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Targeting Histone Deacetylase 4/Ubiquitin-Conjugating Enzyme 9 Impairs DNA Repair for Radiosensitization of Hepatocellular Carcinoma Cells in Mice

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Several strategies to improve the efficacy of radiation therapy against hepatocellular carcinoma (HCC) have been investigated. One approach is to develop radiosensitizing compounds. Because histone deacetylase 4 (HDAC4) is highly expressed in liver cancer and known to regulate oncogenesis through chromatin structure remodeling and controlling protein access to DNA, we postulated that HDAC4 inhibition might enhance radiation's effect on HCC cells. HCC cell lines (Huh7 and PLC5) and an ectopic xenograft were pretreated with HDAC inhibitor or short hairpin RNA to knock down expression of HDAC4 and then irradiated (2.5-10.0 Gy). We evaluated cell survival by a clonogenic assay; apoptosis by Annexin V immunofluorescence; γH2AX, Rad51, and HDAC4 by immunofluorescence staining; HDAC4, Rad51, and ubiquitin-conjugating enzyme 9 (Ubc9) in HCC cell nuclei by cell fractionation and confocal microscopy; physical interaction between HDAC4/Rad51/Ubc9 by immunoprecipitation; and the downstream targets of HDAC4 knockdown by immunoblotting. Both HDAC4 knockdown and HDAC inhibitor enhanced radiation-induced cell death and reduced homologous recombination repair of DNA double-strand breaks and protein kinase B activation, leading to increased apoptosis. HDAC4 knockdown with or without an HDAC inhibitor significantly delayed tumor growth in a radiation-treated xenograft model. Radiation stimulated nuclear translocation of Rad51 in an HDAC4-dependent manner and the binding of Ubc9 directly to HDAC4, which led to Ubc9 acetylation. Moreover, these effects were accompanied by HDAC4/Ubc9/Rad51 complex dissociation through inhibiting nuclear translocation. *Conclusion:* HDAC4 signaling blockade enhances radiation-induced lethality in HCC cells and xenografts. These findings raise the possibility that HDAC4/Ubc9/Rad51 complex in DNA repair may be a target for radiosensitization of HCC. (HEPATOLOGY 2018;67:586-599).

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Hepatocellular carcinoma (HCC) is one of the most prevalent and lethal malignancies worldwide.[1,2] Radiation treatments (RTs), including selective internal RT and external beam RT, are currently being integrated into multimodality treatments for HCC.[3,4] The radiotherapeutic effects of intra-arterial yttrium-90 administration and high-dose stereotactic body RT remain unsatisfactory.[5] One strategy to enhance

Abbreviations: Akt, protein kinase B; BSA, bovine serum albumin; CI, combination index; DAPI, diamidino-2-phenylindole; DSB, double-strand break; FDG, 2-fluoro-2-deoxy-D-glucose; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; HIF-1α, hypoxia inducible factor 1 alpha; HR, homologous recombination; IgG, immunoglobulin G; IHC, immunohistochemical; miRNA, microRNA; NHEJ, nonhomologous end-joining; pAkt, phospho-Akt; PBS, phosphate-buffered saline; PET/CT, positron emission tomography/computed tomography; PI3K, phosphoinositide 3-kinase; PIAS, protein inhibitor of activated STAT; PVDF, polyvinylidene difluoride; RT, radiation treatment; SCID, severe combined immunodeficiency; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; shRNA, short hairpin RNA; SUMO, small ubiquitin-related modifier; Ubc9, ubiquitin-conjugating enzyme 9; WT, wild type.

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their effects on hepatic tumors is to inhibit radiation-activated signals to overcome sublethal damage repair.

Histone deacetylases (HDACs) remove the acetyl moiety from the lysine residues of histones and other proteins, causing gene transcriptional down-regulation and subsequent changes in signal transduction. HDAC inhibitors (HDACi) are epigenetic drugs with potentially useful clinical activities, either as single agents or in combination with other therapies including RT.[6,7] HDAC4 mRNA is up-regulated in human HCC tissues compared with adjacent noncancerous hepatic tissues.[8] By targeting HDAC4, microRNA (miRNA)-22 suppresses cell proliferation and tumorigenicity and thereby improves prognosis.[9]

Double-strand breaks (DSBs) elicited by ionizing radiation are repaired in mammalian cells predominantly by either homologous recombination (HR) or nonhomologous end-joining (NHEJ). Rad51 is the key molecule involved in HR repair and has recombinase activity, which allows it to strand search and invade.[10] Rad51 is recruited to DSB sites where it induces nuclear focus formation. Abolishment of Rad51 focus formation has been widely used as a functional assay to indicate HR repair deficiency.[11,12] DNA damage sensor proteins 53BP1 and Rad51 have been shown to colocalize with HDAC4 in DNA damage-induced nuclear foci, and cells with silenced HDAC4 exhibit marked radiosensitivity.[13,14] This indicates that HDAC4 plays a functional role in DNA repair and HDACi-mediated apoptosis in response to radiation.[15] However, it remains unclear whether HDAC4 participates in DNA repair in irradiated HCC cells.

Sumoylation is catalyzed by E1, E2, and E3 enzymes. Ubiquitin-conjugating enzyme 9 (Ubc9) is the E2 enzyme required for transfer of activated small ubiquitin-related modifier (SUMO) to its target protein (i.e., for sumoylation of the target protein).[16,17] HDAC4 is reported to bind to Ubc9, which regulates sumoylation of myocyte enhancer factor 2.[18] Recent studies revealed the accumulation of SUMO proteins together with Ubc9 at DSB sites,[19] the requirement for Ubc9 before Rad51 can accumulate at sites of DNA damage in human cells,[20] and the interaction of Rad51 with Ubc9 and SUMO-1 before modification by SUMO-1.[21-23] Taken together, these findings suggest that Ubc9-mediated sumoylation is involved in Rad51 accumulation at DSB sites and is regulated by HDAC4.

On the basis of these findings, we evaluated the radiosensitizing role of HDAC4 in DNA damage repair of two human HCC cell lines. We showed that the radiosensitivity of HCC cells *in vitro* was increased by knocking down HDAC4 or by HDACi, and led to a significant delay in ectopic HCC tumor growth following RT. We proposed that the mechanism underlying this delay involves the dissociation of HDAC4/Rad51/Ubc9 complex to inhibit the HR pathway of DNA repair. Thus, future strategies that target and blockade HDAC4 signals may have radiotherapeutic value for HCC.

Materials and Methods

CELL CULTURE

Human HCC cell lines, Huh7 and PLC5, were obtained from the Japanese Collection of Research Bioresources Cell Bank (Okayama, Japan) and American Type Culture Collection (Manassas, VA), respectively. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 50 U/mL of penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Plasmid encoding short hairpin RNA (shRNA) for HDAC4 (pLKO.1-shHDAC4) and control plasmids

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for the RNA interference experiments (pLKO.1) were obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan).

COLONY-FORMATION ASSAY

Wild-type (WT) or shRNA-transfected HCC cells (500-1,000/well) were seeded in six-well plates and treated with different doses of radiation (2.5-10.0 Gy) following 24-hour pretreatment with various doses of HDACi (panobinostat [5-25 nM]; provided by Novartis Pharma AG, NIBR, Cambridge, MA) or dimethyl sulfoxide vehicle. Cells were cultured for 10 days, fixed, and stained with crystal violet. All colonies (clusters of more than 50 cells) visible to the naked eye were counted. Results are presented as means ± SEM of three independent experiments, with duplicate samples for each treatment condition. The combination index (CI) was calculated to determine the synergistic, additive, or antagonistic effect of the combined treatment (Supporting Fig. S1A).

