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Cancer Biology and Signal Transduction

Molecular Cancer Therapeutics

The Role of HMGB1 in Radioresistance of Bladder Cancer

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Abstract

Although radical cystectomy surgery is the standard-of-care for muscle-invasive bladder cancer, it entails complete removal of the bladder and surrounding organs which leads to substantial loss in the quality-of-life of patients. Radiotherapy, which spares the bladder, would be a more appropriate treatment modality if we can utilize molecular markers to select patients with better response to radiation. In this study, we investigate a protein called high mobility group box protein 1 (HMGB1) as a predictive marker for radiotherapy response in bladder cancer. Our *in vitro* results indicate a positive correlation between higher levels of HMGB1 protein and resistance to radiation in various cell lines. Upon HMGB1 protein knockdown, highly significant (>1.5-fold) sensitization to radiotherapy was achieved. We saw that loss of HMGB1 was associated with at least two times higher ($P < 0.001$) DNA damage in cell lines postradiation. Our results also depicted that autophagy was inhibited more than 3-fold ($P < 0.001$) upon HMGB1 knockdown, implicating its role in autophagy as another cause of bladder cancer radioresistance. Further validation was done *in vivo* by conducting mouse tumor xenograft experiments, where HMGB1 knockdown tumors showed a significantly better ($P < 0.001$) response to radiotherapy and decreased autophagy (shown by P62 staining) as compared with controls. The cumulative findings of our *in vitro* and *in vivo* studies highlight the significance of HMGB1 as a radiation response marker as well as its utility in radiosensitization of bladder cancer.
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Introduction

In 2014, bladder cancer accounted for approximately 450,000 new cases and 165,000 deaths worldwide (1). As 90% of bladder cancer mortality burden falls on muscle-invasive disease, it necessitates finding better ways of invasive disease management and control. Even today, radical cystectomy, which involves the complete removal of the bladder and surrounding organs, remains the "gold standard" treatment for invasive bladder cancer (2). Although surgery relates to good survival outcomes, it leads to a significantly lower quality-of-life index in patients due to loss of urinary and sexual functions.

An alternate treatment modality is radiotherapy, which is a localized and noninvasive technique that will preserve the bladder and the surrounding organs, predicting a higher quality-of-life index in patients (3). However, lack of local control of the disease, in the form of recurrence and distant metastasis, remains a major problem in implementation of this therapy. As such, it is essential to develop radiosensitization techniques that would increase the efficacy of radiotherapy in treating bladder cancer. This entails finding specific molecular markers which would predict response to radiotherapy in patients with invasive disease, allowing selection of patient groups that can avoid surgery as well as those who need additional radiosensitization therapy.

In the current study, we recognize HMGB1 as a protein capable of predicting and modulating bladder cancer response to radiotherapy. High mobility group box protein 1 (HMGB1) is a 25 kDa multifunctional protein that hails from the HMG protein superfamily and contains two DNA-binding domains which facilitate its role as a transcription factor. It is a nuclear chromosomal protein that acts as a damage recognition enhancer as well as a chromatin remodeler in various DNA repair processes (4–7). One way in which tumor cells develop radioresistance is by targeting the DNA repair pathways, which enable continued survival and replication despite DNA strand breaks (DSB) caused by the radiation (8, 9). This warrants an investigation of HMGB1's role in postradiation DNA damage as a potential pathway for radiosensitization.

Moreover, HMGB1 is implicated in all characteristic oncogenic processes ranging from innate cell qualities (unlimited replicative potential, evasion of programmed cell death, growth signaling) to extracellular pathways like inflammation, angiogenesis, and metastasis (10). Previous work done with HMGB1 has shown a strong correlation between its overexpression and poor prognosis in patients with various other cancers (10, 11). Having this multifunctional capability to influence cancer progression, HMGB1 becomes a significant target for enabling radiosensitization. Cancer cell autophagy or "programmed survival" is another common occurrence in the development of resistance and an important pathway of HMGB1 action (5, 6, 12). Hence, in our study, we also evaluate the occurrence of autophagy postradiation

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in bladder cancer cells and observe the effect of HMGB1 modulation on this radioresistance pathway.

To our knowledge, this study of HMGB1 in DNA damage and autophagy pathways of bladder cancer radioresistance is novel. Our findings indicate a relationship between modulation of HMGB1 levels and radiosensitization in bladder cancer that has not been observed before.

