

## Original Article

# Inhibition of IGFBP1 induction enhances the radiosensitivity of colon cancer cells in vitro

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**Abstract:** Insulin-like growth factor-binding protein 1 (IGFBP1), also known as placental protein 12 (PP12) is a protein that in humans is encoded by the IGFBP1 gene, plays an important role in the development and progression of cancer. However, the role of IGFBP-1 in colon cancer radiosensitivity remains unclear. Here, we show that IGFBP1 knockdown potentiates irradiation-induced DNA damage and apoptosis in colon cancer cells. Upon exposure to radiation, the human colon cancer cell lines exhibited marked upregulation of IGFBP1 within 24 h. Inhibition of IGFBP1 induction significantly reduced the survival of irradiated radioresistant and -sensitive cells. Interestingly, knockdown of IGFBP1 rendered the colon cancer cells to sustain irradiation-induced DNA damage. Our results suggest that IGFBP1 knockdown sensitizes colon cancer cells radiation in vitro, and thus, could potentially be targeted for therapeutic interventions aimed at radiosensitizing colon cancer.

**Keywords:** IGFBP1, colon cancer, radiosensitivity, cell apoptosis

## Introduction

Recently decades, colon cancer has become the second leading cause of cancer-related mortality in the USA, and a common malignancy in Asia with changes in diet [1]. Although radiotherapy is a standard therapy in the adjuvant treatment of resected colon and rectum cancers [2], and its combination with chemotherapy has been shown to reduce local failure and distant metastasis further, thereby improving the outcome of treatment [3, 4]. The biggest obstacle limiting effective cancer therapy is the occurrence of radio-resistant of cancer cells. Thus, researchers are currently seeking to reverse the resistant phenotype and maximize the toxicity of therapeutic agents toward cancer cells while minimizing it in normal cells [5-7].

The IGF-IGFR-IGFBP axis, which play critical role in differentiation, cell growth, and apoptosis, consists of six well-characterized insulin-like growth factor-binding proteins (IGFBPs), the canonical ligands insulin-like growth factor 1

(IGF1) and insulin-like growth factor 2 (IGF2), IGF receptors (IGF-IR or IGF1R and IGF-IIR or IGF2R), and several IGFBP proteases, including kallikreins, cathepsins, and matrix metalloproteinases (MMPs) [8]. IGFBP1, which located in 7p12.3 has been reported as a potential response marker to selective internal radiation therapy in hepatocellular carcinoma [9]. IGFBP-1 is secreted by liver in a highly phosphorylated form that has higher IGF-I affinity than the non-phosphorylated form, and has been reported to be resistant to proteolysis [10, 11]. Data suggest that expression of IGFBP1 in hepatocytes can be regulated by carnosine, a dietary factor; carnosine down-regulates expression of IGFBP1 in Hep G2 cells; mechanism involves suppression of HIF1A [12]. There was also report that demonstrated IGFBP1 upregulated in response to ionizing radiation in MCF-7 human breast cancer cells [13]. However, to the present front, there is little known about the role of IGFBP-1 in colon cancer radiosensitivity.

In the present study, we found the expression status of IGFBP1 in irradiated colon cancer

## IGFBP1 in colon cancer radiosensitivity

cells, and investigated whether IGFBP1 can modulate the radiosensitivity of colon cancer cells. We found that knockdown of IGFBP1 radiosensitized radioresistant colon cancer cells via persistent DNA damage, and that radiation-induced significant up-regulation of IGFBP1 in colon cancer cells, contributed to their radioresistance by reducing DNA damage. Collectively, we propose that IGFBP1 inhibition might be a good strategy for radiosensitizing colon cancer cells in which IGFBP1 is endogenously overexpressed or induced by radiation. Further, our study may provide a scientific rationale anticancer target for the development of new radiation sensitizers.

### Materials and methods

#### Cell culture

HCT116 were grown in McCoy's 5a Medium (Cat. No. LM005-01; Welgene, Korea), HT29 and LOVO 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<sub>2</sub>.

#### Irradiation and RNA preparation

All cells examined were treated with 2 or 4 Gy of c-irradiation, using a Gamma cell<sup>®</sup>3000 Elan irradiator (<sup>137</sup>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).

