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Inhibition of HAS2 induction enhances the radiosensitivity of cancer cells via persistent DNA damage

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ABSTRACT

Hyaluronan synthase 2 (HAS2), a synthetic enzyme for hyaluronan, regulates various aspects of cancer progression, including migration, invasion and angiogenesis. However, the possible association of HAS2 with the response of cancer cells to anticancer radiotherapy, has not yet been elucidated. Here, we show that HAS2 knockdown potentiates irradiation-induced DNA damage and apoptosis in cancer cells. Upon exposure to radiation, all of the tested human cancer cell lines exhibited marked (up to 10-fold) up-regulation of HAS2 within 24 h. Inhibition of HAS2 induction significantly reduced the survival of irradiated radioresistant and -sensitive cells. Interestingly, HAS2 depletion rendered the cells to sustain irradiation-induced DNA damage, thereby leading to an increase of apoptotic death. These findings indicate that HAS2 knockdown sensitizes cancer cells to radiation via persistent DNA damage, further suggesting that the irradiation-induced up-regulation of HAS2 contributes to the radioresistance of cancer cells. Thus, HAS2 could potentially be targeted for therapeutic interventions aimed at radiosensitizing cancer cells.

1. Introduction

Hyaluronan, a major constituent of the extracellular matrix, is a linear, high molecular glycosaminoglycan composed of a repeating disaccharide unit of glucuronic acid and N-acetyl glucosamine [1,2]. It interacts with several plasma membrane receptors [3] to modulate receptor-mediated physiological changes [3,4]. The synthetic enzymes of hyaluronan, the hyaluronan synthases (HAS1, -2 and -3), control the cellular content of hyaluronan together with the hyaluronidases (HYAL1, -2 and -3), the hyaluronan-degrading enzymes [5].

The hyaluronan synthases each possess distinct enzymatic activities and synthesize hyaluronan polymers of different molecular sizes [6]. Of the three HAS genes, only deletion of HAS2 is embryonic lethal in mice [7]. External stimuli that induce HAS2 expression have been associated with notable changes in the rate of hyaluronan synthesis [8], and various cytokines and growth factors have been shown to control HAS2 promoter activity via the recruitment of transcription regulators [9]. Notably, overexpression of HAS2 and up-regulation of hyaluronan are commonly found in human cancers [10–14], and HAS2 overexpression has been shown to induce an epithelial-mesenchymal transition and transformation in normal epithelial cells [15]. HAS2 overexpression promotes various processes related to cancer progression, including invasion, migration and angiogenesis, in malignant cancer cells [16–18], and HAS2 inducers (e.g., osteopontin and transforming growth factor-β) have been shown to elevate the malignancy of cancer cells [19,20]. Conversely, malignant phenotypes (i.e., invasion, migration, and angiogenesis) are attenuated by HAS2 depletion [21,22]. Thus, the cellular content of HAS2 and hyaluronan are clearly important for carcinogenesis and cancer progression.

Hyaluronan has been suggested to protect normal tissues (e.g., skin and intestine) from the injury and inflammation induced by toxic substances, including UV irradiation [23]. Indeed, the binding of hyaluronan to the Toll-like receptors, TRL2 and TRL4, has been shown to mediate the repair of damaged tissues [24]. Thus, increased levels of hyaluronan, whether due to exogenous addition or HAS activation, appear to protect tissues against injury and inflammation. However, no previous study has examined the potential association of HAS2 with the response of cancer cells to radiotherapy.

In the present study, we investigated whether HAS2 can modulate the radiosensitivity of cancer cells. We found that HAS2 knockdown radiosensitized both radioresistant and -sensitive cancer cells via persistent DNA damage, and that radiation-induced significant up-regulation of HAS2 in cancer cells, contributed to their radioresistance by reducing DNA damage. The biggest obstacle limiting effective cancer therapy is the occurrence of radio- and chemoresistant cancer cells. Thus, researchers are currently

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seeking to reverse the resistant phenotype and maximize the toxicity of therapeutic agents toward cancer cells while minimizing it in normal cells. In this respect, we propose that HAS2 inhibition might be a good strategy for radiosensitizing cancer cells in which HAS2 is endogenously overexpressed or induced by radiation. Further, HAS2 may be a promising anticancer target for the development of new radiation sensitizers.

