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Blockade of SOX4 mediated DNA repair by SPARC enhances radioresponse in medulloblastoma

Chandramu Chetty^a, Ranadheer Dontula^d, Meena Gujrati^b, Dzung H. Dinh^c, and Sajani S. Lakka^{d,*}

^aDepartment of Cancer Biology and Pharmacology, University of Illinois College of Medicine at Peoria, 1 Illini Drive, Peoria, IL 61605, USA

^bDepartment of Pathology, University of Illinois College of Medicine at Peoria, 1 Illini Drive, Peoria, IL 61605, USA

^cDepartment of Neurosurgery, University of Illinois College of Medicine at Peoria, 1 Illini Drive, Peoria, IL 61605, USA

^dSection of Hematology and Oncology (MC713), Department of Medicine, University of Illinois College of Medicine at Chicago, 840 South Wood Street, Suite 820-E, Chicago, IL 60612, USA

Abstract

SPARC is a matricellular glycoprotein and a putative radioresistance-reversal-gene. We therefore explored the possibility of SPARC expression on medulloblastoma radiosensitivity *in vitro* and *in vivo*. The combined treatment of the SPARC and irradiation resulted in increased cell death when compared to cells treated with irradiation alone *in vitro* and *in vivo*. SPARC expression prior to irradiation suppressed checkpoints-1,-2 and p53 phosphorylation and DNA repair gene XRCC1. We also demonstrate that SPARC expression suppressed irradiation induced SOX-4 mediated DNA repair. These results provide evidence of the anti-tumor effect of combining SPARC with irradiation as a new therapeutic strategy for the treatment of medulloblastoma.

Keywords

SPARC; SOX4; Medulloblastoma; Radiosensitization

1. Introduction

Medulloblastoma is the most common primary central nervous system (CNS) tumors in children, comprising ~20% of all pediatric primary CNS tumors [1]. Standard therapy for medulloblastoma patients includes surgery and irradiation followed by chemotherapy, leading to 50–80% 5-yr survival. These tumors are radiosensitive; however, long-term side effects of radiation therapy have led to attempts to decrease the amount of craniospinal and local tumor site irradiation, with resultant disease relapse [2].

The efficiency of radiotherapy for cancer treatment is limited by toxic side effects, which impede dose escalation. Ionizing radiation acts through the induction of double-strand breaks to DNA to induce elimination of cancerous cells via apoptosis [3]. However, cancer

cells often develop radio-resistance mechanisms that are related to the DNA repair response. The interplay of DNA repair with DNA damage response pathways that determine death and survival [4], proliferative signals, and those derived from the tumor microenvironment [5] determines cellular radiosensitivity. Therefore, modulation of DNA damage response network to minimize repair function of tumor cells can potentially sensitize these cells to radiation.

Secreted protein acidic and rich in cysteine (SPARC) is a matricellular glycoprotein that modulates cellular interaction with the extracellular matrix and is expressed in tissues undergoing remodeling [6]. Tumor-derived SPARC is reported to stimulate or retard tumor progression depending on the tumor type. Expression of SPARC by melanoma and meningioma cells has been linked to an invasive phenotype *in vivo* [7,8]. In contrast, overexpression of SPARC by ovarian carcinoma cells led to increased tumor cell apoptosis, and the levels of SPARC were inversely correlated with tumor progression *in vivo* [9]. Studies using SPARC^{-/-} animals revealed that loss of SPARC enhanced the growth of tumor xenografts of pancreatic and lung cancers [10,11]. A high-throughput genomics approach have shown that modulation of SPARC expression affects colorectal cancer sensitivity to radiation and chemotherapy [12], suggesting that SPARC-based gene or protein therapy may ameliorate the emergence of resistant clones and offers a novel approach to treating cancer. The aim of combining gene therapy and radiation is to strengthen the efficiency of radiation by inhibiting DNA repair, thereby overcoming the clonogenic survival in irradiated cells and in turn apoptotic resistance.

We have previously shown that expression of SPARC inhibits the growth of medulloblastoma through autophagy and cell death [13]. In this study, we evaluated the potential of a SPARC gene-therapy approach using plasmid expressing SPARC cDNA to enhance the response of medulloblastoma tumors to X-ray irradiation (IR). We show that SPARC expression significantly enhanced the medulloblastoma cell radiosensitivity.

2. Materials and methods

2.1. Antibodies and reagents

The primary antibodies against SOX4, phospho-MPM2 (Ser/Thr/Pro), FoxM1 (MPM2), phospho-HistoneH3 (Ser-10) (Millipore Corporation, Billerica, MA), phospho-p53 (Ser-15), p53, SPARC, XRCC1, Caspase-3, Chk1, Chk2, Cdc2, phospho-Cdc2 (Thr14/Tyr15), 14-3-3, GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), phospho- γ -H2AX (Ser-139), PARP (EMD Biosciences, San Diego, CA), phospho-Cdc25C (Ser-216), α -tubulin, Caspase-8, and Caspase-9 (Cell Signaling, Boston, MA) were used. HRP-conjugated secondary antibodies, mouse-IgG (Santa Cruz Biotechnology, Santa Cruz, CA); Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA), DAB peroxidase substrate (Sigma, St. Louis, MO), TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end- labeling) detection kit (Roche Molecular Biochemicals, Indianapolis, IN), Apoptosis Detection Kit (BioVision Mountain View, CA); HuSH 29-mer siRNA Constructs against SOX4 in pRFP-C-RS vector (OriGene, Rockville, MD) were also used in this study.