Materials and Methods

Cell culture

Eight human urothelial carcinoma cell lines were used: UM-UC1, UM-UC3, UM-UC5, UM-UC6, UM-UC13, RT4, 253-JP, and 253J-BV. The UM-UC series of urothelial carcinoma cell lines and RT4 were obtained in 2007 from the Specimen Core of the Genitourinary Specialized Programs of Research Excellence in bladder cancer at the MD Anderson Cancer Center (Houston, TX; ref. 13). The 253-JP and 253J-BV were kindly provided to us in 2007 by Dr. Colin P.N. Dinney from the MD Anderson Cancer Center (Houston, TX; ref. 14). The details of the characterization can be found in the references. All cell lines were routinely tested for cellular morphology and microbial presence by microscopic observation. Last cell line authentication was done in June 2015 via Promega services that utilizes short tandem repeat analysis and complies with ATCC standards. For experiments, cells were routinely passaged at 70% to 80% confluency and cultured in Eagle's Minimum Essential Medium (EMEM; Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent).

Protein extraction and quantification

Cells were washed with cold PBS and then lysed with ice-cold RIPA buffer for 45 minutes at 4°C. Extracts were then centrifuged at 12,000 × *g* for 15 minutes at 4°C to collect the supernatant containing total extracted proteins. BCA Protein Assay Kit (Pierce Scientific Ltd) was used for quantification. β-Actin was used as a loading reference.

Immunoprecipitation

Antibodies (5 μg: p53 and Beclin-1, Cell Signaling Technology; IgG, Santa Cruz Biotechnology) were incubated with the Protein A beads for 30 minutes at room temperature. Crosslinking was performed using BS3 (Life Technologies) according to the manufacturer's instructions. Then, cell lysates (750 μg proteins) were added and the mix was incubated for at least an hour at 4°C, before washing, elution, and SDS–PAGE separation. Experiments were performed at least twice.

Western blot analysis

After electrophoretic separation, transfer of proteins to nitrocellulose membranes was done using the TurboBlot (Bio-Rad). Blocking was done with 5% milk in TBS with Tween 20 (TBST). Primary antibodies were added to the membranes for incubation at 4°C overnight (Cell Signaling Technology, 1:1,000 dilution: HMGB1, β-actin, LC3, p53, Beclin-1, H2AX, and phosphoH2AX; Abcam HMGB1 used at 1:25,000 dilution). Membranes were washed and then incubated with secondary antibody (HRP-linked) at 1:2,000 dilution in 5% milk in TBST for 1 hour at room temperature. ECL chemiluminescence [Amersham Biosciences (GE)] was used to detect protein bands. Experiments were done thrice. Manual band quantification was carried out using Spectrum Image and GelQuant software. Error bars indicate SEM unless otherwise indicated.

RNA extraction and RT-PCR

The total RNA was extracted using Qiagen RNeasy Mini Kit. Reverse transcription was done using iScript kit (Bio-Rad). Using the Quantitect SYBR Green PCR Kit, quantitative real-time RT-PCR was performed. GAPDH was used as the housekeeping gene. Each RT-PCR assay was run with 12.5 ng of freshly prepared cDNA and contained duplicates for average calculations. HMGB1 primers used were: 5′-GTATCCCCAAAAGCGTGAGC-3′ and 5′-CTCGTTTCCTGAGCAGTCCA-3′. Quantification was done using the $2^{-\Delta\Delta C_t}$ method (15).

Lentiviral system shRNA knockdown of HMGB1

The HMGB1 shRNA and scramble plasmids were obtained as a collaboration gift from Gerardo Ferbeyre at University de Montreal (Montreal, Quebec, Canada). Lentiviral particles for each plasmid were produced via transfection of HEK293T cells. TransDux (SBI) was used to transduce chosen cell lines with the lentiviral particles. Seventy-two hours after transduction, antibiotic selection with puromycin was done for a week to create stable cell lines.

Clonogenic assay

Cells were seeded in a 6-well plate at a density of 200 cells per well and irradiated the next day using a Faxitron X-Ray machine (2–8 Gy). After radiation, the plates were kept at 37°C/5% CO_2 for a week to allow for colony formation. A cutoff of 50 cells per colony was chosen as the stop point for each experiment. Before counting, cell colonies were fixed for 15 minutes in 3.7% formaldehyde in PBS, stained with 0.4% crystal violet, and air dried. Each experiment had duplicates and was performed three times. The survival curve was plotted by fitting the equation: $S = e^{(-\alpha D - \beta D^2)}$ in the Prism software. Dose modifying factor (DMF) was determined by a ratio of dose required to reach a 50% survival fraction in cells stably transfected with scrambled shRNA to the dose required to reach a 50% surviving fraction in HMGB1 shRNA–transfected cells.