2. Materials and methods

2.1. Cell culture

HCT116 and U2OS cells were grown in McCoy's 5a Medium (Cat. No. LM005-01; Welgene, Korea) and H460 cells were grown in RPMI (Cat. No. LM011-01; Welgene). All media were supplemented with 10% (v/v) fetal bovine serum (Cat. No. 43640; JRS, CA) and 1% (w/v) penicillin/streptomycin (Cat. No. 15140; Gibco, CA). Cells were incubated at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2.

2.2. Irradiation and RNA preparation

All cells examined were treated with 2 or 4 Gy of γ-irradiation, using a Gammacell® 3000 Elan irradiator (^{137}Cs A-ray source; MDS Nordin, ON, Canada), The irradiated cells were maintained for 12 or 24 h, and total RNA was extracted with Ribospin columns (Cat. No. 304-150; GeneAll, Korea). The total RNA concentration was quantified using an ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA) and the quality was checked using a Bioanalyzer 2100 (Agilent Technology, Santa Clara, CA, USA). The recommended RNA quality parameters were an OD 260/280 ratio in the range of 1.8–2.0.

2.3. RT-PCR

Complementary DNA (cDNA) was synthesized from the total RNA using a Maxime RT PreMix kit (Cat. No. 25081; Intron Biotechnology, Korea). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a Maxime PCR PreMix kit (i-StarTaq; Cat. No. 25167; Intron Biotechnology). The following primer sequences were used: HAS2, forward 5′-AGA GCA CTG GGA CGA AGT GT-3′ and reverse 5′-TCC CAG AGG TCC ACT AAT GC-3′; p21, forward 5′-GGA AGA CCA TGT GGA CCT GT-3′ and reverse 5′-GGC GTT TGG AGT GGT AGA AA-3′; and β-actin, forward 5′-AGC GAG CAT CCC CCA AAG TT-3′ and reverse 5′-GGG CAC GAA GGC TCA TCA TT-3′.

2.4. Real-time RT-PCR

The obtained cDNA was also subjected to real-time PCR amplification using the iQ SYBR Green supermix (BioRad, Hercules, CA, USA) and the following gene-specific primers: HAS2, forward 5′-CAG AAT CCA AAC AGA CAG TTC-3′ and reverse 5′-TAA GGT GTT GTG TGT GAC TG-3′; and β-actin, forward 5′-GCA AGC AGG AGT ATG ACG AG-3′ and reverse 5′-CAA ATA AAG CCA TGC CAA TC-3′. The data were processed by the comparative cycle threshold (Ct) method and expressed as the fold increase relative to the basal transcription level. The amount of target mRNA was normalized with respect to that of the β-actin mRNA.

2.5. Western blot analysis

Cells were lysed in TNN buffer [120 mM NaCl, 40 mM Tris–HCl, pH 8.0, 0.5% (w/v) NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 100 mM sodium fluoride, and 1 μg/ml each of leupeptin, aprotinin and pepstatin]. Equal amounts of total proteins were separated by 10% (w/v) SDS–PAGE gel electrophoresis and transferred to nitrocellulose membranes (Cat. No. 10401396; Whatman, Maidstone, UK). The membranes were blocked with 5% (w/v) non-fat dry milk in TBST for 1 h at room temperature and probed with antibodies against HAS2 (Cat. No. sc-365262; Santa Cruz Biotechnology, CA), p21 (Cat. No. sc-397; Santa Cruz Biotechnology) or β-actin (Cat. No. sc-47778; Santa Cruz Biotechnology). Protein-antibody complexes were visualized using an enhanced chemiluminescence kit (Cat. No. sc-2048; Santa Cruz Biotechnology).

2.6. SiRNA-mediated knockdown

HAS2-siRNA #1 (UUGCAGCCCUGAGAGCCAG), HAS2-siRNA #2 (AGAAUCCAGUGAUAAUCGC) and control-siRNA were purchased from Bioneer (Daejeon, Korea) and transfected into cells using the Lipofectamine RNAi MAX reagent (Cat. No. 13778-150; Invitrogen, CA). At 24 h post-transfection, the cells were irradiated and subjected to analysis of cell survival, apoptosis, γ-H2AX foci, etc.