#### Western blot

The samples were shaken at 4°C for 1 h followed by centrifugation at 40000×g at 4°C for another 1 h. Protein concentration was determined by the Bradford method using bovine serum albumin (BSA). 40 µg protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) and blotted onto nitrocellulose membranes. After incubation with the specific primary antibodies (Cell Signaling Technology, Danvers, MA) in blocking solution overnight at 4°C, the blots were probed with a

secondary horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz, CA, USA) and developed with enhanced chemiluminescence reagents. The relative amount of the target protein was normalized to β-actin and analyzed with a Gel Pro Analyzer (Media Cybernetics, Silver Spring, MD, USA).

#### Quantitative real-time PCR

The obtained cDNA was also subjected to real-time PCR amplification using the iQ SYBR Green supermix (BioRad, Hercules, CA, USA). Actin was used as an endogenous control. ALL measurements were performed in triplicate. The real-time PCR data were analyzed using the ΔΔCT method.

#### Apoptosis analysis

Cells were irradiated, incubated for 48 h, and harvested. Thereafter, 1×10<sup>6</sup> 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).

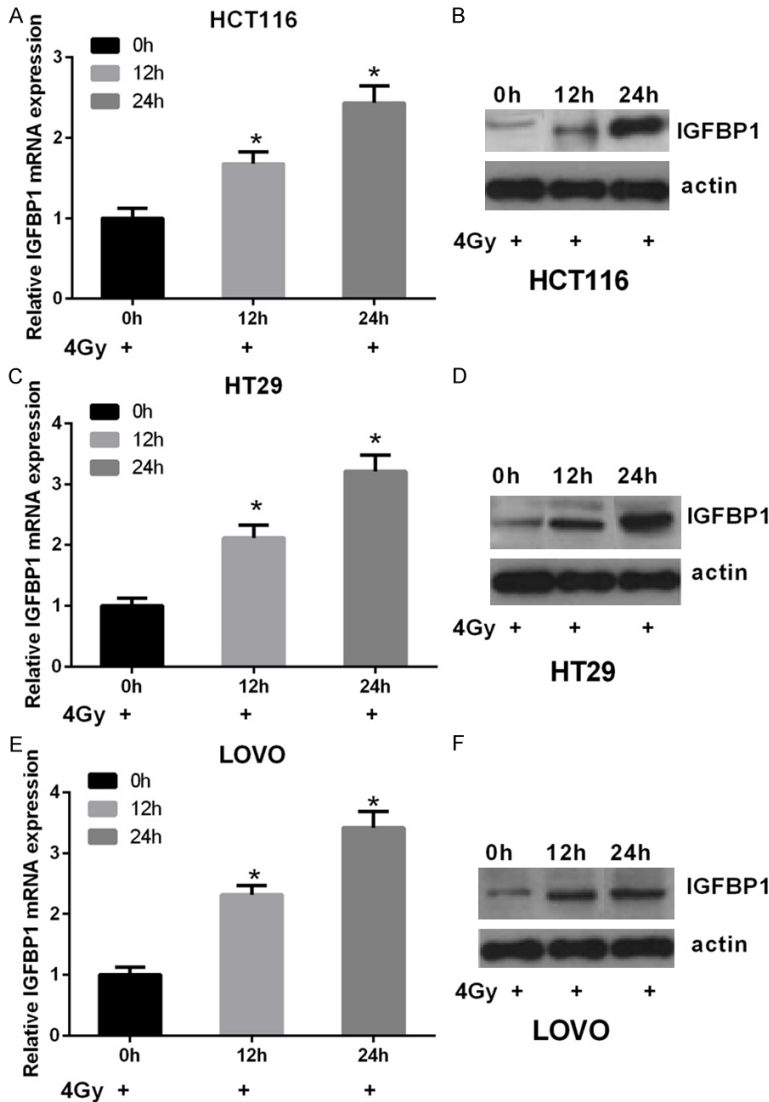
#### Analysis of c-H2AX foci

Cells were grown on coverslips in 35-mm dishes, treated with si-IGFBP1 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-c-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 40,6-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.

#### Statistical analysis

The data are reported as the mean ± S.D. of at least three times of independent experiments with the double-sided Student's t test. A *P* value of less than 0.05 was considered significant (\**P* < 0.05).