Comet assay

Cells were irradiated 24 hours before running the Alkaline CometAssay (Trevigen) as per the manufacturer's instructions. Two trials were done, with 10 image sections taken from each trial. All 20 image sections for each treatment arm were analyzed by CaspLab software to obtain the average olive tail moment (OTM; ref. 16). The comet parameter considered was OTM (defined as the product of the percentage of total DNA in the tail and the distance between the centers of gravity of the head and tail regions) as it has been shown to be a good measure of DNA damage in cells (17, 18).

Immunofluorescence

Twenty-four hours after radiation, cells were fixed with 4% formaldehyde in PBS. All steps were carried out according to the Cell Signaling Technology immunofluorescence protocol. HMGB1 (Abcam) antibody was used at 1:1,000 dilution and LC3 (Cell Signaling Technology) antibody was used at 1:400 dilution. To visualize fluorescence, an inverted IX-81 microscope

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(Olympus) was used. Images were captured using the ImagePro$^+$ software (Media Cybernetics).

In vivo xenograft model

The McGill University Health Center FACC approved our animal protocol #5428. All ethical standards were fully followed and respected. The animals were maintained in state-of-the-art facilities with stringent procedures in place for conducting animal research. Female athymic mice (Nu/Nu strain, 4–6 weeks old; Charles River Laboratories) were used for our xenograft bladder cancer model, as previously reported (19). Briefly, mice were randomized into two groups (UC3 Scramble or UC3 HMGB1 shRNA). To facilitate tumor taking, Matrigel (BD Biosciences) was added to cell suspensions prior to injection. Mice were subcutaneously injected with cells (10^6 cells per injection site) on both flanks. Tumors were allowed to grow for 1 week prior to randomization into further arms (control and radiation), of 7 to 8 mice each. Radiation was given in a fractionated dosage totaling 6 Gy (2×3 Gy). Mice were followed for 3 weeks from the onset of treatments. Tumors were measured twice a week using a Vernier caliper to calculate volumes, $V = [(\text{length} \times \text{width}^2) \times (\pi/6)]$. After euthanasia, tumors were harvested, immediately weighed, and formalin fixed and paraffin embedded (FFPE) or snap frozen for further studies.

Immunohistochemistry

Sections of FFPE tumor blocks (4–5 μm) were rehydrated. Antigen retrieval was performed by boiling these sections in Tris-EDTA buffer. Overnight incubation with a HMGB1 (Abcam, dilution 1:250) or a P62 (Abcam dilution 1:100) antibody was done. IgG secondary antibody kit (Santa Cruz Biotechnology) and DAB substrate (Sigma Aldrich) was used for detection and color development. Slides were viewed under a Leica inverted microscope and pictures were captured using a Leica Application Suite. Analysis was based on an average of 5 foci, at 40× magnification, showing viable cells, and a computed H-score was calculated by summing the products of the percentage cells stained (0–100) and the staining intensity (0 for negative staining, 1 for low staining, 2 for moderate staining, and 3 for high staining).

Statistical analysis

All statistical data analyses were done using the Student *t* test, with the significance set at 5% and null hypothesis being rejected at $P < 0.01$. Data points were obtained from at least duplicate measurements, and each experiment was performed at least twice. *** represents $P < 0.001$.

Results

Baseline HMGB1 expression and inherent radiation response characterization of urothelial carcinoma cell lines *in vitro*

With the goal of establishing HMGB1's role in radiation response of bladder cancer, our first objective was to obtain a baseline characterization of HMGB1 levels and radiation response in different cell lines. We quantified HMGB1 mRNA and protein levels across a panel of eight human urothelial carcinoma cell lines (Fig. 1A). Although each cell line presented with unique HMGB1 expression, a strong linear correlation ($r^2 = 0.91$) between mRNA and protein levels was observed, indicating a good association between transcription and translation levels of HMGB1 across all tested cell lines. Average values from all trials established the two extremes of HMGB1 expression among the different bladder cancer