2.2. Cell lines and culture conditions

We used D425 and UW228 cell lines (containing wild type p53) [14,15], and H2411 primary cells for this study. D425 and H2411 cells were kindly provided by Dr. Darell D. Bigner (Duke University Medical Center); and UW228 cells were kindly provided by Dr. Ali-Osman (Duke University Medical Center). The cells were authenticated on the basis of *c-myc* amplification, chromosomal aberrations [16]. At the 3rd or 4th passage of cells were frozen and these frozen stocks were used for further experimental studies up to the 10th passage to obtain consistent results. D425 and H2411 cells were cultured in Improved-MEM

(Zn Option) and UW228 cells were cultured in RPMI-1640 media. The media were supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37 °C.

2.3. Construction of pSPARC, cell transfections and irradiation

An 1100-bp cDNA of human SPARC was amplified by Reverse Transcription-PCR using synthetic primers and cloned into a pcDNA3.1 vector (Invitrogen, San Diego, CA) in sense orientation as described earlier [17]. Cells were transfected with pcDNA3.1 plasmid containing full-length cDNA of SPARC (pSPARC) or empty vector (pEV) using FuGene®HD (Roche, Indianapolis, IN) as per manufacturer's instructions. After 4–6 h of transfection, the necessary amount of serum containing medium was added. After 24 h of incubation, cells were irradiated with X-ray irradiation at a dose of 8 Gy (using The RS 2000 Biological Irradiator; Rad Source Technologies, Inc., Boca Raton, FL), the medium replaced, and cells were incubated for a further 6 h or for the indicated times.

2.4. Immunoblot analysis

D425 and UW228 cells were transfected and irradiated (8 Gy) as above. Whole cell lysates were prepared by lysing cells in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% IGEPAL; 1 mM EDTA; 0.25% sodium deoxycholate; 1 mM sodium fluoride; 1 mM sodium orthovanadate; 0.5 mM PMSF; 10 µg/ml aprotinin; 10 µg/ml leupeptin), as described earlier [13]. Equal amounts of protein fractions were resolved over SDS-PAGE and transferred onto the PVDF membrane. Proteins were detected with primary antibodies followed by HRP-conjugated secondary antibodies. ECL plus western blotting detection reagents were used and visualized signals using FluorChemQ (Alpha Innotech, San Leandro, CA). Comparable loading of proteins on the gel was verified by re-probing the blots with an antibody specific for the housekeeping gene, *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*.

2.5. Reverse transcription-PCR (RT-PCR)

Total RNA was extracted using TRIZOL reagent (Life Technologies, Grand Island, NY) according to manufacturer's protocol, and RT-PCR was performed as described previously [13]. PCR products were resolved on 2% agarose gels and were visualized by ethidium bromide staining. To normalize for the amount of input RNA, RT-PCR was performed with primers for the *GAPDH*. The specific primers used in this study were as follows: SPARC, forward 5'-GGAAGAACTGTGGCAGAGG-3' and reverse 5'-ATTGCTGCACACCTTCTCAA-3'; *GAPDH*, forward 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and reverse 5'-CATGTGGGCCATGAGGTCCACCAC-3'. To determine the quantity of PCR products on the agarose gel, images were generated by Alpha Innotech Image Acquisition and Analysis Software (Alpha Innotech, San Leandro, CA) and processed for display.

2.6. Clonogenic assay

Cells were transfected with mock, pEV, and pSPARC for 24 h. Cells were trypsinized, irradiated (8 Gy) and seeded in 100 mm Petri dishes (5×10^2 cells). On day 10 after irradiation, cells were fixed in cold methanol and stained with Giemsa and colonies (>50 cells) were counted. The plating efficiency (PE) is defined as the number of colonies observed/the number of cells plated. Surviving fraction (SF) is the colonies counted divided by the number of colonies plated with a correction for the plating efficiency.

$$\text{Survival fraction (SF)} = \frac{\text{Colonies counted}}{\text{Cells seeded} \times (\text{PE}/100)}$$

2.7. Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick labeling (TUNEL) assay

To evaluate the apoptotic response of pSPARC and irradiation (IR), we performed TUNEL using the commercially available *in situ* cell death detection kit (Roche, Indianapolis, IN), as per the manufacturer's instructions. Briefly, cells were seeded onto 8-well chamber slides (5×10^3) and transfected with mock, pEV or pSPARC and irradiated as described above. The cells were washed and fixed with 4% buffered para-formaldehyde and permeabilized with 0.1% Triton-X100, 0.1% sodium citrate solution followed by incubation with TUNEL reaction mixture for 1 h at 37 °C in a humidified chamber. The incorporated biotin-dUTP was detected under a fluorescence microscope. For the paraffin-embedded tissue sections, slides were dewaxed, rehydrated, and permeabilized according to the standard protocols and processed as above. The apoptotic index was calculated as follows: apoptotic index (%) = $100 \times (\text{apoptotic cells}/\text{total cells})$.