## IGFBP1 in colon cancer radiosensitivity



**Figure 1.** IGFBP1 expression is induced by irradiation of human colon cancer cell lines in a time-dependent manner. (A, B) HCT116 cells were irradiated and IGFBP1 expression was examined by real-time-PCR and Western blot analyses at the indicated time points (0 h, 12 h and 24 h). actin was used as an internal control. (C, D) HT29 cells were irradiated and IGFBP1 expression was examined by real-time-PCR and Western blot analyses at the indicated time points (0 h, 12 h and 24 h). actin was used as an internal control. (E, F) LOVO cells were irradiated and IGFBP1 expression was examined by real-time-PCR and Western blot analyses at the indicated time points (0 h, 12 h and 24 h). actin was used as an internal control. The data are reported as the mean  $\pm$  S.D. of at least three times of independent experiments (\* $P < 0.05$ ).

### Results

#### *IGFBP1 expression is up-regulated in irradiated cells in a time dependent manner*

To examine whether IGFBP1 affects the cellular response to radiation, we first assessed the expression levels of IGFBP1 in HCT116 colon

cancer cells before and after irradiation. Real time-PCR and Western blot analyses revealed that the Mrna (**Figure 1A**) and protein (**Figure 1B, 1D and 1F**) levels of IGFBP1 were dramatically up-regulated in colon cancer cells following 4-Gy irradiation, evidently detecting at 12 h post-irradiation and continuing for 24 h thereafter. To further evaluate the radiation-induced up-regulation of IGFBP1, we examined this effect in HT29 and LOVO colon cancer cells. These two colon cancer cell lines also exhibited up-regulation of IGFBP1 mRNA and protein expression at 12 and 24 h after 4-Gy irradiation (**Figure 1C-F**).

#### *IGFBP1 expression is up-regulated in irradiated cells in a dose-dependent manner*

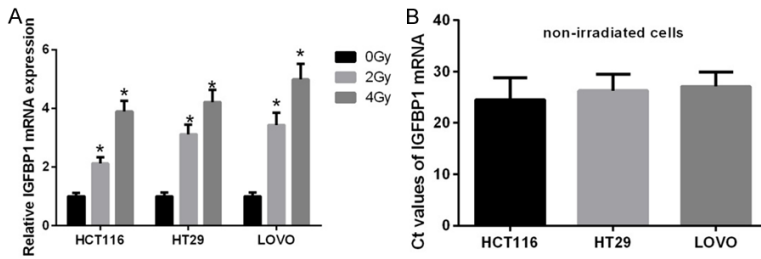
The other real time-PCR was used to indicate IGFBP1 mRNA expression was also up-regulated following a much lower dose of 2-Gy irradiation in the same cells (**Figure 2A**). To further evaluate the irradiation-induced up-regulation of IGFBP1, we used real-time-PCR to quantitatively analyze the IGFBP1 mRNA levels in non-irradiated cells. Among the non-irradiated cells, the basal levels of IGFBP1 mRNA were similar among the three tested cell lines (**Figure 2B**). These quantitative results indicate that irradiation significantly increases IGFBP1

levels in these colon cancer cell lines within in a dose-dependent manner.

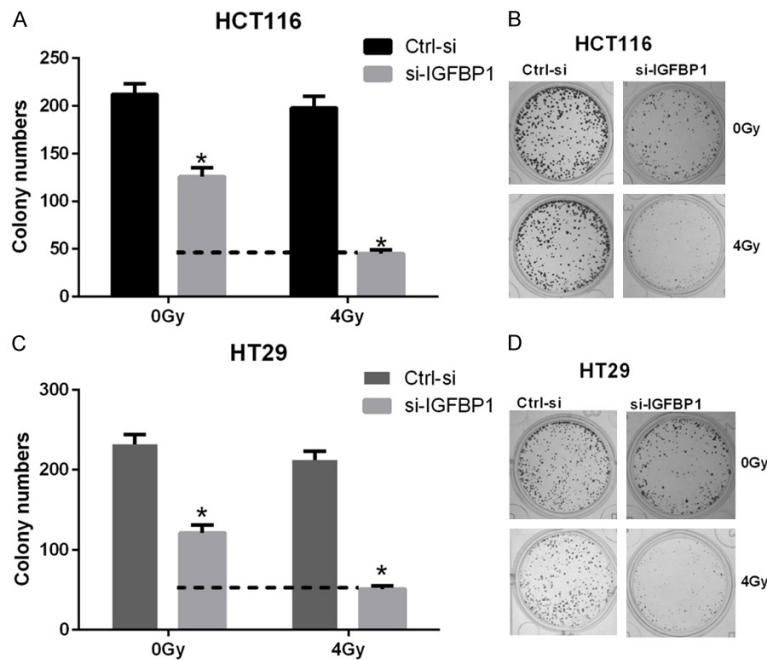
#### *IGFBP1 knockdown increases radiosensitivity*