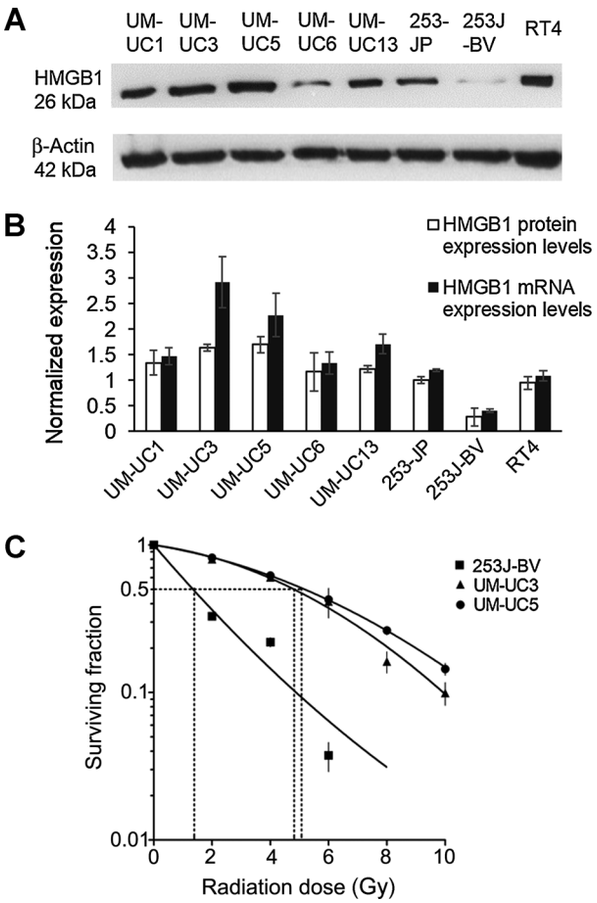

Figure 1.
Baseline characterization of HMGB1 expression and inherent radiation response. A, Western blot analysis representing HMGB1 protein levels across a panel of eight different urothelial carcinoma cell lines. B, bar graph showing the average protein and mRNA levels across all cell lines. Error bars indicate SD. C, clonogenic assay for high (UM-UC3 and UM-UC5) and low (253J-BV) HMGB1-expressing cell lines.

cell lines: UM-UC3 and UM-UC5 as two cell lines with consistently high HMGB1 protein levels, whereas 253J-BV as a cell line with very low HMGB1 protein levels (Fig. 1B).

Using our clonogenic assay data (19), we correlated the response to radiation of five urothelial carcinoma cell lines to the levels of HMGB1 protein expression in each of them. The Spearman correlation resulted in a value of 0.900 (test of null hypothesis: HMGB1 and radiation dose were independent, $P = 0.0374$), concluding that HMGB1 protein levels had a significant association with the inherent radiation response of bladder cancer cells. Next, to mature this correlation into causality, we chose to evaluate the change in radiation response post-HMGB1 level modulation. For the baseline control, inherent radiation response of high (UM-UC3, UM-UC5) and low (253J-BV) HMGB1 expression cell lines was tested via clonogenic assays (Fig. 1C). The survival curves of UM-UC3 and UM-UC5 cell lines showed a gradual decrease in surviving fraction over increasing doses of radiation, with more than 30% cells still surviving at 8 Gy. Moreover, both cell lines required a radiation dose of about 5 Gy (4.82 Gy for UM-UC3 and 5.20 Gy for UM-UC5) to reach a 50% surviving fraction. On the other hand, the survival curve for

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253J-BV was much steeper. The requirement of only 1.43 Gy for 50% survival fraction proved that these cells were highly sensitive to radiation.

HMGB1 protein knockdown sensitizes bladder cancer cells to radiation

Manipulation of HMGB1 protein level was carried out in high expression bladder cancer cell lines (UM-UC3 and UM-UC5) to detect its direct effect on radiation responsiveness of cells. Stable control (untransfected and scrambled shRNA transfected) and knockdown (HMGB1 shRNA transfected) cell lines were established (Fig. 2A and D). Quantification analysis revealed a highly successful HMGB1 knockdown in both cell lines. More than 70% HMGB1 protein knockdown was seen in shRNA-transfected UM-UC3 cells, whereas 50% HMGB1 knockdown was noticed in the shRNA-transfected UM-UC5 cells (Fig. 2B and E, respectively). As expected, the control transfection of scramble vectors displayed no such decrease in HMGB1 levels, indicating efficiency.