2.8. Fluorescence Activated Cell Sorting (FACS) analysis

Propidium iodide (PI) positive cells were determined as described earlier [13]. Briefly, cells were transfected and irradiated as described above. Cells were trypsinized, washed with cold PBS, and incubated with 5 μ l of PI in $1 \times$ binding buffer at room temperature for 10–15 min in the dark. FACS analysis was performed using a FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA) with an excitation wavelength of 488 nm and emission wavelength of 530 nm. Data acquisition and analysis were performed using CellQuest software (BD Biosciences, San Jose, CA).

2.9. Animal experiments

D425 cells stably transfected for luciferase expression and (1×10^5 cells/ 10μ L) were stereotactically implanted as described previously [17]. Twelve days after tumor cell implantation, the animals were randomized into six groups (10/group). ALZET osmotic pumps (model 1007D; ALZET Osmotic Pumps, Cupertino, CA) containing 100 μ l volume of Mock (PBS; Groups 1 and 4), pEV (Groups 2 and 5) or pSPARC (Groups 3 and 6) were implanted for plasmid delivery (dose: 3–6 mg/kg body weight) as described previously [18]. The Groups 4–6 received 2 doses of irradiation (4 Gy $\times$ 2) on days 15 and 17 after cell implantations. Animals which lost 20% of body weight or had trouble ambulating, feeding, or grooming were sacrificed. Four animals from each group were euthanized 2 days after last radiation treatment (19th day) and brains were collected and fixed in 10% buffered formalin. Remaining animals (6/group) were monitored for up to 4 weeks after treatments, which is when we arbitrarily terminated the experiment. Tumor burden was quantified by luciferase *in vivo* imaging (Bioluminescence imaging) using an IVIS-200 Xenogen imaging system (Xenogen Corporation, CA, USA) until the study termination. Mice brains were fixed in 10% buffered formalin and embedded in paraffin. The brain sections were stained with hematoxylin and eosin, and the tumor volume was calculated as described previously with a few modifications [19]. Briefly, the tumor area from every fifth hematoxylin and eosin stained tumor section (5 μ m) was calculated digitally with the aid of microscope attached to a computer and Image Pro Discovery Program software (Media Cybernetics, Inc., Silver spring, MD). The total tumor volume was calculated as the sum of the tumor area on all sections multiplied by the width of the sections.

2.10. Immunohistochemical analysis

The fixed tissue samples were then processed into paraffin blocks. Tissue sections (5 μm) were subjected to immunostaining for SPARC, SOX4 and XRCC1 as described earlier [17]. Briefly, after deparaffinization and rehydration, tissue sections were permeabilized in 0.1% TritonX-100 in PBS. Endogenous peroxidase activity was quenched by 3% H_2O_2 in methanol for 20 min followed by PBS rinses. Non-specific binding was blocked for 1 h at room temperature with 1% BSA in PBS followed by incubation for 1 h at room temperature with primary antibody or anti-mouse IgG (negative control). The slides were rinsed in PBS and incubated for 1 h with HRP-conjugated secondary antibody. The slides were washed and stained with diaminobenzidine (DAB) peroxidase substrate solution and then counterstained with hematoxylin. Mounted specimens were observed and photographed using light microscopy.

2.11. Analysis

Protein bands were quantified using ImageJ software (from the NIH), and all bands were normalized to their GAPDH controls. All data are presented as mean $\pm$ SE of at least three independent experiments, each performed at least in triplicate. Statistical analysis was performed using the student's *t*-test or a one-way analysis of variance. $p < 0.05$ was considered significant.

3. Results

3.1. Irradiation inhibits SPARC expression in medulloblastoma cells

We previously demonstrated that human medulloblastoma tissue samples expressed very low or minimal levels of SPARC when compared with normal cerebellum [20]. SPARC was shown as a putative therapy resistance reversal gene whose expression was significantly decreased in resistant cancer cells [12]. To examine the effect of irradiation on SPARC expression, we determined SPARC protein levels in UW228 and D425 medulloblastoma cells. Fig. 1A indicates that SPARC expression levels were reduced with radiation in a dose dependent manner. SPARC expression was inhibited by 60–65% at 8 Gy compared to the control.