To examine the biological impact of the radiation-induced upregulation of IGFBP1, we used siRNA to block this up-regulation and examined

## IGFBP1 in colon cancer radiosensitivity



**Figure 2.** IGFBP1 expression is induced by irradiation of human colon cancer cell lines in a dose-dependent manner. A. IGFBP1 mRNA levels were determined in HCT116, HT29 and LOVO cells at 24 h post-irradiation (0 Gy, 2 Gy and 4 Gy). B. The basal levels of IGFBP1 mRNA in non-irradiated HCT116, HT29 and LOVO cells are presented as Ct-values obtained from real-time-PCR analyses. actin was also used as an internal control for real-time-PCR. The data are reported as the mean  $\pm$  S.D. of at least three times of independent experiments (\* $P < 0.05$ ).



**Figure 3.** IGFBP1 knockdown decreases survival among irradiated colon cancer cells. HCT116 (A, B), HT28 (C, D) cells were transfected with si-IGFBP1 or control-siRNA, and then subjected to 4-Gy irradiation. The survival rates were determined by clonal analyses in which we calculated the number of colony numbers in irradiated versus non-irradiated cells. A representative picture of surviving crystal-violetstained colonies is shown, and the data are presented as means  $\pm$  SD from three independent experiments (\* $P < 0.05$ ).

cell survival (colony formation assay). The IGFBP1 knockdown siRNAs were applied to HCT116 and HT29 cells prior to irradiation. The cell colony numbers were significantly decreased in all tested cell lines upon IGFBP1 depletion followed by irradiation or not (**Figure 3A-D, 0 Gy group**). Specifically, treatment of HCT116 or HT29 cells with IGFBP1-siRNA reduced much more of colony numbers following 4