IN VIVO ECTOPIC TUMOR MODEL

Male nonobese diabetic/severe combined immunodeficiency (SCID) mice (6 weeks of age) were obtained from the National Laboratory Animal Center and used for ectopic xenograft implantation. Body weights were measured weekly. Ectopic tumors were established by subcutaneous injection of Huh7 cells (1×10^6) into the right hind leg. Tumor dimensions were measured with a set of calipers and their volumes calculated using a standard formula: width^2×length/2. All experimental procedures using these mice were performed in accord with protocols approved by the National Taiwan University Institutional Animal Care and Use Committee. As the tumors became established, mice were randomized into six groups to receive the following treatments: (1) methylcellulose/Tween 80 vehicle on days 1-7; (2) panobinostat (25 mg/kg/day of body weight) on days 1-7; (3) methylcellulose/Tween 80 vehicle plus 7.5 Gy/day of RT on days 3-5; (4) panobinostat (25 mg/kg/day of body weight) on days 1-7 plus RT (7.5 Gy/day) on days 3-5; (5) shHDAC4 transfected Huh7 cells with vehicle; and (6) shHDAC4 transfected Huh7 cells with vehicle plus 7.5 Gy/day of RT on days 3-5. Small animal positron emission tomography/computed tomography (PET/CT) scans with [^{18}F]-2-fluoro-2-deoxy-D-glucose (FDG) were performed 1 day before and on day 9 of treatment. Mice were intravenously injected with 14 MBq (378 Ci) of ^{18}F-FDG in saline by the tail vein.

HISTOLOGICAL EVALUATION

Mice from each group were sacrificed on day 10. The tumor was fixed in 10% neutral buffered formalin and processed for histopathological and immunohistochemical (IHC) staining. After fixation, tumor tissues were embedded in paraffin blocks and sectioned (10 μm). Tumor cells were identified in representative stained sections. Expressions of HDAC4 and Rad51 were evaluated after IHC staining using specific antibodies.

IMMUNOFLUORESCENCE STAINING ANALYSIS

WT or shRNA transfected HCC cells were plated on four-well chamber slides, allowed to attach overnight, exposed to ionizing irradiation of 10 Gy either alone or combined with 20 nM of panobinostat, fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes, and then permeabilized in 0.5% Triton X-100 with 3% bovine serum albumin (BSA) in PBS for 20 minutes. Fixed cells were incubated for 16 hours at 4°C with primary antibodies against Rad51, Ubc9, and HDAC4 (200 in PBS containing 1% BSA; Santa Cruz Biotechnology, Santa Cruz, CA), then with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin G (IgG; 1:1,000) and rhodamine-conjugated sheep anti-mouse antibodies (1:1,000; Santa Cruz Biotechnology) and, finally, with diamidino-2-phenylindole (DAPI) to stain the chromosomes. A Zeiss Axio Imager A1 fluorescence microscope (Zeiss, Germany) or a Leica confocal SP5 microscope (Wetzlar, Germany) was used for visualization.

γH2AX IMMUNOFLUORESCENCE MICROSCOPY

After treatment, HCC cells were incubated for 24 hours, washed three times with ice-cold PBS, fixed in 4% formaldehyde/PBS for 20 minutes, permeabilized in 0.5% Triton X-100 with 3% BSA in PBS for 20 minutes at room temperature, then incubated with the antibody (FITC-conjugated anti-phospho-histone γH2AX [Ser139; 1:500]; Millipore, Billerica, MA) for 16 hours at 4°C in the dark, washed with PBS, and mounted in Vectashield mounting medium containing

DAPI (Vector Laboratories, Burlingame, CA). γH2AX foci were observed using a Zeiss Axio Imager A1 fluorescence microscope. In each sample, the number of γH2AX foci per nucleus was counted at high magnification using an automated foci counter, and approximately 150 nuclei were analyzed. The average number of γH2AX foci per nucleus represents the number of DSBs.

IMMUNOPRECIPITATION

Whole protein lysates containing 1 mg of protein were prepared using radioimmunoprecipitation assay lysis buffer and immunoprecipitated at room temperature for 2 hours using the Catch and Release v2.0 Reversible Immunoprecipitation System (Millipore). HDAC4 or Ubc9 was immunoprecipitated with primary antibody diluted 1:100 in a total volume of 500 μL. Protein complexes were eluted from the column using 70 μl of 1× denaturing elution buffer in accord with the manufacturer's directions. Approximately 30 μL of eluted proteins were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% resolving gel, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed overnight with primary antibodies to HDAC4, Ubc9, Rad51, and acetyl-lysine (Santa Cruz Biotechnology).

SUBCELLULAR FRACTIONATION

Cytoplasmic and nuclear extracts were prepared according to the instructions supplied by the manufacturer of the nuclear/cytosol fractionation kit (BioVision, Mountain View, CA). The resulting supernatants were then resolved by SDS-PAGE and visualized by western blotting analysis. Rad51, Ubc9, HDAC4, laminin B, and α-tubulin were detected using their specific antibodies (Santa Cruz Biotechnology) diluted at 1:1,000.

WESTERN BLOTTING ANALYSIS

Proteins (50 μg of protein/aliquot) in cell lysate aliquots were separated by SDS-PAGE (8%-15% polyacrylamide), transferred to PVDF membranes, and immunoblotted with various antibodies. Bound antibodies were detected using the appropriate peroxidase-coupled secondary antibodies, followed by enhanced chemiluminescence detection. Antibodies to phospho-Akt (pAkt) and protein kinase B (Akt) were obtained from Cell Signaling, Inc. (Burlingame, CA), and beta-actin and HDAC4 from Santa Cruz Biotechnology, and used as the loading control.

DETERMINATION OF APOPTOSIS WITH FLUORESCENCE MICROSCOPY

Apoptotic cells were detected using the Annexin V/FITC apoptosis detection kit, AVK050 (Strong Biotech, Taipei, Taiwan), according to the manufacturer's instructions. Annexin V–positive cells were examined using a Zeiss Axio Imager A1 fluorescence microscope.

STATISTICAL ANALYSIS

The tumor volume data satisfied the assumptions of normality and homogeneity required for parametric analysis; thus, group means for the ectopic tumor models were compared with a one-way analysis of variance followed by Fisher's least significant difference method for multiple comparisons. Differences were considered significant at $P < 0.05$.

Results

HDAC4 KNOCKDOWN OR TREATMENT WITH HDAC INHIBITOR RADIOSENSITIZES HCC CELLS AND INHIBITS THE REPAIR OF DNA DSBs

Two human HCC cell lines (Huh7 and PLC5) were transfected using shRNA targeting HDAC4 (shHDAC4) before irradiation. Both HCC cell lines were significantly ($P < 0.05$) radiosensitized under HDAC4 down-regulation by shHDAC4 versus the nonspecific shControl (Fig. 1A). Similar to shHDAC4, a pan-HDAC inhibitor significantly suppressed HDAC activity ($P < 0.05$), thereby reducing cell survival in 2.5-10.0 Gy irradiated HCC cells (Fig. 1B). To determine whether the effect of combining HDACi with radiation was synergistic (greater than the sum of the component effects), CIs were calculated from the dose-response data. In Huh7 and PLC5 cells treated with combined irradiation and HDACi, CI values of <1 were achieved and indicative of synergism (Supporting Fig. S1A,B). In contrast, the interaction between HDACi and irradiation was not synergistic in hepatocyte CL48 cells, indicating the potential therapeutic ratio (Supporting Fig. S1C,D). We found a significantly higher number of ϒH2AX foci