Next, clonogenic assays were carried out on control and knockdown cell lines. When the shRNA-transfected cells were subjected to radiation, the effect of HMGB1 loss could be seen in cell survival across all tested doses. Both the shRNA cell lines had a steeper curve than their respective control and scramble cell lines (Fig. 2C and F). For a surviving fraction of 50%, UM-UC3 scramble cell line required a dose of 4.69 Gy, whereas its HMGB1 shRNA cell line required only 1.62 Gy, giving a $DMF_{0.5}$ value of 2.9. Similarly, for a surviving fraction of 50%, UM-UC5 scramble cell line required a dose of 4.94 Gy, while its HMGB1 shRNA cell line required only 2.84 GY. This difference, indicated by the $DMF_{0.5}$ value of 1.74, measured the increased radiosensitization because of HMGB1 loss in the cell line. Overall, the knockdown of HMGB1 triggered an increased radiation response in both UM-UC3 and UM-UC5 cell lines as illustrated by highly significant $DMF_{0.5}$ ratios. Furthermore, adding recombinant HMGB1 (extraction and clonogenic assay use of recombinant HMGB1 detailed in the Supplementary Methods) to UM-UC3 and UM-UC5 HMGB1 shRNA cell lines reverses this radiosensitization, giving further proof that HMGB1 is a key modulator of bladder cancer radiosensitivity (Supplementary Fig. S1).

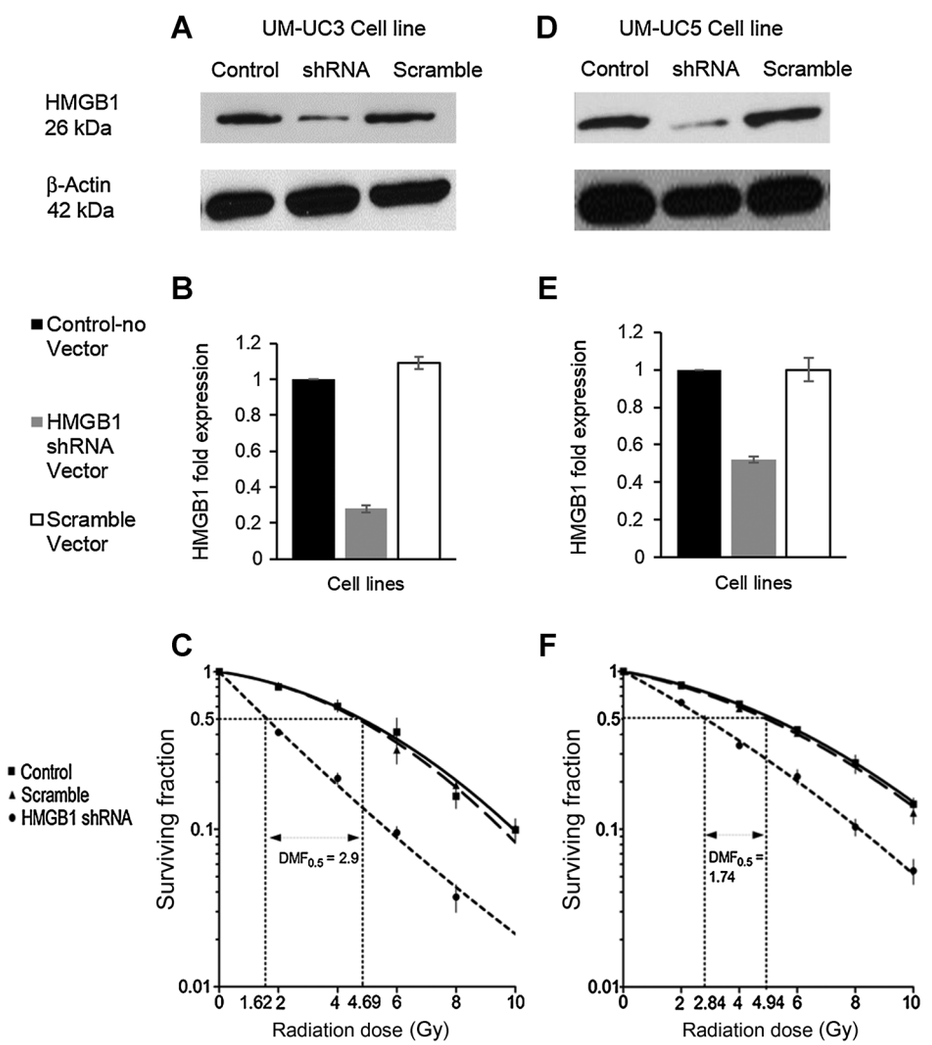

Figure 2.
HMGB1 protein knockdown. A and D, Western blot analysis indicating the levels of HMGB1 after shRNA knockdown of HMGB1 protein in high-expressing cell lines UM-UC3 and UM-UC5, respectively. B and E, bar graphs depicting average value of HMGB1 fold expression. C and F, clonogenic assay survival curves for stable cell lines.