3.2. SPARC expression enhances medulloblastoma cell death to radiation

Initially, we constructed a plasmid vector expressing SPARC full-length cDNA (pSPARC). To confirm the expression of SPARC from the engineered plasmid, we transfected medulloblastoma cells with pSPARC for 24 h and irradiated (8 Gy), and incubated for a further 6 h. Immunoblot and RT-PCR analyses of SPARC protein and mRNA, respectively, in medulloblastoma cells revealed that pSPARC enhanced the expression of SPARC protein and mRNA in a dose-dependent manner as compared with mock and pEV-transfected controls. Fig. 1B shows that SPARC protein and mRNA levels increased up to 3–4-folds in both D425 and UW228 cell lines transfected at a 2 $\mu\text{g}/\text{ml}$ of pSPARC concentration. We performed colony-forming assay to determine the effect of SPARC expression on cell survival in irradiated cells. Fig. 1C indicates that overexpression of SPARC conferred increased sensitivity to radiation, as fewer colonies were formed compared to cells treated with radiation alone. No significant changes in cell growth were observed for the combination of pEV (Empty vector) and radiation when compared to treatment with radiation alone.

3.3. SPARC expression augments γ -H2AX phosphorylation in irradiated cells

Histone γ -H2AX phosphorylation induces DNA fragmentation and degradation through activated DNase, which indicates that γ -H2AX phosphorylation at Ser-139, is a prerequisite

for the cell death process [21]. γ -H2AX is rapidly phosphorylated at the Ser139 residue in response to IR or other agents that introduce DNA double-strand breaks [22]. We therefore evaluated whether SPARC expression enhanced irradiation induced DNA damage in medulloblastoma cells. Fig. 2A show a 10–12-fold ($p < 0.01$) increase in phospho- γ -H2AX (S-139) in cells treated with pSPARC and IR treatments compared to cells that received IR alone (2–2.5-folds; also see Suppl. Fig. S1). We next evaluated the kinetics of DNA repair gene XRCC1 in cells that received pSPARC and IR. XRCC1 expression was suppressed at initial time points but was restored to control levels by 36–48 h in cells treated with IR alone. However, cells that received pSPARC treatment before IR significantly decreased the expression of XRCC1 protein level at 48 h time point (Fig. 2B; also see Suppl. Fig. S1). These results show that SPARC expression suppressed DNA repair in irradiated medulloblastoma cells.

3.4. SPARC expression suppresses checkpoints expression and p53 phosphorylation in irradiated cells

In response to DNA damage, an elaborate network of signaling pathways, collectively called the DNA damage checkpoint, are activated to prevent the damaged DNA from being replicated or transmitted to the next generation. The main function of the DNA damage checkpoint is to halt the cell cycle progression until the damaged DNA is repaired [23,24]. There was a significant increase in both Chk1 and Chk2 levels in medulloblastoma cell lines and primary cells treated with IR compared to controls, whereas pSP-ARC treatment prior to IR suppressed IR induced Chk1 and Chk2 levels (Fig. 2C and Suppl. Fig. S1). Both of these kinases phosphorylate multiple sites in the N-terminal domain of p53 which in turn binds to an array of gene promoters, such as p21/waf1, which are responsible for DNA damage induced cell cycle arrest [25]. Fig. 2C shows that expression and phosphorylation of p53 were increased in irradiated cells; while, SPARC expression prior to radiation strongly inhibited radiation-induced phosphorylation of p53 (also see Suppl. Fig. S1).

3.5. SPARC expression alleviates irradiation induced G2/M arrest

Phosphorylation of Cdc25C at Ser-216 by Chk1/Chk2 both, inactivates its phosphatase activity directly and creates a 14-3-3-binding site. Binding of 14-3-3 masks a proximal nuclear localization sequence and anchors Cdc25C in the cytoplasm, preventing efficient access of Cdc25C to cyclin-B1-Cdc2 complex [25]. Corresponding to these studies, we show that radiation induced phosphorylation of Cdc25C on Ser-216 (Fig. 3A). Activation of Cdc2 kinase requires dephosphorylation at Thr-14/Tyr-15 residues [25]. We therefore determined the Thr-14/Tyr-15 phosphorylation status of Cdc2 in cells treated with radiation and pSPARC. Fig. 3A indicates the accumulation of the phosphorylated-Cdc2 (Thr-14/Tyr-15) (negatively regulates cell cycle progression) in irradiated cells. However, SPARC expression in combination with radiation decreased radiation-induced phosphorylation of Cdc25C (Ser-216), which in turn results in activation of Cdc2 (dephosphorylates Cdc2) leading to cell cycle progression through G2 phase. We, next immunoprecipitated 14-3-3 proteins from total cell lysates of pSPARC and radiation treated cells using pan-14-3-3 antibody and performed immunoblot analysis for Cdc25C. As shown in Fig. 3B, showed high band intensities for 14-3-3 bound phospho-Cdc25C (Ser-216) in irradiated samples compared to mock and pEV transfected cells. However, 14-3-3 bound phospho-Cdc25C (Ser-216) band intensities were drastically reduced with pSPARC transfection prior to irradiation. Subsequently, we performed FACS analysis for DNA content and results indicated a significant increase in the proportion of G2/M cells with IR treatment alone; while there was a significant decrease in the proportion of G2/M population in cells which received a combination treatment of pSPARC and IR (Fig. 3C). Taken together, these results suggest that SPARC expression prior to radiation released 14-3-3 bound phospho-Cdc25C

which in turn dephosphorylated Cdc2 at Thr-14/Tyr15, leading to cell cycle progression through G2 phase.