2.7. Clonogenic survival assay

Cells were plated at a low density of 2×10^3 in 60-mm dishes and then irradiated as described above. Twenty-four hours later, the medium was changed, and then the cells were incubated until they formed colonies. After that, the colonies were fixed with methanol and stained with crystal violet. The survival rate was calculated as: (the number of colonies in irradiated cells/the number of colonies in non-irradiated cells).

2.8. Apoptosis analysis

Cells were irradiated, incubated for 48 h, and harvested. Thereafter, $\sim 1 \times 10^6$ cells/group were stained with Annexin V-FITC (BD Biosciences, Franklin, NJ, USA), and the apoptotic population was analyzed by fluorescence-activated cell sorting (BD Biosciences).

2.9. Analysis of γ-H2AX foci

Cells were grown on coverslips in 35-mm dishes, treated with HAS2- or control-siRNA for 24 h, irradiated at 4 Gy, and then further incubated for 48 h. Afterwards, the cells were fixed with PBS containing 4% (w/v) paraformaldehyde for 15 min and permeabilized with 0.1% (w/v) NP-40 in PBS for 15 min at room temperature. The cells were then treated with blocking buffer [10% (v/v) FBS, 0.1% (w/v) NP-40/PBS] at room temperature, incubated with an anti-γ-H2AX antibody (Millipore, MA, USA) for 2 h at room temperature, and then incubated with Alexa Fluor 555 (Cat. No. A21425; Invitrogen) for 45 min at room temperature. The nuclei of the labeled cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min, and the stained samples were mounted and visualized under an LSM 710 microscope (Carl Zeiss, Oberkochen, Germany). Cells displaying 10 or more foci were counted as positive regarded as having DNA damage.

2.10. Statistics

The data are expressed as means ± standard deviations unless otherwise indicated. Differences between groups were assessed by the Student's *t*-test. $p < 0.05$ was taken as reflecting a statistically significant difference.

3. Results

3.1. HAS2 expression is up-regulated in irradiated cells

To examine whether HAS2 affects the cellular response to radiation, we first assessed the expression levels of HAS2 in HCT116

cancer cells before and after irradiation. Semi-quantitative RT-PCR and Western blot analyses revealed that the mRNA and protein levels of HAS2 were dramatically up-regulated in HCT116 cells following 4-Gy irradiation, evidently detecting at 12 h post-irradiation and continuing for 24 h thereafter (Fig. 1A, left panel). HAS2 expression was also up-regulated following a much lower dose of 2-Gy irradiation in the same cells (Fig. 1A, right panel). To further evaluate the radiation-induced up-regulation of HAS2, we examined this effect in U2OS cancer cells, which are relatively radioresistant, and H460 cancer cells, which are relatively radiosensitive. These two cancer cell lines also exhibited up-regulation of HAS2 mRNA expression at 12 and 24 h after 4-Gy irradiation (Fig. 1B). These results indicate that irradiation triggers an up-regulation of HAS2 expression in cancer cells within 24 h.

To further evaluate the irradiation-induced up-regulation of HAS2, we used real-time RT-PCR to quantitatively analyze the HAS2 mRNA levels in 4-Gy-irradiated cells versus non-irradiated cells. In HCT116, H460, and U2OS cells, the HAS2 mRNA levels were 9.4-, 7.6-, and 1.8-fold higher at 12 h post-irradiation, and 10.5-, 7.5-, and 2.9-fold higher at 24 h post-irradiation, respectively, compared to the levels in non-irradiated cells (Fig. 1C). Among the non-irradiated cells, the basal levels of HAS2 mRNA were similar among the three tested cell lines (Fig. 1D). These quantitative results indicate that irradiation significantly increases HAS2 mRNA levels in these cancer cell lines within 24 h.

3.2. HAS2 knockdown increases radiosensitivity

To examine the biological impact of the radiation-induced up-regulation of HAS2, we used siRNA to block this up-regulation and examined cell survival (i.e., radiosensitivity). Two different HAS2-siRNAs were applied to HCT116, H460, and U2O2 cells prior to irradiation. The cell survival rates were significantly decreased in all tested cell lines upon HAS2 depletion followed by irradiation, irrespective of which HAS2 siRNA was used (Fig. 2). Specifically, treatment of HCT116 cells with HAS2-siRNA #1 and #2 reduced