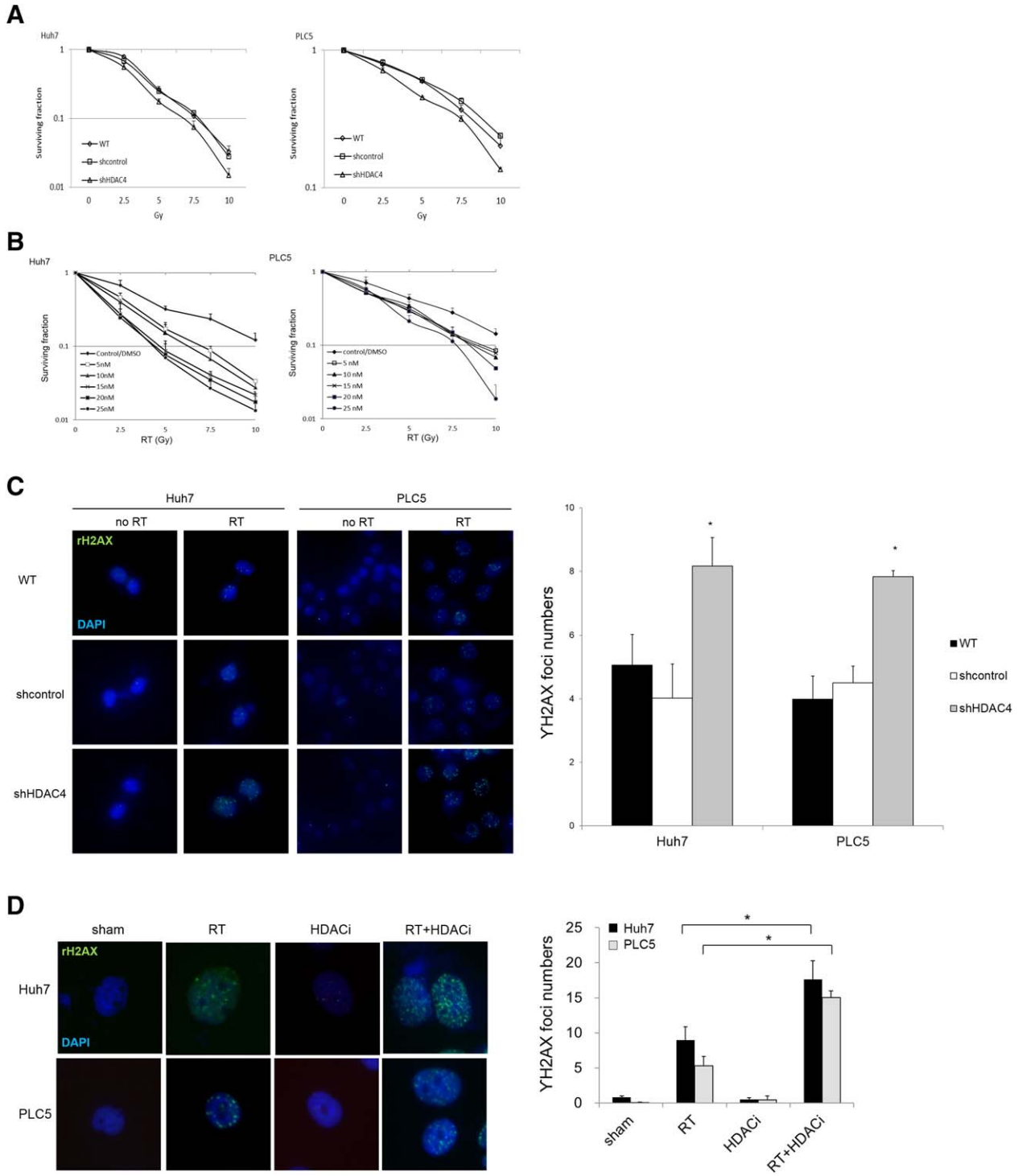

FIG. 1. Both HDAC4 knockdown and the treatment with HDACi enhance radiosensitization of HCC cells. (A) Sensitization of irradiation (RT)-treated Huh7 and PLC5 cells following HDAC4 shRNA transfection. (B) Exponentially growing HCC cells were treated with different doses of HDACi for 24 hours before exposure to different doses of RT. The results are shown as the mean ± SD calculated from three independent experiments. The number of ϒH2AX foci/cell was counted in a minimum of 150 cells per treatment with (C) HDAC4 knockdown (cells plated for 6 hours before RT) or (D) HDACi. The representative images of Huh7 and PLC5 cells were taken at 24 hours after RT. The average number of ϒH2AX foci/cell is shown. Error bars indicate SD, $^*P < 0.05$.

in shHDAC4-treated cells than shControl-treated cells at 24 hours after irradiation (Fig. 1C). Consistently, the number of ϒH2AX foci per cell was significantly increased after combined treatment with irradiation and HDACi (Fig. 1D). The results demonstrated that either knockdown of HDAC4 or HDACi enhanced radiosensitivity and reduced DNA repair activity in HCC cells. Next, we determined whether HDACi induced hyperacetylation of histones in HCC cells. Huh7 and PLC5 cells were treated with HDACi of different doses, and histone H3 acetylation was analyzed by western blotting. HDACi treatment significantly increased the acetylated histone, H3, in a dose-dependent manner (Supporting Fig. S2A). Combined RT (10 Gy) and HDACi (20 nM) significantly increased the acetylation of histone H3 compared to either treatment alone (Supporting Fig. S2B).

HDAC4 KNOCKDOWN OR TREATMENT WITH HDAC INHIBITOR ENHANCES *IN VIVO* RADIOSENSITIVITY OF HCC ECTOPIC XENOGRAFTS

Subsequently, we conducted therapeutic studies in mice bearing Huh7 ectopic xenografts. Mice bearing subcutaneous WT Huh7 tumors or HDAC4 knockdown tumors were randomized into six groups to receive (1) sham treatment, (2) RT, (3) intraperitoneal injection of HDACi, and (4) combined RT and HDACi to WT xenografts, and (5) sham treatment and (6) RT to HDAC4 knockdown xenografts. The combined HDACi and RT suppressed the growth of WT tumors to a significantly greater extent than RT alone or HDACi alone. RT significantly delayed the growth of HDAC4 knockdown xenografts compared to WT xenografts (Fig. 2A). The number of days until the tumor volume reached 500 mm^3 was 23 and 57 in the WT xenograft groups receiving RT and combined HDACi + RT, respectively, and 42 in the HDAC4 knockdown xenograft group receiving RT. Two days after treatment, ^{18}F-FDG micro-PET/CT images showed that tumor viability was much decreased after combined HDACi and RT, compared to either RT alone or sham treatment (Fig. 2B). Metabolic tumor volume was not reduced by HDACi alone, but partially reduced by RT alone. To determine whether HDAC4 expression is a feature of Rad51 focus formation, we evaluated tumor expression of HDAC4 and Rad51 in each treatment group. HDAC4 (Fig. 2C) and Rad51 (Fig. 2D) expressions in nuclei of Huh7 cells were increased after RT and decreased after RT + HDACi. The percentages of HDAC4-positive and Rad51-positive nuclei were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD). The results showed that the addition of HDACi to RT significantly reduced nuclear foci of HDAC4 (38.2 ± 7.1% vs. 63.6 ± 6.8%; $P = 0.01$) and Rad51 (25.2 ± 2.9% vs. 57.8 ± 3.8%; $P = 0.0003$) induced by RT alone (Supporting Fig. S3).

HDAC INHIBITOR ENHANCES *IN VIVO* RADIOSENSITIVITY OF HCC ORTHOTOPIC XENOGRAFTS COMPARED TO NORMAL LIVER

IHC staining (Supporting Fig. S4B,C) and western blottings (Supporting Fig. S4D,E) of cleaved caspase-3 revealed that pretreatment with HDACi increased the expression of this apoptotic marker in irradiated orthotopic xenograft, but not in normal liver tissues. With high or low radiation doses, the percentages of apoptotic cells were not different in irradiated and nonirradiated livers between the non-tumor-bearing mice with and without pretreament of HDACi (Supporting Fig. S5).

HDAC4 KNOCKDOWN OR TREATMENT WITH HDAC INHIBITOR STRONGLY INHIBITS HOMOLOGOUS RECOMBINATION REPAIR AS DETERMINED BY Rad51 FOCUS FORMATION

We tested whether HDAC4 acts as a mediator of RT-induced Rad51 and 53BP1 focus formation in HCC cells. Treatment of HCC cells with HDACi resulted in a significant reduction in the number and size of Rad51 foci in both Huh7 and PLC5 cells (Fig. 3A). In contrast, 53BP1 foci after irradiation were not affected by HDACi (Fig. 3B). Huh7 and PLC5 cells in which HDAC4 was depleted by shRNA were used to evaluate whether HDAC4 regulates Rad51 focus formation. The results showed that HDAC4 depletion limited the number and size of Rad51 foci formed upon irradiation (Fig. 3D). To determine whether HDAC4 truly controls Rad51 accumulation in the nucleus, we examined the cellular location of Rad51 in HCC cells. In both HCC cell lines, the nuclear fraction of Rad51 increased after irradiation and this increase was down-regulated by HDAC4 knockdown or pretreatment with HDACi (Fig. 3C,D).