3.6. SPARC expression enhances sub-G1 population and increases TUNEL positive cells in irradiated medulloblastoma cells

The flow cytometric analysis of cells indicated a significant increase in the Sub-G1 population in cells that received a combination of pSPARC and IR treatments (Fig. 3C). We next determined whether SPARC expression was sufficient to cause mitotic catastrophe-mediated apoptosis in medulloblastoma cell lines. We found that giant multinucleated cells that are compatible with mitotic catastrophe in cells treated with pSPARC and IR (Fig. 4A). Further, pSPARC and IR-treated cells exhibited a significant increase in the population of multipolar spindles as observed with double labeling with anti- α -tubulin and DAPI (Fig. 4B) while, mitotic catastrophe was only minimally present with the highest incidence (being < 5%) with pSPARC or IR alone (data not shown). We next performed immunoblot analysis for mitosis-specific markers, phospho-histoneH3 (Ser-10) and phospho-MPM2 (Ser/Thr/Pro) in cells treated with pSPARC and IR. Fig. 4C demonstrated that there was a 70–80% increase in the expression of phospho-histoneH3 and phospho-MPM2 in IR-treated cells compared with cells treated with mock and empty vector. However there was ~70% decrease in the expression of phospho-HistoneH3 and phospho-MPM2 in cells treated with both pSPARC and IR. To determine whether SPARC expression enhanced IR-induced apoptosis in D425 and UW228 medulloblastoma cell lines and H2411 primary cells, we performed TUNEL assay. Apoptotic cell death is minimum (7–10%) following treatment with radiation alone, this proportion increased up to 60% in cells treated with pSPARC and IR (Fig. 5A, B and also see Suppl. Fig. S1). Taken together, these results suggest that cells receiving IR alone were able to exit the G2 phase, enter the M phase, and be arrested in M, while pSPARC treatment before caused cells to exit mitosis but generally failed to complete cytokinesis, eventually resulting in apoptosis in irradiated cells.

3.7. SRY-related HMG-box-4 (SOX4) contributes to SPARC-mediated radiosensitivity

We next sought to determine the molecular mechanisms underlying SPARC induced radiosensitivity, SOX4 is required for p53 activation in response to DNA damage and was shown to promote cell cycle arrest in a p53-dependent manner [26]. Since D425 and UW228 cells express wild type p53 [14,15], we next investigated the involvement of SOX4 in pSPARC enhanced radiosensitivity of medulloblastoma cells. We treated medulloblastoma cells with pSPARC and IR alone and in combination, and performed immunoblot analysis for SOX4 and p53 proteins. IR induced SOX4 protein levels paralleled with the induction of p53 protein by 2–2.5-fold (Fig. 5C; $p < 0.01$; and Suppl. Fig. S1). Interestingly, pSPARC treatment along with IR blocked radiation-induced SOX4 expression (Fig. 5C; and Suppl. Fig. S1). To evaluate the functional role of SOX4 in DNA repair in radiated cells, we used RNA interference to down regulate the endogenous SOX4 expression. A pool of siRNA constructs, directed against SOX4, efficiently depleted cells of endogenous SOX4 (Fig. 5D). Radiation-induced increases of p53 (S-15) phosphorylation and XRCC1 proteins were dramatically decreased with high levels of phospho- γ -H2AX levels in cells treated with SOX4 siRNA and radiation compared with cells subjected to radiation alone, suggesting that SOX4 functions upstream of p53-mediated DNA repair (Fig. 5D). Taken together, these results suggest that SPARC expression blocks radiation-induced SOX4 expression, which in turn inhibits DNA repair leading to increased cell death.

3.8. Overexpression of SPARC increases radiosensitivity of medulloblastoma cells in an intracranial model in vivo

We next attempted to determine whether SPARC expression could enhance tumor growth regression in radiated tumors *in vivo* using tumor xenografts in nude mice. Following