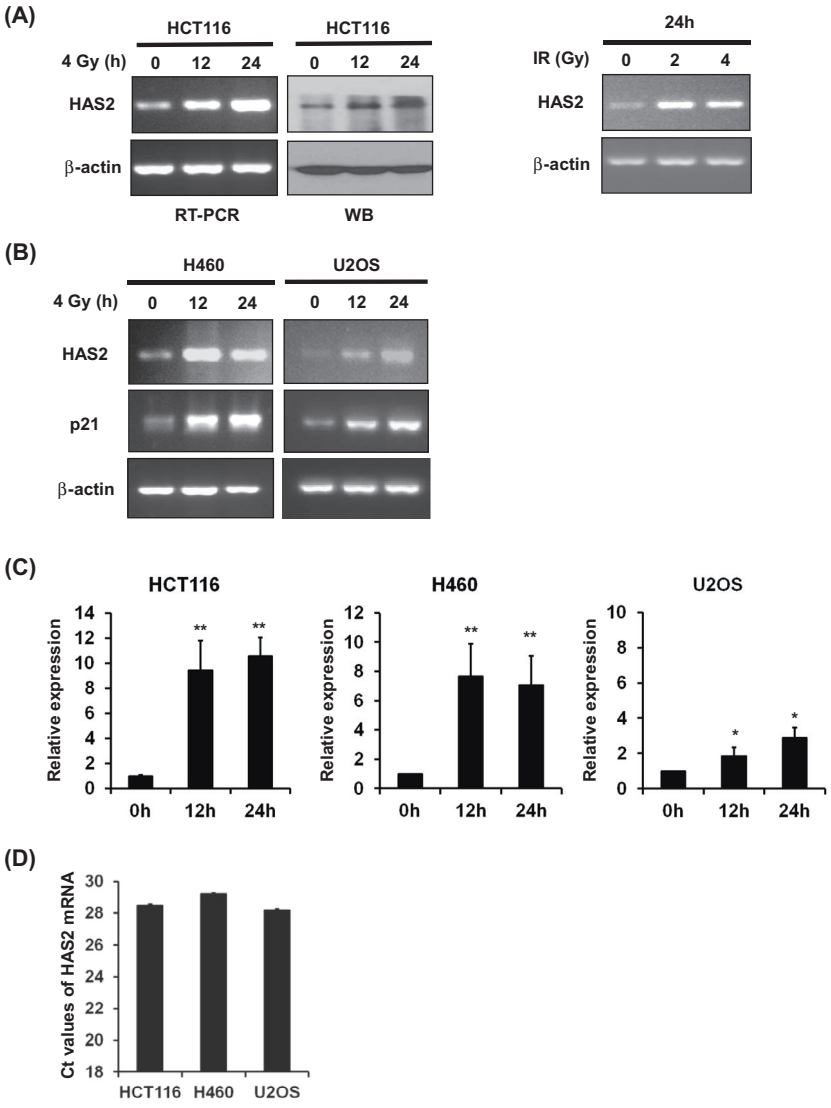

Fig. 1. HAS2 expression is induced by irradiation of human cancer cell lines. (A) HCT116 cells were irradiated and HAS2 expression was examined by semi-quantitative RT-PCR and Western blot analyses at the indicated time points (left panel) and doses (right panel). β-actin was used as an internal control. (B) HAS2 mRNA levels were determined in H460 and U2OS cells at 12 and 24 h post-irradiation (4 Gy). p21 was used as a control of radiation response. (C) The levels of HAS2 mRNA were quantitatively assessed by real-time RT-PCR analysis of HCT116, H460 and U2OS cells. HAS2 mRNA expression was evaluated as the fold-change between the irradiated and non-irradiated states. (D) The basal levels of HAS2 mRNA in non-irradiated HCT116, H460 and U2OS cells are presented as Ct-values obtained from real-time RT-PCR analyses. β-actin was also used as an internal control for real-time RT-PCR.

the survival rate from 53.2% (in irradiated cells not subjected to HAS2 depletion) to 4.3% and 4.7%, respectively (Fig. 2A). Similar results were obtained in H460 cells (Fig. 2B), U2OS cells (Fig. 2C), and HeLa cells (Supplementary Fig. 1). These results indicate that HAS2 depletion reduces cell survival following irradiation, further suggesting that the above-described radiation-induced up-regulation of HAS2 contributes to radioresistance in cancer cells.