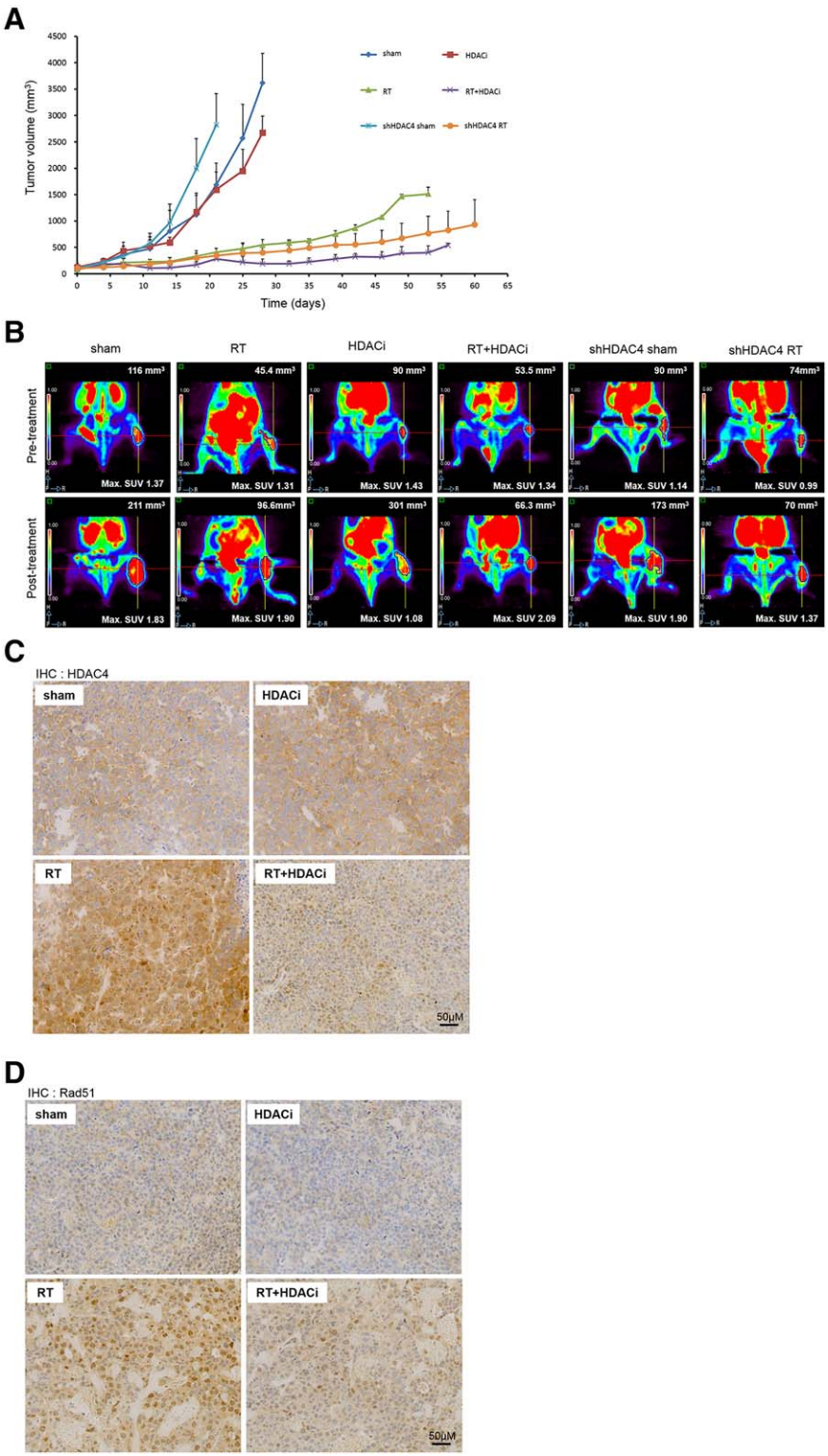

FIG. 2. *In vivo* radiosensitizing effect of HDAC4 knockdown (shHDAC4) and the treatment with HDACi. Tumors grown (subcutaneously) to 100 mm^3 in the hind legs of SCID mice were randomized to the following treatments (n = 5 in each group): (1) sham treatment (sham), (2) radiotherapy (RT), (3) HDACi, (4) the combination of RT and HDACi to WT Huh7 ectopic xenografts, as well as (5) sham treatment (shHDAC4 sham) and (6) RT to shHDAC4 xenografts (shHDAC4 RT). (A) Tumor growth curves of different treatment groups were plotted. Data are the mean tumor volume from each group measured on the indicated days. (B) PET/CT was performed on day 9. Representative images are shown. The standard uptake value (SUV) and the viable volume of tumor are shown at the upper corners of the image. Tumors from each group were excised on day 10. The IHC staining with (C) HDAC4 and (D) Rad51 of cross-sections are shown.

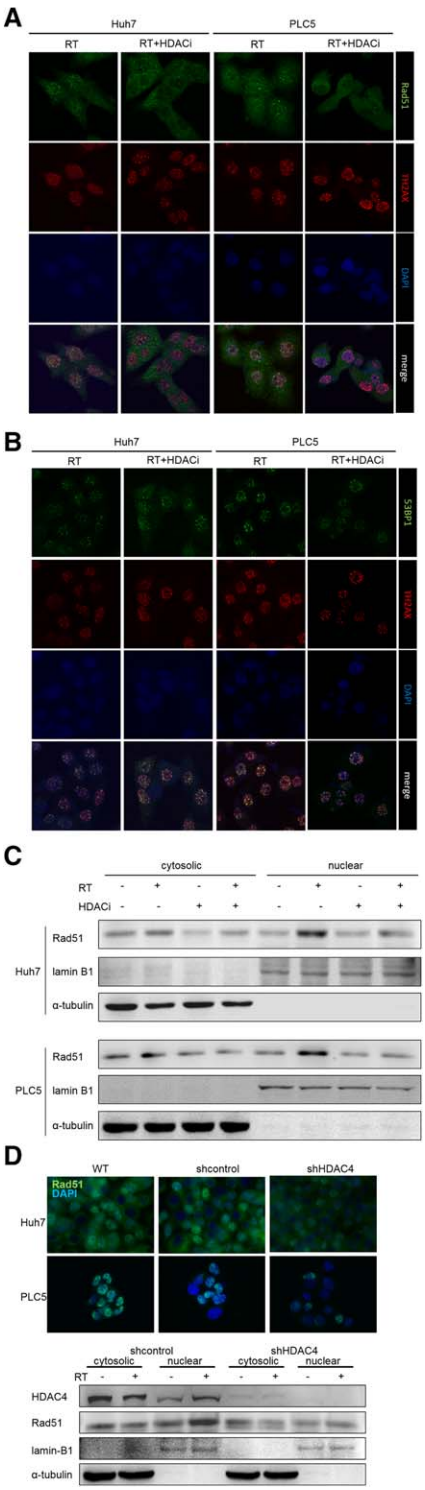

FIG. 3

HDAC4 AND Rad51 INTERACT WITH THE SUMO-CONJUGATING ENZYME, Ubc9

We next determined whether the interaction between Ubc9 and HDAC4 contributes to the cellular response to RT-induced DNA damage. HCC cells were treated with 10 Gy of radiation, and whole cell extracts were prepared and subjected to the immunoprecipitation assay at the indicated intervals. Extracts immunoprecipitated with antibody to HDAC4 contained Ubc9 (Fig. 4A), and extracts immunoprecipitated with antibody to Ubc9 contained HDAC4 and Rad51 (Fig. 4D). The amount of HDAC4 and Rad51 present in the immunoprecipitates was increased 4 hours after irradiation and reduced by pretreatment with HDACi (Fig. 4B) or by knocking down HDAC4 (Fig. 4D). Double-labeling immunofluorescence experiments revealed the translocation of green fluorescence–labeled HDAC4 and red fluorescence–labeled Ubc9 into the nucleus following irradiation, and the reduction of this translocation by pretreatment with HDACi. Yellow fluorescence in the nucleus formed by the overlap of red and green fluorescence indicated that the colocalization of HDAC4 and Ubc9, respectively, was induced after irradiation and was reduced by HDACi (Fig. 4C). The amount of acetylated Ubc9 in the immunoprecipitates was substantially increased by HDACi (Fig. 4B) or HDAC4 depletion (Fig. 4D). These results demonstrate that Ubc9 is linked to HDAC4 and Rad51, and this association is enhanced by exposure of cells to RT.