implantation of D425 cells, the animals were exposed to 2 cycles of 4 Gy irradiation along with intratumoral injection of pSPARC using ALZET mini-osmotic pumps. One set of animals were euthanized and collected brains from each treatment after 2 days of post-radiation. Brain tissue sections from mice that received radiation alone showed intense staining for SOX4 and XRCC1. However, SOX4 and XRCC1 expression was decreased significantly in tissue sections from mice treated with pSPARC and radiation, thereby confirming our *in vitro* results (Fig. 6A). Another set of mice were euthanized 4 weeks after post-radiation treatment and tumor volumes were determined in the brain sections. Histologic analysis of Hematoxylin and Eosin (H&E) stained brain tumor sections showed ~60% reduction of tumor volume (48.13 ± 14.35 vs 21.03 ± 11.85 ; $p < 0.01$ for pEV vs pSPARC) in the brains of mice treated with pSPARC as compared with mock and pEV controls. However, a ~75% reduction in the tumor volumes (48.13 ± 14.35 vs 11.94 ± 14.06 ; $p < 0.01$ for pEV vs pSPARC + IR) was observed in mice treated with pSPARC before radiation compared with IR and pEV-treated controls (Fig. 6B and C). Furthermore, we carried out the TUNEL assay to detect the number of apoptotic cells in tumor sections from mice treated with pSPARC and IR. Corresponding to their growth characteristics, we found very few apoptotic cells (15.16 ± 2.54 and 17.52 ± 3.67 cells/field) in irradiated tumors compared to mock and pEV controls. The number of apoptotic cells in the tumor sections from mice treated with pSPARC was increased to 51.00 ± 6.25 cells/field. However, we found abundant TUNELpositive cells in the tumor sections from mice that received a combination of pSPARC and radiation treatments (229 ± 25.95 cells/field; Fig. 6D).

4. Discussion

Radiation sensitization of tumors has been a long-term goal of radiation oncologists and biologists. The goal of combining gene therapy with radiation is to maximize the selective pressure against cancer cell growth while minimizing treatment-associated toxicity. We have previously shown that expression of SPARC in human medulloblastoma tissue samples was very low when compared with normal cerebellum [20], and expression of SPARC inhibits medulloblastoma tumor growth and angiogenesis *in vitro* and *in vivo* [13,17]. In this study we evaluated the effect of combining SPARC expression along with radiation in an orthotopic medulloblastoma tumor. We found that SPARC expression before radiation augmented the effects of radiation and successfully regressed tumors. This study not only gives an understanding of the mechanism, but also reveals the fact that the SPARC expression increases the effectiveness of treatment when combined with radiation. We demonstrate that SPARC expression enhanced radiation-induced DNA damage by blocking DNA repair. Our studies also highlight SOX4, a member of the SOX (SRY-related HMG-box) transcription factor family, as a potential key factor in SPARC-induced radiosensitivity.

Double-strand breaks are produced under physiologic circumstances in normal tissues, but at a very low level, and using targeted radiation therapy to selectively increase their representation within tumors offers a possibility for achieving a therapeutic advantage. Because of the propensity of ionizing radiation to cause double-strand breaks, inhibition of these repair pathways in particular promises to enhance the efficacy and selectivity of standard tumor radiation therapy. The pattern of decreasing SPARC mRNA and protein expression with the radiation and suppression of DNA repair gene *XRCC1*, with the concomitant increase in DNA damage as demonstrated by an increase in γ -H2AX phosphorylation at Ser-139 in cells treated with pSPARC and IR, suggests that SPARC may play a role in regulating DNA repair. A variety of approaches have been used to target DNA double-strand break repair molecules for radiosensitization, including small interfering RNA (siRNA), aptamers and antisense [27–30]. Mitotic catastrophe is a cell death mode occurring either during or shortly after a dysregulated/failed mitosis and can be accompanied by

morphological alterations, including micronucleation and multinucleation [31]. Recently, mitotic catastrophe has been widely reported as an effect of conventional chemotherapeutic drugs, such as the anti-microtubule agents, taxans and vinca alkaloids, and by IR and DNA-damaging drugs [32]. We observed that giant multinucleated cells that are compatible with mitotic catastrophe were often present in medulloblastoma cells treated with pSPARC and IR. Mitotic catastrophe occurs as a result of DNA damage or deranged spindle formation coupled to the debilitation of different checkpoint mechanisms that would normally arrest progression into mitosis and hence suppress catastrophic events until repair has been achieved [32]. Therefore, the combination of checkpoint deficiencies and specific types of damage would lead to mitotic catastrophe. Our data demonstrate that SPARC expression suppressed checkpoints-1 and -2 in medulloblastoma cells leading to mitotic catastrophe following by apoptosis. Previous studies demonstrate that mitotic catastrophe induced by histone acetyltransferases such as CBP and p300 depletion appears to be mediated by γ -H2AX phosphorylation, which ultimately leads to DNA fragmentation condensation. The pharmacological inhibition or genetic suppression of several G2 checkpoint genes such as ATM, Chk1 and Chk2 can promote DNA-damage-induced mitotic catastrophe [33–35]. The inhibition of Chk2 was shown to abolish the cell-cycle arrest, thereby facilitating apoptosis induction in doxorubicin-treated HCT116 cells [33]. Activation of Chk1 and Chk2 results in phosphorylation of Cdc25C at Ser-216, which promotes the binding of Cdc25C to 14-3-3 proteins. Thus bound, Cdc25C is sequestered in the cytoplasm, unable to dephosphorylate and activate Cdc2. With Cdc2's inhibitory phosphorylation intact, cells undergo G2 arrest [33]. Our data indicates that SPARC expression prior to IR suppresses binding of Cdc25C to 14-3-3 protein allowing cell cycle progression. We also show that pSPARC expression sustains the suppression of the DNA repair gene XRCC1 whereas, XRCC1 expression is restored to control levels in cells within 36–48 h with IR treatment alone, suggesting that SPARC expression alters IR induced cell cycle arrest and DNA repair.