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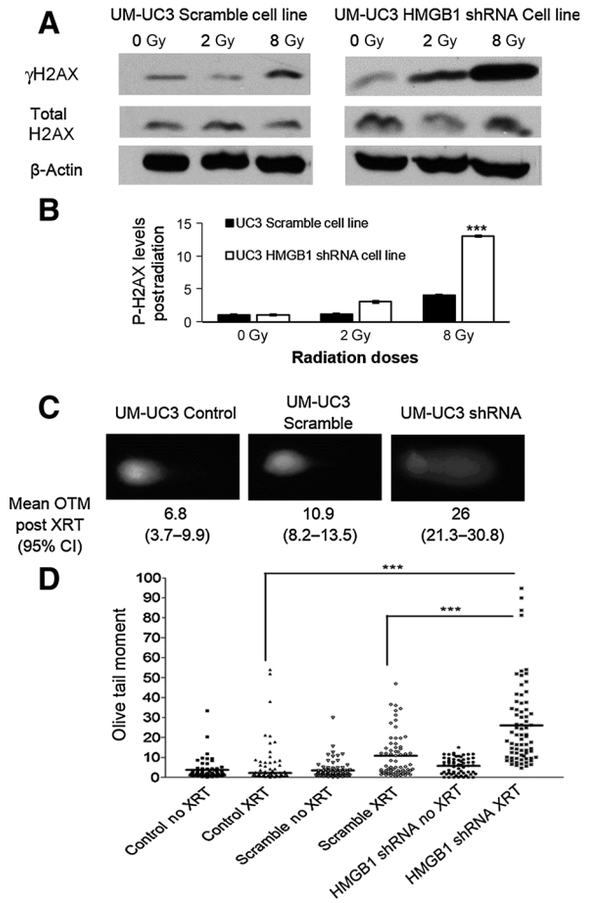

Figure 3.
DNA damage analysis *in vitro*. A, Western blots of phospho-H2AX levels indicating DNA damage postradiation. B, graph representing average phosphorylation of H2AX after radiation in both cell lines across two different radiation doses. C, representative pictures of comets formed by different cell lines postradiation. D, graph summarizing the entire range of OTM values among the three cell lines and their averages. CI, confidence interval; XRT, radiation. ***, $P < 0.001$.

Loss of HMGB1 in bladder cancer cells leads to higher DNA damage postradiation

We further went on to elucidate the pathways through which HMGB1 acts in bladder cancer radioresistance. With the known facts that radiotherapy induces cell death by targeting DNA and creating single and double-strand DNA breaks (20), recent reports on HMGB1's involvement in DNA repair processes inclined our attention to look at HMGB1's involvement in DNA damage postradiation. To evaluate this, two assays were chosen: expression levels of H2AX phosphorylation and alkaline comet assay.

As the phosphorylation occurs soon after DNA damage and peaks at 1 hour after treatment, γH2AX is currently the most easily detectable and quantitative marker for *in vitro* DNA damage testing (21). For our experiments, Western blots for γH2AX were done with protein extractions made 3 hours after radiation. Results showed expected increase in levels of γH2AX at incremental radiation doses for the UM-UC3 scramble–transfected cell line, but three times higher γH2AX levels for the UM-UC3 HMGB1 shRNA–transfected cell line (Fig. 3A and B). Similar results were seen in UM-UC5 (Supplementary Fig. S2). This indicated a significantly higher DNA damage postradiation associated with HMGB1 loss. Next, we tested DNA damage at the level of individual cells via the alkaline comet assay. The analysis for UM-UC3 control and scramble cell lines depicted mean OTM values of approximately at 7 and 11, respectively, indicating the extent of DNA damage by 5 Gy of radiation in these cells (Fig. 3C and D). UM-UC5 HMGB1 shRNA cell line also had four times higher OTM values as compared with the controls (Supplementary Fig. S3); whereas for the UM-UC3 HMGB1 shRNA cell line, a significantly ($P < 0.001$) higher mean OTM of 26 was observed at the same dose. These findings showed that HMGB1 knockdown leads to higher DNA damage postradiation, and hence DNA damage pathway seems to be an important part of HMGB1-induced radioresistance in bladder cancer cells.