FIG. 3. HDAC4 is involved in the nuclear relocation of Rad51, but not 53BP1, after the DNA damage. Co-immunofluorescence staining with antibodies against (A) Rad51 and γH2AX, and (B) 53BP1 and γH2AX in HCC cells at 4 hours after 10 Gy of irradiation (RT). Pretreatment with 20 nM of HDACi strongly inhibited focus formation of Rad51, but not 53BP1. DNA was stained with DAPI. Rad51, γH2AX, and the nucleus were visualized in green, red, and blue, respectively. (C) With/without pretreatment with HDACi, irradiated HCC cells were processed at 4 hours to isolate and quantify the cytosolic and nuclear protein fractions. Nuclear protein (15 μg) and cytosolic protein (30 μg) were used for western blotting analysis with antibodies against Rad51. (D) HDAC4 knockdown HCC cells were irradiated and stained with DAPI and Rad51 at 4 hours (upper panel). Cytosolic and nuclear protein fractions were isolated and quantified from irradiated HDAC4 knockdown Huh7 cells at 4 hours (lower panel). Lamin B1 (a nuclear protein) and α-tubulin (a cytoplasmic protein) were used as the controls for cell fractionation. DNA damage by RT induced an increase in nuclear levels of Rad51 and genetic knockdown of HDAC4 induced a reduction in nuclear levels of Rad51.

RADIATION ACTIVATES HDAC4 AND Ubc9 NUCLEAR LOCALIZATION

Immunofluorescence microscopy with antibodies that specifically bind HDAC4 confirmed the radiation-activated nuclear translocation of HDAC4 in both Huh7 and PLC5 cells (Fig. 5A). To evaluate the nuclear localization of HDAC4 and Ubc9, the separate cytoplasmic and nuclear protein fractions of irradiated cells with or without pretreatment of HDACi were extracted. As shown in western blotting assays (Figure 5B), pretreatment with HDACi decreased the amount of HDAC4 and Ubc9 in the nuclear fraction of HCC cells. We also investigated the subcellular localization of other class I or class II HDACs. With or without HDACi, irradiation failed to alter class I (HDAC-1, HDAC2, and HDAC3) or class II HDAC (HDAC5 and HDAC6) levels (Fig. 5C).

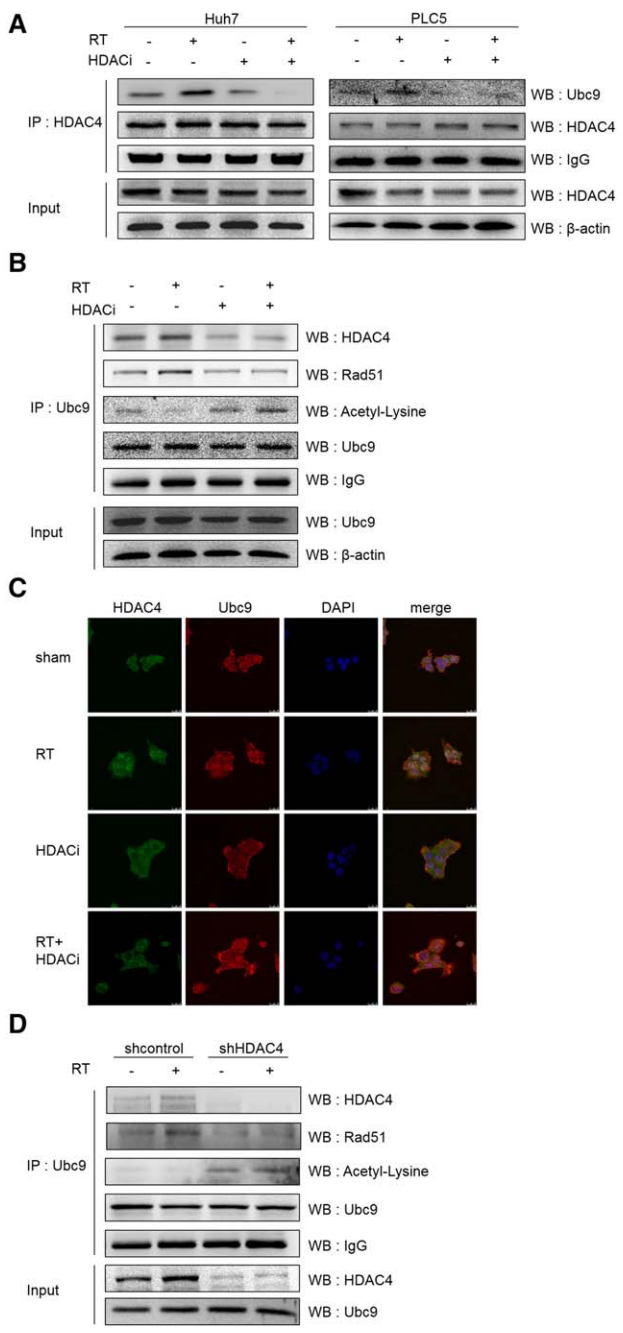

FIG. 4

HDAC4 KNOCKDOWN OR TREATMENT WITH HDAC INHIBITOR SUPPRESSES RADIATION-INDUCED Akt PHOSPHORYLATION AND INCREASES APOPTOSIS IN HCC CELLS

Given that RT activates phosphoinositide 3-kinase (PI3K)/Akt signaling, we investigated whether HDAC4 depletion or pretreatment with HDACi suppresses RT-activated Akt phosphorylation. At 4 hours, the RT-induced phosphorylation of Akt was inhibited by HDAC4 depletion (Fig. 6A) or HDACi (Fig. 6B). Subsequently, cells were stained with FITC-labeled Annexin V to identify apoptotic cells. The percentage of early apoptotic cells treated with HDACi increased

FIG. 4. HDAC4 inhibits the binding of Ubc9 to Rad51 and increases Ubc9 acetylation. Immunoprecipitation, immunoblotting, and immunofluorescence analyses were performed with the indicated antibodies. (A) Huh7 and PLC5 cells were pretreated with or without HDACi 24 hours before radiation (RT). Cells were collected 4 hours after RT. HDAC4 and Ubc9 were detected by western blotting (WB) in immunoprecipitates (IP) with HDAC4 antibody. (B) Huh7 cells were pretreated with or without HDACi 24 hours before RT. Cells were collected 4 hours after RT. HDAC4, Rad51, Ubc9, and acetyl-lysine were detected by WB in IP with Ubc9 antibody. (C) Confocal scanning laser microscopy was used to confirm that RT increases colocalization of HDAC4 and Ubc9 in the nucleus. Huh7 cells grown on coverslips were pretreated with or without HDACi 24 hours before 10 Gy of RT. Following fixation and permeabilization, cells were immunostained with antibodies against HDAC4 (green) and Ubc9 (red). These pictures have been merged with images of the nuclei stained with DAPI (blue). (D) A control or HDAC4 shRNA was stably expressed in Huh7 cells. Huh7 cells with or without expression of HDAC4 shRNA were treated with or without 10 Gy of RT and then incubated for 4 hours. HDAC4 and Ubc9 were detected by WB in IP with antibody to HDAC4. Abbreviation: sh, short hairpin RNA.