SOX4 is a critical developmental transcription factor in vertebrates and is required for precise differentiation and proliferation in multiple tissues [36–38]. In addition, *SOX4* is overexpressed in many other types of human cancers including medulloblastoma [39,40]. Down-regulation of *SOX4* expression in prostate cancer cell lines resulted in a strong decrease in cell viability and a corresponding increase in apoptosis [41]. *SOX4* induction has also been shown to impair cell viability and induce apoptosis [42]. The contradictory effect of *SOX4* expression on apoptosis in different cell types suggests that *SOX4*, like *c-Myc*, has both anti- and pro-apoptotic activities. The balance between anti- and pro-apoptotic signals induced by *SOX4* expression was suggested to be tissue-specific and dependent on external signals. However, the precise mechanism by which *SOX4* is involved in tumorigenesis remains largely unknown. We demonstrate that *SOX4* induces p53-mediated DNA repair in cells treated with radiation alone. The significance of the *SOX4* induction in this event lies in the fact that suppression of *SOX4* in pSPARC-treated cells impairs p53 activation and consequently DNA repair functions leading to cell death. Collectively our results suggest that SPARC expression modulates *SOX4* mediated DNA repair.

In conclusion, we established the SPARC expression suppressed *SOX4* induction in irradiated cells which is an important p53 regulator involved in DNA repair and exerts its inhibitory role on DNA repair leading to mitotic catastrophe-mediated apoptosis and suggests a new strategy for improving radiosensitivity. SPARC gene therapy combined with radiotherapy may have potential clinical application to improve tumor control for medulloblastoma patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.canlet.2012.04.014>.

Fig. 1.

X-ray radiation inhibits SPARC expression in medulloblastoma cell lines. (A) D425 and UW228 cells were irradiated (IR) with X-ray (0–14 Gy), incubated for 6 h, and cells were collected. SPARC expression was determined by immunoblot (IB) analysis in cell lysates. The blots were stripped and reprobed with an antibody against the housekeeping gene *GAPDH* to verify equal loading. (B) Cells were transfected with mock, pEV (2 µg/ml) or pSPARC (1 or 2 µg/ml). After 24 h of incubation, cells were irradiated (8 Gy) and incubated for a further 6 h. *Upper panel*: Total cell lysates were used to determine SPARC levels by immunoblot (IB) analyses. GAPDH was also immunodetected as a control to confirm equal loading of cell lysates. *Bottom panel*: Total RNA was used to determine SPARC mRNA transcription levels by RT-PCR with gene-specific primers. Expression of GAPDH was verified for the uniform levels of cDNA. (C) After 24 h of transfection, cells were irradiated (IR; 8 Gy) and clonogenic assay was performed as described in Section 2. After 10 days the colonies (>50 cells) were counted. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.05$, significant difference from mock; ** $p < 0.01$, significant difference from IR.

Fig. 2. SPARC expression aggravates radiation-induced DNA damage response *via* DNA repair gene inhibition. D425 and UW228 cells were transfected with mock, pEV or pSPARC. After 24 h of incubation, cells were irradiated (IR; 8 Gy) and incubated for a further 6 h. (A) Cellular extracts were subjected to immunoblotting with antibodies specific for phospho-γ-H2AX (Ser-139), a marker for DNA double strand breaks and XRCC1. The blots were stripped and reprobed with an antibody against the housekeeping gene GAPDH to verify equal loading. Densitometric analysis of phospho-γ-H2AX and XRCC1 bands against corresponding GAPDH protein band density is shown. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.01$, significant difference from IR. (B) Cells were collected 0–48 h after IR and cellular extracts were subjected to immunoblotting with antibodies specific for phospho-γ-H2AX and XRCC1. The blots were stripped and reprobed with an antibody against the housekeeping gene GAPDH to verify equal loading. Densitometric analysis of phospho-γ-H2AX bands against corresponding GAPDH band density is shown. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.01$, significant difference from IR. (C) Cellular extracts were subjected to immunoblotting with antibodies specific for Chk1, Chk2, p53 and phospho-p53 (Ser-15). The blots were stripped and reprobed with an antibody against the housekeeping gene GAPDH to verify equal loading. Densitometric analysis of phospho-p53 (Ser-15) bands against GAPDH band density is shown. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.01$, significant difference from IR.

Fig. 3.