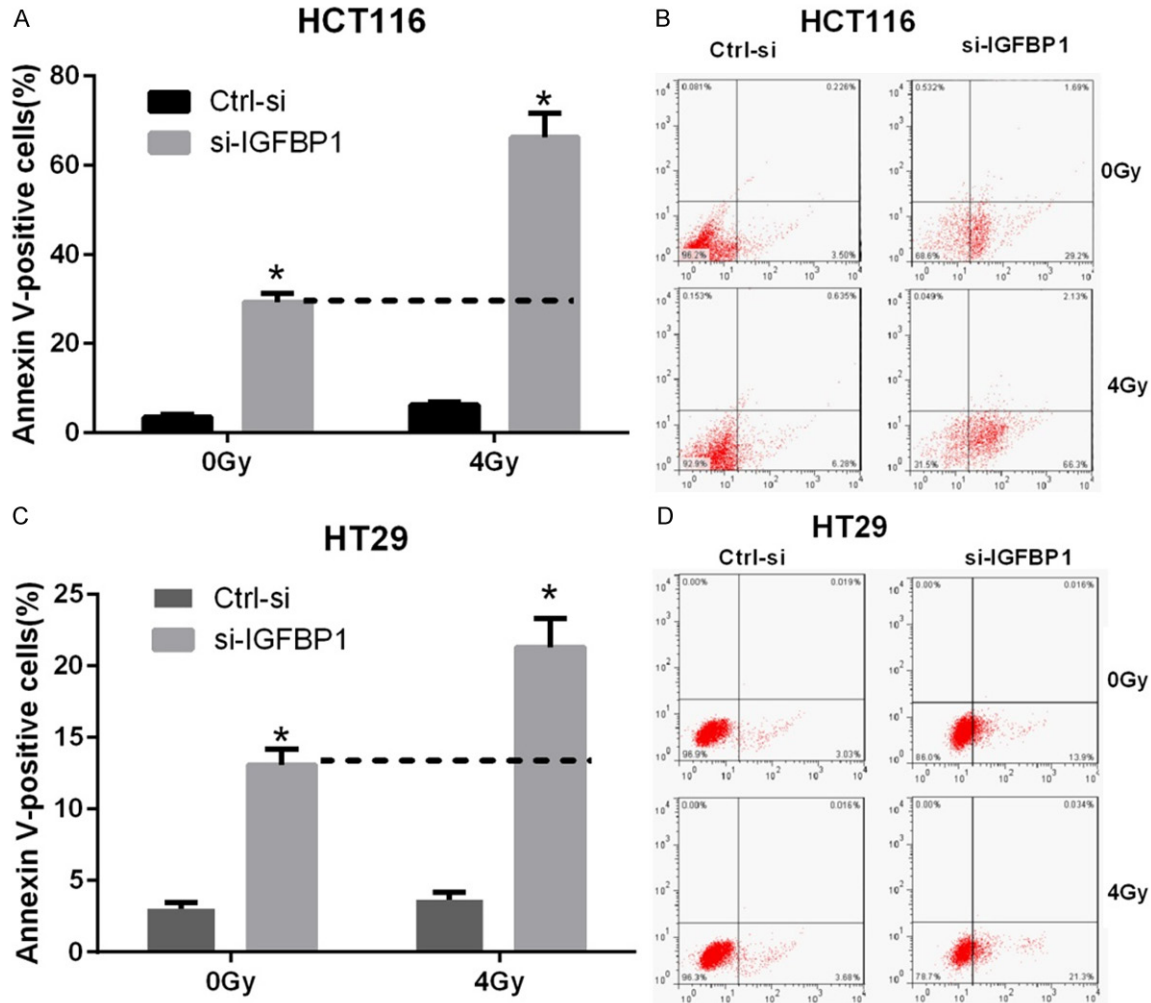
Gy-dose irradiation (**Figure 3A-D, 4 Gy group**). These results indicate that IGFBP1 depletion reduces cell survival following irradiation, further suggesting that the above-described radiation-induced up-regulation of IGFBP1 contributes to radioresistance in colon cancer cells.

### *IGFBP1 knockdown increases colon cancer cells apoptosis*

Next, we used Annexin V staining to examine the apoptotic populations and determine whether the IGFBP1-depletion-mediated decrease in cell survival was associated with increased apoptosis among irradiated HCT116 and HT29 cells. FACS analysis of Annexin V-stained cells revealed that IGFBP1 depletion greatly shifted the cell population toward higher levels of fluorescent intensity, reflecting increased apoptosis with or without irradiation (**Figure 4A-D, 0 Gy group**). Then, similar to the results of our survival analysis, IGFBP1 depletion significantly alter the apoptotic profile of colon cancer cells prior to irradiation (**Figure 4A-D, 4 Gy group**). Thus, our results indicate that IGFBP1 knockdown increases apoptosis and enhances radiosensitivity in the tested cell lines.

### *IGFBP1 knockdown increases irradiation-induced DNA damage*

As hyaluronic acid protects cells from the cellular stresses, including DNA damage, we measured nuclear c-H2AX foci (a sign of DNA damage) in our system. Fluorescence microscopy showed that the number of c-H2AX-positive HCT116 and HT29 cells was greatly increased at 12 and 24 h post-irradiation (**Figure 5A, 5B**) in both control and IGFBP1-depleted cells. These results suggest that IGFBP1 depletion



**Figure 4.** IGFBP1 knockdown increases apoptosis among irradiated colon cancer cells. (A, B) HCT116 cells transfected with si-IGFBP1 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. The percentages of Annexin-V-positive apoptotic cells were determined by analysis of the histograms are shown. (C, D) The representative picture of scatter diagram from each group. The data are reported as the mean  $\pm$  S.D. of at least three times of independent experiments. (\* $P < 0.05$ ).

potentiates radiation-induced DNA damage in colon cancer cells, perhaps by decreasing the recovery from DNA damage.