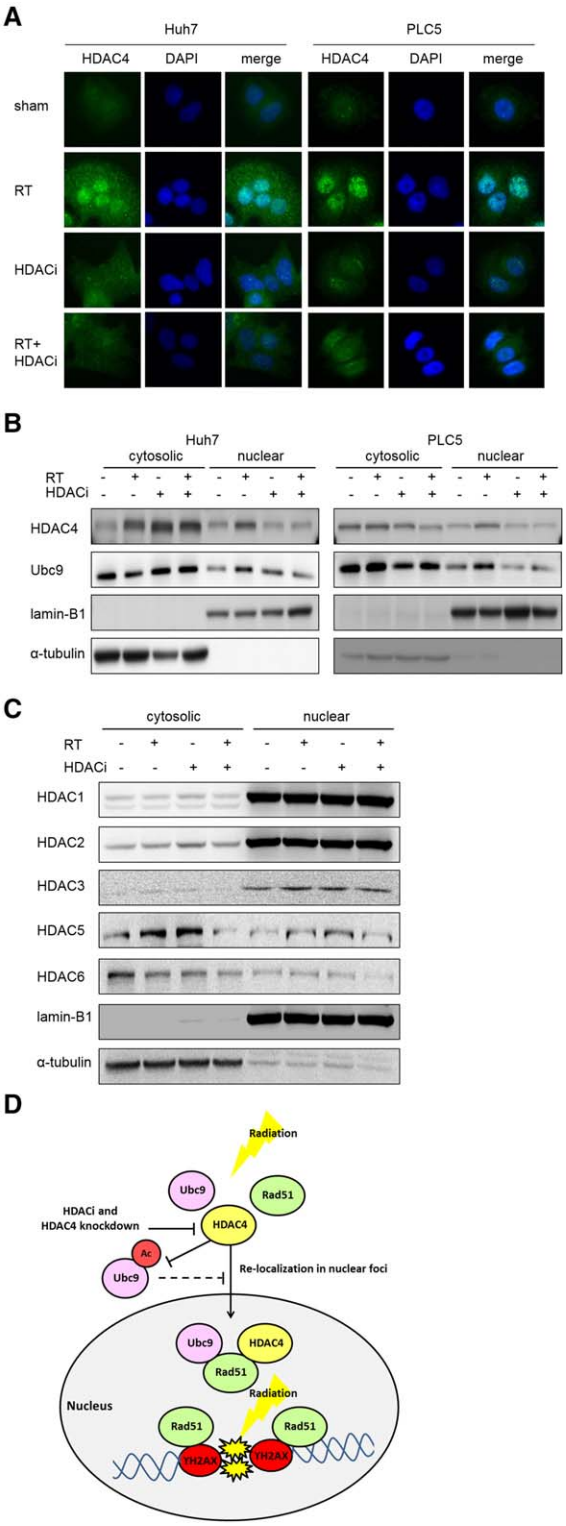

FIG. 5

significantly at 24 hours after RT when compared with sham-irradiated cells and RT-alone cells (Fig. 6C).

Discussion

Increasing use and technological advances have gradually led to the establishment of RT as an alternative treatment regimen for HCC.[24] Selective internal RT using yttrium-90 microspheres introduces radiation into hepatic tumors by an intra-arterial route, whereas stereotactic body RT targets localized hepatic tumor(s) with a high radiation dose. However, partial regression is the most common sublethal response of HCC to RT.[3] To increase safety and reduce the side effects of RT, medicinal compounds have been used in combination to increase tumor cell radiosensitization.[25]

Few HDACi are used clinically as sensitizers of RT, mainly because their role in radiosensitization remains unclear. We previously reported that a phenylbutyrate-derived HDACi, AR-42, synergized with radiation to inhibit HCC cell survival and the growth of ectopic xenografts.[26] In this study, inhibition of HDAC4 either genetically by shRNA or pharmaceutically by an HDACi (panobinostat) acted with RT to reduce growth of HCC cells and ectopic xenografts. Panobinostat, a pan-HDACi, radiosensitized HCC cells at relatively low concentration and low cytotoxicity. Panobinostat is a novel cinnamic hydroxamic acid analogue HDACi. It is currently approved for treatment of hematological malignancies and its use with radiation enhances apoptosis in human non-small-cell lung

FIG. 5. HDACi inhibited the radiation (RT)-induced recruitment of HDAC4 and Ubc9 to the nucleus. (A) Immunofluorescence microscopy was used to confirm that RT increases nuclear levels of HDAC4. Huh7 and PLC5 cells grown on coverslips were irradiated with 10 Gy. Following fixation and permeabilization, cells were immunostained with antibodies against HDAC4 (green). These pictures have been merged with images of the nuclei stained with DAPI (blue). These results were from three independent experiments performed in duplicate. Cytosolic and nuclear protein fractions are from irradiated Huh7 and PLC5 cells exposed at 4 hours. Nuclear protein (15 μg) and cytosolic protein (30 μg) were taken for western blotting analysis with antibodies against (B) HDAC4 and Ubc9; (C) HDAC1, HDAC2, HDAC3, HDAC5, and HDAC6 proteins. Purity of the nuclear and cytosolic fractions was demonstrated by probing for the presence or absence of the nuclear protein, lamin B, or cytosolic α-tubulin. Experiments were performed in duplicate cell cultures and are representative of three independent experiments. (D) Effects of HDAC4 depletion or treatment with HDACi on the homologous recombination DNA repair pathway in HCC cells treated with RT.

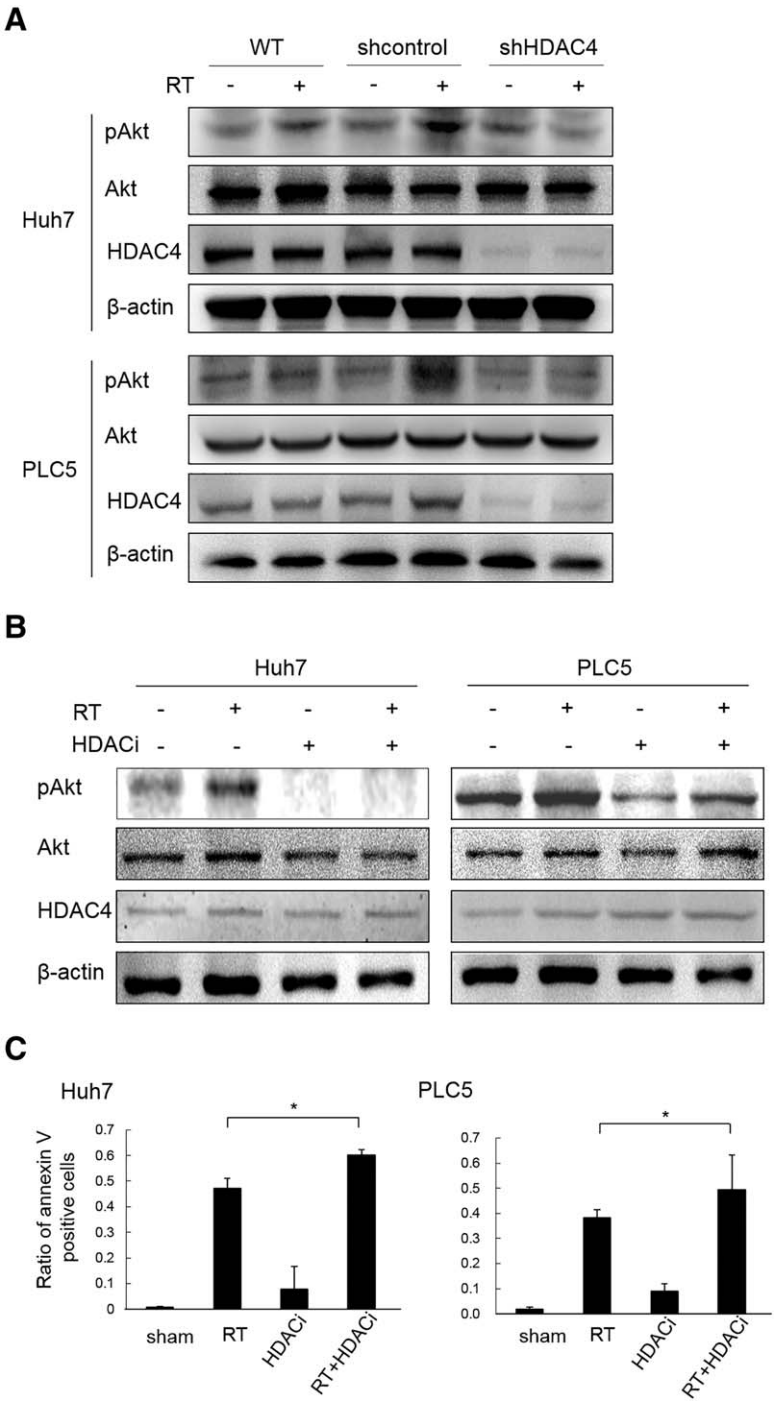

FIG. 6. Down-regulation of HDAC4 by an HDAC4-specific shRNA (shHDAC4) or HDACi treatment results in loss of radiation (RT)-induced Akt phosphorylation in both Huh7 and PLC5 cells. (A) Huh7 and PLC5 cells were transfected with vector control (shcontrol) or shHDAC4 followed by RT. At 4 hours, whole cell protein was extracted and pAkt expression was detected by western blotting analyses and normalized to β-actin. (B) Western blotting showed that levels of pAkt increased after RT and were inhibited by HDACi. (C) Early apoptotic changes in Huh7 and PLC5 cells are identified using labeled Annexin V–FITC. Percentage of Annexin V–positive cells in Huh7 and PLC5 cells was calculated at 24 hours. Error bars indicate SD, $^*P < 0.05$.

cancer and prostate cancer cells.[27,28] In contrast to the increased cell apoptosis of irriadated orthotopic xenografts with pretreatment of HDACi, no apoptotic difference was observed in normal liver of mice from different treatment groups. Moreover, the synergistic effect of adding HDACi to RT was not obvious in hepatocyte CL48 and normal liver irradiated with either high or low doses. Notably, technical advancement of radiotherapy better conforms higher doses to liver tumor while sparing normal liver. This evidence suggests a therapeutic role for HDACi in radiosensitization.