Next, we used Annexin V staining to examine the apoptotic populations and determine whether the HAS2-depletion-mediated decrease in cell survival was associated with increased apoptosis among irradiated HCT116 cells. FACS analysis of Annexin V-stained cells revealed that HAS2 depletion prior to irradiation greatly shifted the cell population toward higher levels of fluorescent intensity, reflecting increased apoptosis (Fig. 3A and Supplementary Fig. 2). Similar to the results of our survival analysis, HAS2 depletion alone did not significantly alter the apoptotic profile in the absence of irradiation ($p = 0.336$; Fig. 3A and B). In irradiated cells, however, the Annexin-V-positive populations comprised 50.6% and 19.2% of the HAS2-depleted and undepleted cell populations, respectively ($p = 0.009$; Fig. 3B). Thus, our results indicate that HAS2 knockdown increases apoptosis and enhances radiosensitivity in the tested cell lines.

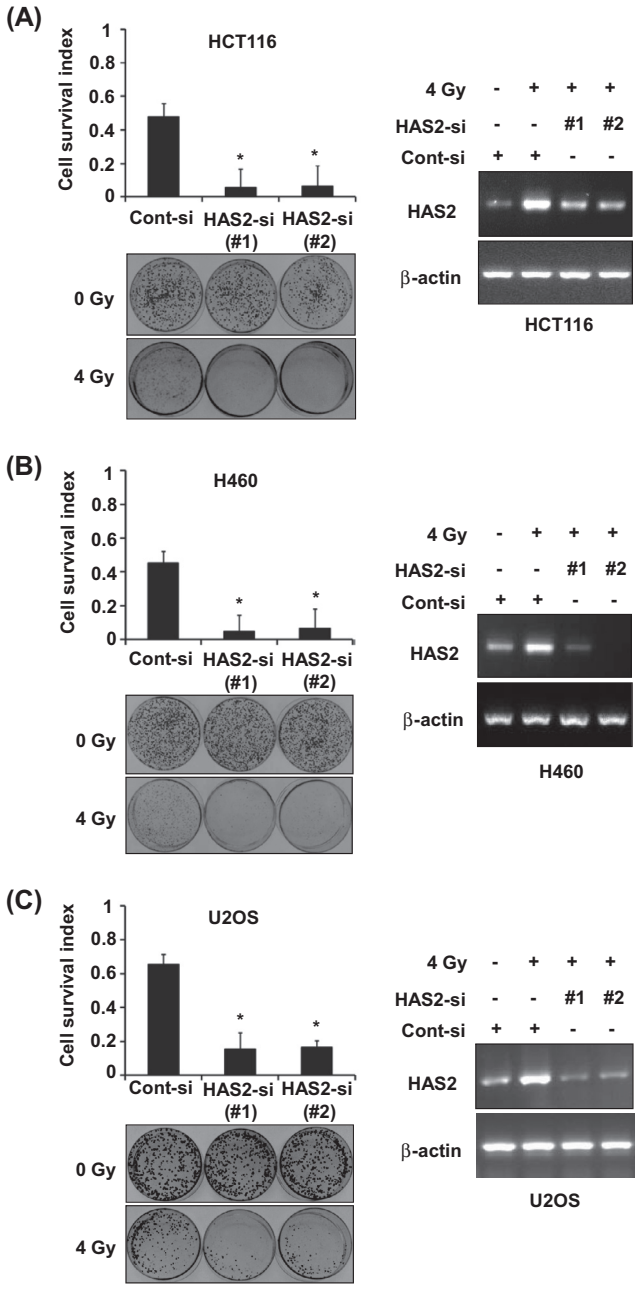

Fig. 2. HAS2 knockdown decreases survival among irradiated cancer cells. HCT116 (A), H460 (B) and U2OS (C) cells were transfected with HAS2- (#1 and #2) or control-siRNA, and then subjected to 4-Gy irradiation. HAS2 mRNA levels were assessed by semi-quantitative RT-PCR, and survival rates were determined by clonal analyses in which we calculated the ratio of colony numbers in irradiated versus non-irradiated cells. A representative picture of surviving crystal-violet-stained colonies is shown at the bottom of each figure, and the data are presented as means ± SE from three independent experiments.