**Discussion**

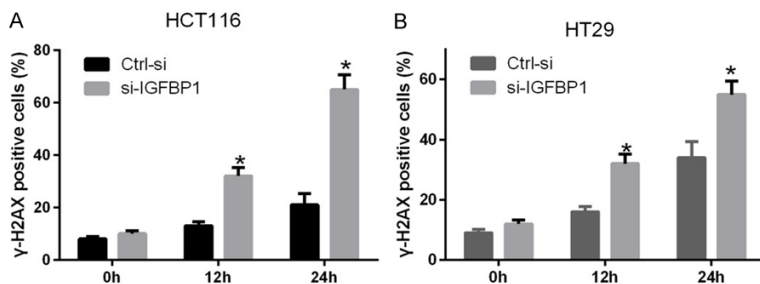
As a important member of IGFBPs, IGFBP1 has been reported to have both inhibitory and potentiating effects on IGF-I action [14-17] and Zhao et al. [18] and Jyung et al. [19] showed that it was required in addition to IGF-I to stimulate tissue repair in vivo. Meanwhile, the complexity of IGFBP-1 has also been demonstrated in malignancies. IGFBP-1 promotes apoptosis of cancer cells under some conditions but not under others [20, 21]. Furthermore, IGFBP-1

expression has been documented to be both positively and negatively correlated with cancer risk [20, 22]. However, there is limited evidence from the literature concerning the role of IGFBP-1 in colon cancer radioresistance. Here in, we demonstrate that irradiation of human colon cancer cell lines triggers up-regulation of IGFBP1, whereas knockdown of it potentiates DNA damage and increases apoptosis in these cells. Thus, IGFBP1 may be a promising anti-cancer target for therapeutic strategies aimed at radiosensitizing colon cancer cells.

The remaining cancer cells after surgery would be sufficient for recurrences of many types of cancer. Chemotherapy and radiotherapy have



## IGFBP1 in colon cancer radiosensitivity



**Figure 5.** IGFBP1 knockdown potentiates c-H2AX foci in irradiated colon cancer cells. A. The results showing that the formation of c-H2AX foci following irradiation is increased in IGFBP1-knockdown cells compared to controls. HCT116 cells grown on coverslips were transfected with si-IGFBP1 or control-siRNA and then exposed to 4-Gy irradiation. Irradiated and non-irradiated cells were fixed, stained with an antibody against c-H2AX, and then counterstained with DAPI. Fluorescent microscopy was used to examine the presence of c-H2AX foci in nuclei ( $\times 400$ ). The percentages of cells with c-H2AX foci were determined from fluorescent microscopic images. Cells displaying 10 or more nuclear foci were counted as positive for c-H2AX. At least more than 70 cells were assessed per group. B. Similar tendency was observed in HT29 cells. The data are reported as the mean  $\pm$  S.D. of at least three times of independent experiments. (\* $P < 0.05$ ).

been used for cancer treatments. However, they result in some serious side effects, producing radiation- and drug-resistant cells, for instance. Therefore, cancer immunotherapy would be potentially effective because it could enhance patients' immune surveillance and fight against cancer cells without any side effects. Cancer immunotherapy has been more effectively used to detect and destroy cancer cells. The immune system must be triggered by a specific "tumor antigen" or "tumor associated antigens" on the surface of cancer cells [23]. For instance, The IGF family can stimulate DCs to secrete different cytokine profiles [24]. Combined therapy with IGF-1R, HER-2, and HER-1 peptides induces synergistic antitumor effects against breast and pancreatic cancer cells [25]. Our results demonstrate IGFBP1 was involved in colon cancer tumorigenesis, and also may imply its role in colon cancer immunotherapy. Further study will be explored the role of IGFBP1 in colon cancer immunotherapy.

Studies have been suggested radiotherapy is one of the major therapeutic strategies for cancer treatment and results in DNA damage, including double-strand break (DSBs), which in turn initiates a variety of signaling events in cancer cells [26]. Then the DSBs process could lead to the phosphorylation of H2AX. According to these procedures, numbers of researchers have used this as a marker for the cellular response to radiation-induced DNA damage

[27-29]. In the present study, our results showed that the knockdown of IGFBP1 in irradiated colon cancer cells delayed the clearance of  $\gamma$ H2AX, suggesting that knockdown of IGFBP1 prevents DNA repair and hence increases radio-sensitivity.

In summary, we herein report for the first time that knockdown of IGFBP1 can reverse radioresistance in colon 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 IGFBP1-induced radioresistance.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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## IGFBP1 in colon cancer radiosensitivity

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