Aberrant expression of HDACs has been observed in different types of cancers, including HCC.[29-31] HDAC mRNAs (HDAC2, HDAC4, and HDAC11) were significantly up-regulated in HCC compared to normal liver.[31] Previous studies have shown that specific inhibition of seven isotypes (HDAC1, HDAC3, HDAC4, HDAC6, HDAC7, HDAC10, and HDAC11) enhanced radiation lethality in head and neck cancer cell line SQ20B. Geng et al.[28] reported translocation of HDAC4 from the cytoplasm into the nucleus of lung cancer cells following irradiation. Among class I and II HDACs, we showed that only HDAC4 is up-regulated in the nucleus with irradiation and down-regulated with HDACi (Fig. 6C). Because recent discoveries have highlighted the role of HDAC4 in the biological responses of HCC, we focused on HDAC4 in the present study.

Down-regulated miRNA-200a and miRNA-22 expression, by elevating HDAC4 expression, promoted HCC carcinogenesis and progression.[8,9] HDAC4 was found to bind hypoxia inducible factor 1 alpha (HIF-1α) and, when suppressed, to augment HIF-1α acetylation in HCC cell lines.[29] Moreover, knockdown of HDAC4 or treatment with HDACi was found to inhibit cell proliferation in HCC cell lines.[9] Consistently, our *in vitro* and *in vivo* data showed that inhibition of HDAC4 in HCC cells had a radiosensitizing effect. Notably, radiation-induced nuclear influx of HDAC4 was inhibited by HDACi and shown previously to result from increased beta-adrenergic signaling.[30] Dephosphorylation and nuclear import of HDAC4 was also shown to accompany melatonin-induced apoptosis of human colon adenocarcinoma cells.[31]

Mechanistic investigations revealed that panobinostat disrupts Rad51, but not 53BP1, responses to irradiation and may cause inhibition of HR repair. In our study, panobinostat significantly inhibited Rad51 focus formation and colocalization of Rad51 foci with ϒH2AX foci in irradiated HCC cells. The reduction of Rad51/ϒH2AX foci observed in irradiated HCC cells after panobinostat pretreatment likely indicates that panobinostat inhibits Rad51 recruitment to DSBs and thereby reduces DNA repair. Panobinostat pretreatment resulted in the persistence of ϒH2AX foci at 24 hours after irradiation in HCC cells, which was thought to reflect the lethality of the DNA damage.[32,33] Many proteins, in addition to Rad51 and 53BP1, have been shown to form nuclear foci following DNA damage, including ataxia telangiectasia mutated, breast cancer 1, and MRE11/RAD50/NBS1 complex.[34-36] HDAC inhibitors have also been reported to decrease the amount of Rad51 protein and inhibit HR repair in response to irradiation.[37-39] Given that our data showed the greater radiosensitizing effect of pan-HDACi than of HDAC4 knockdown, additional inhibitory mechanisms from HDACs other than HDAC4 are likely to exist.

In this study, either treatment with HDACi or HDAC4 knockdown reduced the interaction of Ubc9 and Rad51 proteins in irradiated HCC cells. The SUMO E2 (Ubc9) protein is required for Rad51 focus formation,[20] overexpressed in several malignancies, such as lung adenocarcinoma, colorectal cancer, breast adenocarcinoma, and HCC,[40-42] and up-regulated to promote tumor progression by suppressing the acute cellular stress response associated with the accumulating DNA damage from (but not limited to) radiation exposure.[43] Ubc9 has been reported to functionally interact with the coiled-coil domains of HDAC4 and HDAC5[18] and to interact with Rad51.[21,44] Ubc9 and E3 ligases, protein inhibitor of activated STAT (PIAS) 1 and PIAS4, were required for Rad51 accretion at sites containing DNA damage in human cells.[20] Acetylation of Ubc9 at residue K65 attenuated Ubc9 binding to substrates and reduced substrate sumoylation.[45] Notably, our data showed that HDAC4 inhibition enhanced Ubc9 acetylation. Unlike SUMO E1 expression (which has been associated with worse survival of HCC patients), SUMO E2 (Ubc9) expression has a still unknown role in prognosis. The role of Ubc9 in selecting HCC patients with a maximum response to RT remains to be investigated.

Several studies have shown that activation of the PI3K/Akt signaling pathway is notably linked to the radioresistance of many cancer cell lines. HDAC inhibitors were shown to mediate Akt dephosphorylation in glioblastoma and prostate cancer cells through a histone acetylation-independent mechanism.[46] Treatment with panobinostat led to acetylation of heat shock protein 90, which induced down-regulation of the EGFR and decreased levels of pAkt.[47] Panobinostat has also been reported to induce apoptosis and delay DNA damage repair in lung cancer cells.[28] Again, we showed that the mechanism of radiosensitization by panobinostat may involve inhibition of Akt phosphorylation/up-regulation and apoptosis in irradiated HCC cells.

This study explored the biological effect of HDAC4 inhibition and HDAC/Rad51/Ubc9 dissociation on radiosensitization of HCC. Our results suggest that blockade of HDAC4 signaling may be a potential

strategy for modulating a tumor cell's response to radiation, and used as the basis of a clinical trial of combined treatment with HDACi and RT in patients with HCC.