3.3. HAS2 knockdown increases irradiation-induced DNA damage

As hyaluronic acid protects cells from the cellular stresses, including DNA damage [25,26], we measured nuclear γ-H2AX foci

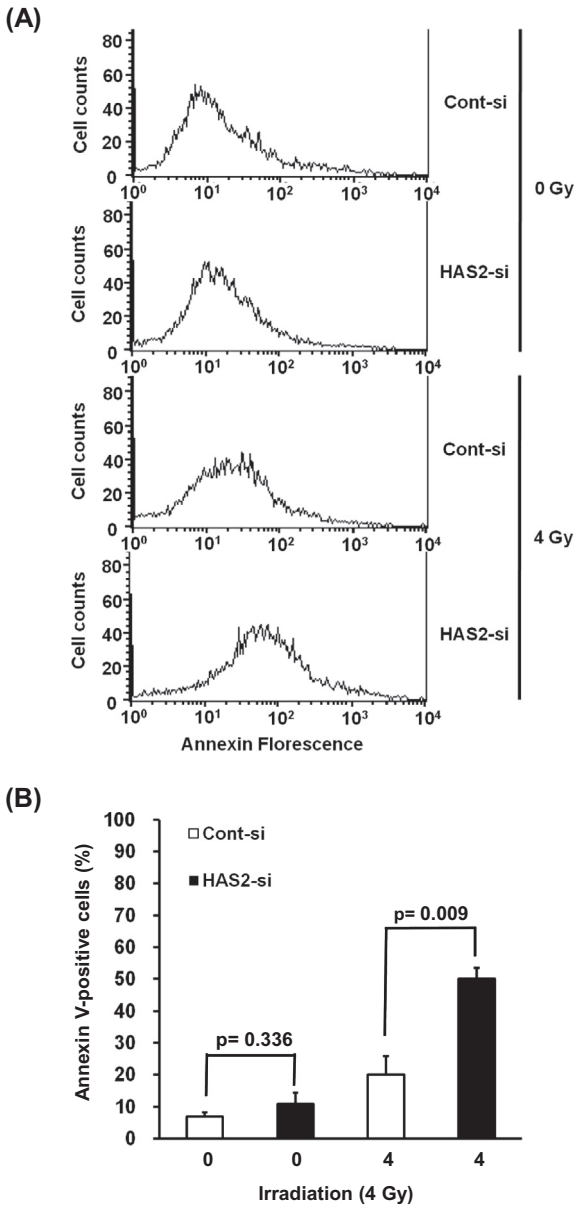

Fig. 3. HAS2 knockdown increases apoptosis among irradiated cancer cells. (A) HCT116 cells transfected with HAS2- or control-siRNA were irradiated with 4 Gy and subjected to Annexin V staining. Flow cytometry was used to compare the apoptotic (Annexin-V-positive) cell profiles before and 48 h after radiation. (B) The percentages of Annexin-V-positive apoptotic cells were determined by analysis of the histograms shown in (A).

