 gene expression is directly activated by the β-catenin/TCF transcriptional complex (16). Using a ChIP assay specific for the β-catenin/TCF-binding sites of the ALDH1A1 gene promoter, we revealed that irradiation induced tri-methylation of Lys 36 in histone H3 (H3K36me3) on the ALDH1A1 promoter. This modification has been associated with activation of ALDH1A1 gene transcription. Our studies suggest that irradiation induction of different genes involved in the regulation of epigenetic modifications including expression of the lysine methyltransferase EZH2. Overexpression of EZH2 has been associated with a number of cancers, including prostate, breast cancer, and melanoma (43–46). Recent studies revealed that EZH2 enhances WNT/β-catenin–mediated gene transactivation (47). Moreover, expression of EZH2 was inhibited by siRNA-mediated knockdown of β-catenin, suggesting a direct link between irradiation-induced activation of the WNT/β-catenin signaling pathway and epigenetic reprogramming of the tumor cells. In accordance with this observation, expression of ALDH1A1 and EZH2 genes has a tendency toward co-occurrence with expression of the CTNNB1 (β-catenin) gene in prostate tumor specimens (48). The chemical inhibition of EZH2 with DZNep, which acts as a broad methyltransferase inhibitor resulted in the downregulation of ALDH1A1 expression and led to a significant increase in cell radiosensitivity and inhibition of the tumorigenic properties. A compound such as DZNep decreases H3K27me3 and H3K9me2 repressive methylation mediated by EZH2 as well as the active histone mark H3K4me3 and H3K36me3, therefore preventing a broad epigenetic resetting of the tumor cells (49). Noteworthy, all-trans retinoid acid (ATRA), which is the only chemical drug in clinical use for tumor cell differentiation and is an inhibitor of ALDH activity, was recently shown to reduce the proliferation of prostate cancer DU145 cells through downregulation of EZH2-mediated methylation of the HOXB13 gene, which encodes Hoxb13 anti-proliferative transcription factor (50). Several studies have demonstrated that the level of EZH2 expression is positively correlated with prostate cancer aggressiveness, and EZH2 is not only overexpressed in metastatic prostate cancer but also in the localized cancers with a high risk of recurrence after radical prostatectomy (22, 46, 51, 52). A high EZH2 expression in esophageal squamous...
cell carcinoma and rectal cancer was significantly correlated with the lack of clinical complete response to chemoradiotherapy (53, 54). Another study has demonstrated that EZH2 protects glioma stem cells from irradiation-induced cell death (55). Taken together, these findings demonstrate that EZH2 bears significant potential to serve as a diagnostic and prognostic marker for prostate cancer and other cancers and as a therapeutic target for tumor radiosensitization. In support of this, recent clinical studies of the various epigenetic inhibitors including EZH2 inhibitors EPZ-6438 and CPI-1205 for the treatment of patients with advanced solid tumors or lymphomas (ClinicalTrials.gov identifier NCT01897571 and NCT02395601), as well as the inhibitors of histone deacetylases, which are already approved for clinical use or currently being clinically investigated, are paving the way to improved effectiveness of cancer treatment by the inhibition of tumor cell reprogramming.

This study is, to our best knowledge, the first investigation that demonstrates that radioresistant populations within prostate cancer cells undergo a phenotypic switch during the course of irradiation, rather than being selected from the preexisting cell pool. We revealed that irradiation drives histone modifications on the promoter sequence of ALDH1A1 genes, which regulate cancer cell tumorigenicity and radioresistance. Our findings demonstrated that treatment of tumor cells with DZNep resulted in a significant increase in DNA damage, cell apoptosis, and radiosensitivity, and cells with a higher radioresistance are more sensitive to DZNep treatment.

Future research is needed to validate our findings by using additional experiments on primary cultures and tumor biopsies and by combination of the drug with radiotherapy in patient-derived prostate cancer xenograft mouse models. Our data suggest that compounds such as DZNep that inhibit EZH2 and prevents a broad resetting of histone methylation marks may be useful as epigenetic cotherapy to prevent tumor cell reprogramming into more aggressive and therapy-resistant state (Fig. 7).

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An Epigenetic Reprogramming Strategy to Resensitize Radioresistant Prostate Cancer Cells
Claudia Peitzsch, Monica Cojoc, Linda Hein, et al.
Cancer Res 2016;76:2637-2651. Published OnlineFirst March 16, 2016.

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