HMGB1 is involved in radiation-induced autophagy of bladder cancer cells

Another interesting feature of HMGB1 is its potential role in autophagic induction, characterized by the protein's translocation from the nucleus to the cytoplasm. As autophagy is a "cell survival" process implicated in almost all cancers, we looked into HMGB1's role in induction of postradiation autophagy in bladder cancer cells.

First, the expression and subcellular localization of LC3 and HMGB1 was seen in control UM-UC3 cells by immunofluorescence (Fig. 4A and B). Results showed an increase in levels of cytoplasmic LC3 as well as number of punctae, both indicators of autophagy induction (22). Moreover, translocation of HMGB1 from nucleus to cytoplasm was observed postradiation (5 Gy), giving evidence to HMGB1-induced autophagy in bladder cancer cells. To test the direct implications of HMGB1 manipulation on autophagy levels postradiation in bladder cancer cells, LC3 expression levels were evaluated in UM-UC3 and UM-UC5 HMGB1 shRNA cell lines (Fig. 4C and Supplementary Fig. S4). Quantification of LC3II to LC3I ratio depicted at least four times greater conversion in UM-UC3 scramble cells as compared with UM-UC3 HMGB1 shRNA cells ($P < 0.001$; Fig. 4D). This signified high levels of autophagy in control cells that seemed highly attenuated in the HMGB1 knockdown cells postradiation. Furthermore, expression levels of PARP cleavage, a marker of apoptosis, were enhanced in HMGB1 shRNA cells.

Recently, dissociation from p53 and association with Beclin-1 have also been established as modulators of the autophagy process (23, 24). In our immunoprecipitation analysis, we demonstrated the following postradiation: approximately four times higher association of HMGB1 with Beclin-1 and three times lower binding to p53 in UM-UC3 cells, further giving validity to HMGB1's role in bladder cancer autophagy (Fig. 4E). Cumulative evidence from these three experiments concluded that HMGB1 is a proautophagic protein promoting postradiation autophagy and that its loss results in autophagy inhibition and possibly an increase in early apoptosis of bladder cancer cells postradiation.

HMGB1 knockdown delays bladder cancer cell tumor growth *in vivo*

The fact that *in vitro* HMGB1 loss alone could increase DNA damage and inhibit autophagy in bladder cancer cells exposed to radiation proved its importance as a radioresistant protein. To get further insight into the proposed relevance of HMGB1's role in

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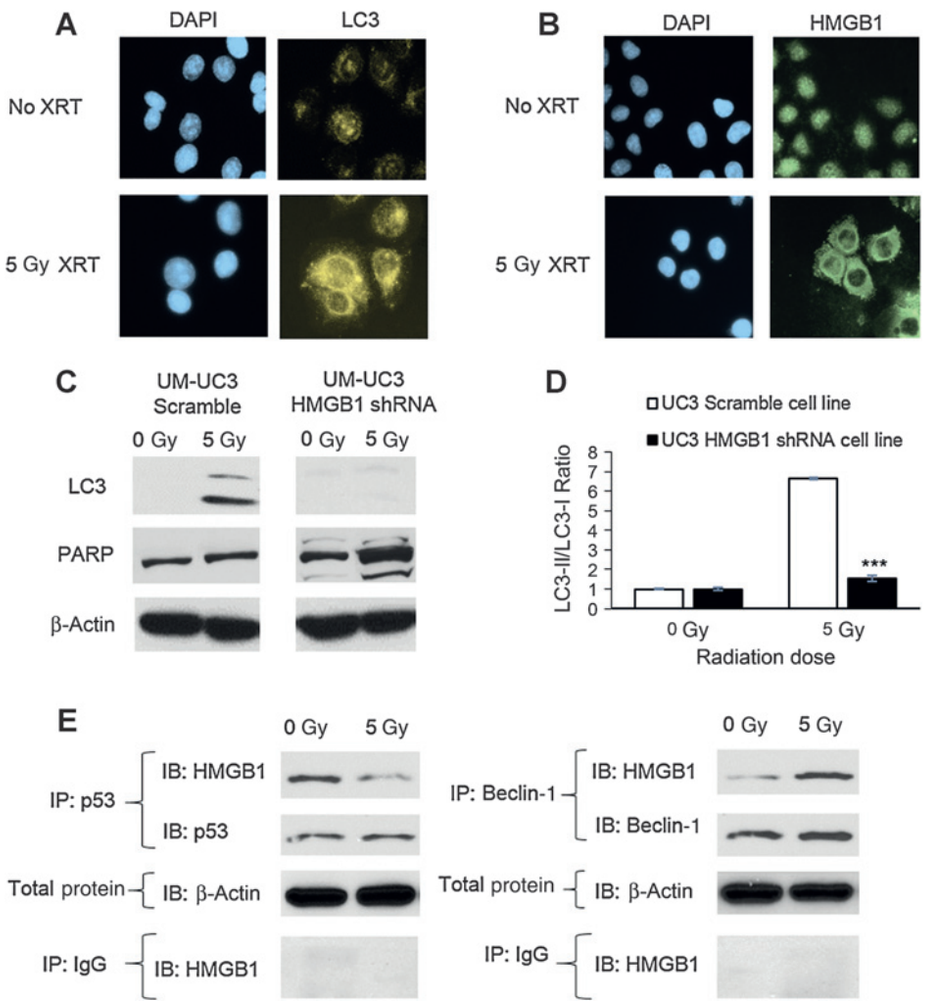