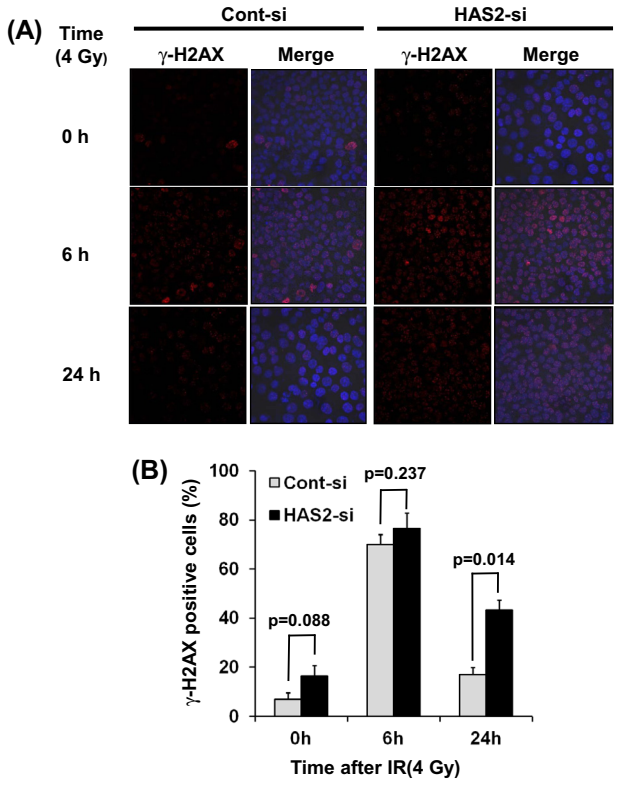

Fig. 4. HAS2 knockdown potentiates γ-H2AX foci in irradiated cancer cells. (A) Representative photomicrographs showing that the formation of γ-H2AX foci following irradiation is increased in HAS2-knockdown cells compared to controls. HCT116 cells grown on coverslips were transfected with HAS2- or control-siRNA and then exposed to 4-Gy irradiation. Irradiated and non-irradiated cells were fixed, stained with an antibody against γ-H2AX, and then counterstained with DAPI. Fluorescent microscopy was used to examine the presence of γ-H2AX foci in nuclei (x 400). (B) The percentages of cells with γ-H2AX foci were determined from fluorescent microscopic images. Cells displaying 10 or more nuclear foci were counted as positive for γ-H2AX. At least more than 70 cells were assessed per group.

(a sign of DNA damage) in our system. Fluorescence microscopy showed that the number of γ-H2AX-positive HCT116 cells was greatly increased at 6 h post-irradiation (Fig. 4A) in both control and HAS2-depleted cells (70.3% versus 76.5%, respectively; $p = 0.237$; Fig. 4B). Twenty-four hours post-irradiation, the positive population was greatly decreased in control cells, but this decrease was much less evident in HAS2-depleted cells (17.5% versus 43.4% positive, respectively; $p = 0.014$; Fig. 4B), indicating that HAS2-depleted cells maintained a higher level of radiation-induced DNA damage over time. These results suggest that HAS2 depletion potentiates radiation-induced DNA damage in cancer cells, perhaps by decreasing the recovery from DNA damage.

4. Discussion

Hyaluronan is synthesized by three hyaluronan synthases (HAS1∼3) that are induced under various conditions, including growth stimulation, tissue injury, and inflammation. In the present study, we demonstrate that irradiation of human cancer cell lines triggers up-regulation of HAS2 (up to 10-fold at 4 Gy), whereas HAS2 knockdown potentiates DNA damage and increases apoptosis in these cells. Thus, HAS2 may be a promising anticancer target for therapeutic strategies aimed at radiosensitizing cancer cells.

In response to toxic substances, normal tissues (e.g., skin, intestine and bone marrow) experience up-regulation of HAS gene expression [27–29]. These tissues are highly sensitive to radiation, and may be seriously injured during radiotherapy [28,29]; however the administration of exogenous hyaluronan prior to irradiation has been shown to decrease their degree of injury [23,24]. These previous findings suggested that the up-regulation of endogenous HAS and hyaluronan may be insufficient to protect tissues against injury, but also prompted speculation that these agents could be associated with the radiosensitivity of cancer cells. Here, we report for the first time that endogenously increased HAS2 expression contributes to the radioresistance of cancer cells.

One plausible mechanistic basis for the involvement of HAS2 in cancer cell radioresistance is the interaction of hyaluronan with the CD44 receptor, which evokes chemoresistance by recruiting EGFR and activating the EGFR signaling pathway [30]. Hyaluronan-induced cisplatin resistance has been shown to be attenuated by inhibition of the hyaluronan-CD44 receptor interaction [31] and abolished by inhibition of EGFR [32]. Based on these previous results, we hypothesize that HAS2-mediated radioresistance is due to increased hyaluronan synthesis, the interaction of this hyaluronan with the CD44 receptor, and the subsequent activation of EGFR signaling. Another possible mechanism is through ankyrin, a cytoskeletal protein whose recruitment has been associated with the hyaluronan-mediated regulation of cancer cell survival [33,34]. Indeed, it seems possible that the irradiation-induced up-regulation of HAS2 expression and subsequent increase of hyaluronan can stimulate the colocalization of hyaluronan-CD44-ankyrin, which in turn may contribute to radioresistance in cancer cells.

In summary, we herein report for the first time that HAS2 depletion can reverse radioresistance in cancer cell lines, suggesting that this strategy could be useful for the future development of new radiosensitizers. However, further efforts are needed to define the mechanistic basis of HAS2-induced radioresistance.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.026.

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