SPARC downregulates mitosis-associated proteins during induction of cell death through mitotic catastrophe. D425 and UW228 cells were transfected with mock, pEV (2 μg/ml) or pSPARC (1 or 2 μg/ml). After 24 h of incubation, cells were irradiated (IR; 8 Gy) and incubated for a further 6 h. (A) Cellular extracts were subjected to immunoblotting with antibodies specific for SPARC, Cdc2, phospho-Cdc2 (Thr-14/Try-15) and phospho-Cdc25C (Ser-216). The blots were stripped and reprobed with an antibody against the housekeeping gene GAPDH to verify equal loading. (B) 14-3-3 proteins were co-immunoprecipitated (IP) from total cell lysates with pan-14-3-3 antibody. Equal volumes of immunocomplexes were resolved over SDS-PAGE and performed immunoblot (IB) analysis for Cdc25C. The blot was re-probed with pan 14-3-3 antibody for loading control. (C) After 24 h of transfection, cells were irradiated (IR; 8 Gy) and stained with propidium iodide and evaluated DNA content for cell cycle by flow cytometry. Graphic representations of the cell cycle profiles were shown.

Fig. 4.

SPARC expression enhances radiation-induced DNA damage. D425 and UW228 cells were transfected with mock, pEV (2 μ g/ml) or pSPARC (1 or 2 μ g/ml). After 24 h of incubation, cells were irradiated (IR; 8 Gy) and incubated for a further 6 h. (A) Cells were nuclear stained with DAPI and photographed. (B) Immunofluorescence analysis for mitotic spindles (α -tubulin; red), and DNA (DAPI; blue) was performed. (C) Total cell lysates were immunoblotted for mitotic markers; phospho-MPM2 (Ser/Thr/Pro) and phospho-histoneH3 (Ser-10) using specific antibodies. The blots were stripped and reprobed with an antibody against the housekeeping gene GAPDH to verify equal loading. Densitometry normalization of phospho-MPM2 (Ser/Thr/Pro) and phospho-histoneH3 (Ser-10) against corresponding GAPDH protein band density is shown. Columns: mean of triplicate experiments; bars: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.01$, significant difference from IR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

SPARC expression enhances DNA damage and radiosensitivity. D425 and UW228 cells were transfected with mock, pEV or pSPARC and irradiated as described in Section 2. (A) Twelve hours after irradiation, apoptosis was detected using the TUNEL assay. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.01$, significant difference from IR. (B) Total cell lysates were immunoblotted for cleavage of caspases-8, -9 and -3, and PARP1 using specific antibodies. The blots were stripped and reprobed with an antibody against the housekeeping gene *GAPDH* to verify equal loading. (C) Cellular extracts were subjected to immunoblotting with antibodies specific for SOX4. The blots were stripped and reprobed with an antibody against the housekeeping gene *GAPDH* to verify equal loading. Densitometric analysis of SOX4 bands against *GAPDH* band density is shown. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.01$, significant difference from IR. (D) SOX-siRNA augments radiation-induced DNA damage response similar to SPARC expression. D425 and UW228 cells were transfected with mock, pEV, pSPARC, pSOX4-Si or pSPARC + pSOX4-Si. After 24 h of incubation, cells were irradiated (8 Gy) and incubated for a further 6 h. Cellular extracts were subjected to immunoblotting with antibodies specific for SOX4, p53, phospho-p53 (Ser-15), phospho- γ -H2AX (ser-139) and XRCC1. The blots were stripped and reprobed with an antibody against the housekeeping gene *GAPDH* to verify equal loading. Densitometric analysis of SOX4, phospho- γ -H2AX and XRCC1 band densities normalized to corresponding *GAPDH* protein band density are shown. *Columns*: mean of quadruplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.01$, significant difference from IR.

Fig. 6.

SPARC expression enhances radiosensitivity *in vivo*. Mice were implanted D425 cells and treated with pSPARC and radiation (IR) as described in Section 2. (A) Representative tumor sections are shown from mice after a 2-day time point. Hematoxylin and eosin (H&E) staining and immunohistochemical analysis of SPARC, SOX4 and XRCC1 expression are shown (60×). Inset: Negative control. (B) Representative H&E staining of xenograft sections from mice following 4 weeks of treatment are shown (2.5×). (C) Graphical representation of tumor volumes of individual tumors in mice with various treatments. Each diamond represents one animal and bars (red) represent mean ($n = 6$). * $p < 0.05$ (pSPARC vs pSPARC + IR); * $p < 0.01$ (pEV + IR vs pSPARC + IR). (D) TUNEL staining of tissue sections. Also shown is the negative control (Inset). Quantitation of apoptotic cells indicated an increase in number of TUNEL-positive cells in tumor sections from mice treated with pSPARC and IR. *Columns:* mean of triplicate experiments; *bars:* SE; of 8–10 sections from six animals in the same treatment group. * $p < 0.01$ significant difference from mock; ** $p < 0.01$, significant difference from IR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)