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REFERENCES

1) Bruix J, Llovet JM. HCC surveillance: who is the target population? Hepatology 2003;37:507-509.
2) El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007;132: 2557-2576.
3) Cheng JC, Chuang VP, Cheng SH, Huang AT, Lin YM, Cheng TI, et al. Local radiotherapy with or without transcatheter arterial chemoembolization for patients with unresectable hepatocellular carcinoma. Int J Radiat Oncol Biol Phys 2000;47:435-442.
4) Krishnan S, Dawson LA, Seong J, Akine Y, Beddar S, Briere TM, et al. Radiotherapy for hepatocellular carcinoma: an overview. Ann Surg Oncol 2008;15:1015-1024.
5) Cheng JC, Wu JK, Huang CM, Liu HS, Huang DY, Cheng SH, et al. Radiation-induced liver disease after three-dimensional conformal radiotherapy for patients with hepatocellular carcinoma: dosimetric analysis and implication. Int J Radiat Oncol Biol Phys 2002;54:156-162.
6) Wagner JM, Hackanson B, Lubbert M, Jung M. Histone deacetylase (HDAC) inhibitors in recent clinical trials for cancer therapy. Clin Epigenetics 2010;1:117-136.
7) **Groselj B, Sharma NL,** Hamdy FC, Kerr M, Kiltie AE. Histone deacetylase inhibitors as radiosensitisers: effects on DNA damage signalling and repair. Br J Cancer 2013;108:748-754.
8) Yuan JH, Yang F, Chen BF, Lu Z, Huo XS, Zhou WP, et al. The histone deacetylase 4/SP1/microrna-200a regulatory network contributes to aberrant histone acetylation in hepatocellular carcinoma. Hepatology 2011;54:2025-2035.
9) **Zhang J, Yang Y,** Yang T, Liu Y, Li A, Fu S, et al. microRNA-22, downregulated in hepatocellular carcinoma and correlated with prognosis, suppresses cell proliferation and tumourigenicity. Br J Cancer 2010;103:1215-1220.
10) Wang AT, Kim T, Wagner JE, Conti BA, Lach FP, Huang AL, et al. A dominant mutation in human RAD51 reveals its function in DNA interstrand crosslink repair independent of homologous recombination. Mol Cell 2015;59:478-490.
11) Davies AA, Masson JY, McIlwraith MJ, Stasiak AZ, Stasiak A, Venkitaraman AR, West SC. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. Mol Cell 2001; 7:273-282.
12) Ward A, Khanna KK, Wiegmans AP. Targeting homologous recombination, new pre-clinical and clinical therapeutic combinations inhibiting RAD51. Cancer Treat Rev 2015;41:35-45.
13) Kao GD, McKenna WG, Guenther MG, Muschel RJ, Lazar MA, Yen TJ. Histone deacetylase 4 interacts with 53BP1 to mediate the DNA damage response. J Cell Biol 2003;160:1017-1027.
14) Zgheib O, Huyen Y, DiTullio RA, Jr., Snyder A, Venere M, Stavridi ES, Halazonetis TD. ATM signaling and 53BP1. Radiother Oncol 2005;76:119-122.
15) Karagiannis TC, El-Osta A. Modulation of cellular radiation responses by histone deacetylase inhibitors. Oncogene 2006;25: 3885-3893.
16) **Schwarz SE, Matuschewski K, Liakopoulos D,** Scheffner M, Jentsch S. The ubiquitin-like proteins SMT3 and SUMO-1 are conjugated by the UBC9 E2 enzyme. Proc Natl Acad Sci U S A 1998;95:560-564.
17) Johnson ES, Blobel G. Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. J Biol Chem 1997;272:26799-26802.
18) Zhao X, Sternsdorf T, Bolger TA, Evans RM, Yao TP. Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. Mol Cell Biol 2005;25:8456-8464.
19) Galanty Y, Belotserkovskaya R, Coates J, Polo S, Miller KM, Jackson SP. Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. Nature 2009;462:935-939.
20) Shima H, Suzuki H, Sun J, Kono K, Shi L, Kinomura A, et al. Activation of the SUMO modification system is required for the accumulation of RAD51 at sites of DNA damage. J Cell Sci 2013;126:5284-5292.
21) Kovalenko OV, Plug AW, Haaf T, Gonda DK, Ashley T, Ward DC, et al. Mammalian ubiquitin-conjugating enzyme Ubc9 interacts with Rad51 recombination protein and localizes in synaptonemal complexes. Proc Natl Acad Sci U S A 1996;93:2958-2963.
22) Li W, Hesabi B, Babbo A, Pacione C, Liu J, Chen DJ, et al. Regulation of double-strand break-induced mammalian homologous recombination by UBL1, a RAD51-interacting protein. Nucleic Acids Res 2000;28:1145-1153.
23) Ho JC, Warr NJ, Shimizu H, Watts FZ. SUMO modification of Rad22, the *Schizosaccharomyces pombe* homologue of the recombination protein Rad52. Nucleic Acids Res 2001;29:4179-4186.
24) Hawkins MA, Dawson LA. Radiation therapy for hepatocellular carcinoma: from palliation to cure. Cancer 2006;106:1653-1663.
25) Tsai CL, Hsu FM, Cheng JC. How to improve therapeutic ratio in radiotherapy of HCC. Liver Cancer 2016;5:210-220.
26) Lu YS, Chou CH, Tzen KY, Gao M, Cheng AL, Kulp SK, Cheng JC. Radiosensitizing effect of a phenylbutyrate-derived histone deacetylase inhibitor in hepatocellular carcinoma. Int J Radiat Oncol Biol Phys. 2012;83:e181-e189.
27) Xiao W, Graham PH, Hao J, Chang L, Ni J, Power CA, et al. Combination therapy with the histone deacetylase inhibitor LBH589 and radiation is an effective regimen for prostate cancer cells. PLoS One 2013;8:e74253.
28) **Geng L, Cuneo KC,** Fu A, Tu T, Atadja PW, Hallahan DE. Histone deacetylase (HDAC) inhibitor LBH589 increases duration of gamma-H2AX foci and confines HDAC4 to the cytoplasm in irradiated non-small cell lung cancer. Cancer Res 2006; 66:11298-11304.
29) Geng H, Harvey CT, Pittsenbarger J, Liu Q, Beer TM, Xue C, Qian DZ. HDAC4 protein regulates HIF1alpha protein lysine acetylation and cancer cell response to hypoxia. J Biol Chem 2011;286:38095-38102.
30) Liu Y, Schneider MF. Opposing HDAC4 nuclear fluxes due to phosphorylation by beta-adrenergic activated protein kinase A or by activity or Epac activated CaMKII in skeletal muscle fibres. J Physiol 2013;591:3605-3623.
31) Wei JY, Li WM, Zhou LL, Lu QN, He W. Melatonin induces apoptosis of colorectal cancer cells through HDAC4 nuclear import mediated by CaMKII inactivation. Journal of pineal research 2015;58:429-438.
32) Antonelli F, Campa A, Esposito G, Giardullo P, Belli M, Dini V, Meschini S, et al. Induction and repair of DNA DSB as

revealed by H2AX phosphorylation foci in human fibroblasts exposed to low- and high-LET radiation: relationship with early and delayed reproductive cell death. Radiat Res 2015;183:417-431.
33) Van Oorschot B, Oei AL, Nuijens AC, Rodermond H, Hoeben R, Stap J, et al. Decay of gamma-H2AX foci correlates with potentially lethal damage repair and P53 status in human colorectal carcinoma cells. Cell Mol Biol Lett 2014;19:37-51.
34) Choudhary SK, Li R. BRCA1 modulates ionizing radiation-induced nuclear focus formation by the replication protein A p34 subunit. J Cell Biochem 2002;84:666-674.
35) Tauchi H, Kobayashi J, Morishima K, Matsuura S, Nakamura A, Shiraishi T, et al. The forkhead-associated domain of NBS1 is essential for nuclear foci formation after irradiation but not essential for hRAD50[middle dot]hMRE11[middle dot]NBS1 complex DNA repair activity. J Biol Chem 2001;276:12-15.
36) Rothkamm K, Barnard S, Moquet J, Ellender M, Rana Z, Burdak-Rothkamm S. DNA damage foci: meaning and significance. Environ Mol Mutagen 2015;56:491-504.
37) Adimoolam S, Sirisawad M, Chen J, Thiemann P, Ford JM, Buggy JJ. HDAC inhibitor PCI-24781 decreases RAD51 expression and inhibits homologous recombination. Proc Natl Acad Sci U S A 2007;104:19482-19487.
38) **Zhao H, Luoto KR,** Meng AX, Bristow RG. The receptor tyrosine kinase inhibitor amuvatinib (MP470) sensitizes tumor cells to radio- and chemo-therapies in part by inhibiting homologous recombination. Radiother Oncol 2011;101:59-65.
39) Chen X, Wong P, Radany EH, Stark JM, Laulier C, Wong JY. Suberoylanilide hydroxamic acid as a radiosensitizer through modulation of RAD51 protein and inhibition of homology-directed repair in multiple myeloma. Mol Cancer Res 2012;10:1052-1064.
40) Tomasi ML, Tomasi I, Ramani K, Pascale RM, Xu J, Giordano P, et al. S-adenosyl methionine regulates ubiquitin-conjugating enzyme 9 protein expression and sumoylation in murine liver and human cancers. HEPATOLOGY 2012;56:982-993.
41) Wu F, Zhu S, Ding Y, Beck WT, Mo YY. MicroRNA-mediated regulation of Ubc9 expression in cancer cells. Clin Cancer Res 2009;15:1550-1557.
42) Moschos SJ, Smith AP, Mandic M, Athanassiou C, Watson-Hurst K, Jukic DM, et al. SAGE and antibody array analysis of melanoma-infiltrated lymph nodes: identification of Ubc9 as an important molecule in advanced-stage melanomas. Oncogene 2007;26:4216-4225.
43) Romanenko AM, Kinoshita A, Wanibuchi H, Wei M, Zaparin WK, Vinnichenko WI, et al. Involvement of ubiquitination and sumoylation in bladder lesions induced by persistent long-term low dose ionizing radiation in humans. J Urol 2006;175:739-743.
44) Shen Z, Pardington-Purtymun PE, Comeaux JC, Moyzis RK, Chen DJ. Associations of UBE2I with RAD52, UBL1, p53, and RAD51 proteins in a yeast two-hybrid system. Genomics 1996;37:183-186.
45) Hsieh YL, Kuo HY, Chang CC, Naik MT, Liao PH, Ho CC, et al. Ubc9 acetylation modulates distinct SUMO target modification and hypoxia response. EMBO J 2013;32:791-804.
46) Chen CS, Weng SC, Tseng PH, Lin HP. Histone acetylation-independent effect of histone deacetylase inhibitors on Akt through the reshuffling of protein phosphatase 1 complexes. J Biol Chem 2005;280:38879-38887.
47) Kim IA, No M, Lee JM, Shin JH, Oh JS, Choi EJ, et al. Epigenetic modulation of radiation response in human cancer cells with activated EGFR or HER-2 signaling: potential role of histone deacetylase 6. Radiother Oncol 2009;92:125-132.

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