Figure 4.
HMGB1-mediated autophagy postradiation. A and B, immunofluorescence images show LC3 and HMGB1 cytoplasmic accumulation postradiation. DAPI staining marks nuclear DNA. C, Western blot analysis indicates loss of autophagy marker LC3 after HMGB1 knockdown and radiation. Cleavage of PARP was also seen to measure apoptosis levels in the cells. D, quantification results depicting the decreased LC3II/LC3I ratio postradiation in HMGB1 shRNA–transfected cells. E, immunoprecipitation (IP) experiment shows increased HMGB1 binding to Beclin-1 and loss of association with p53 upon radiation. ***, $P < 0.001$.

this radioresistance, we carried out *in vivo* experiments using established stable cell lines.

The *in vivo* pattern of tumor growth suggested a growth delay in the HMGB1 shRNA tumors from both groups (untreated and radiation). More significantly, within the radiation group, the tumors from the HMGB1 shRNA cell line were significantly smaller ($P < 0.001$) in volume at every time point as compared with tumors from the scramble cell line (Fig. 5A), illustrating that loss of HMGB1 leads to a better radiation response *in vivo*. Also, the average tumor weight in the scramble radiation group (0.28 g ± 0.1) was double the weight in the HMGB1 shRNA radiation group (0.15 g ± 0.08, $P < 0.001$), further demonstrating the higher response to radiation in the HMGB1 shRNA cells (Fig. 5B).

Immunohistochemical staining shows reduced levels of autophagy in HMGB1 shRNA tumor tissue sections

Next, HMGB1 staining was done in nontreated (control) tumor tissue to confirm the baseline levels of HMGB1 in the different arms (HMGB1 shRNA and scramble; Fig. 6A). As HMGB1 is majorly a nuclear protein under untreated circumstances, H-score was obtained by looking at the intensity and percentage of nuclear brown staining. Analyzed results showed significantly lower levels of HMGB1 staining in the shRNA control tumors as compared with the scramble controls ($P < 0.001$; Fig. 6C). This validated the efficiency of the stable knockdown *in vivo*.

To evaluate postradiation autophagy in the tumor sections, P62 staining was done (Fig. 6B). P62 is a cytoplasmic protein that gets obliterated with high levels of autophagy (25). Hence, cells with low or absent cytoplasmic P62 staining were considered to undergo autophagy. Quantification of H-score showed that scramble-radiated tumors had the least cytoplasmic P62 staining (highest levels of autophagy), whereas the HMGB1 shRNA–radiated tumors had the highest expression of P62 (autophagy inhibition; Fig. 6D). This difference gave significant ($P < 0.001$) evidence to postradiation autophagy inhibition upon HMGB1 loss *in vivo*.

Discussion

In this study, we provide novel evidence that HMGB1 level in bladder cancer cells is able to predict response to radiation. We performed a knockdown of HMGB1 protein levels in selected urothelial carcinoma cell lines to implicate HMGB1 directly in bladder cancer radioresistance. Moreover, we conducted *in vitro* and *in vivo* experiments that showed that a decrease in HMGB1

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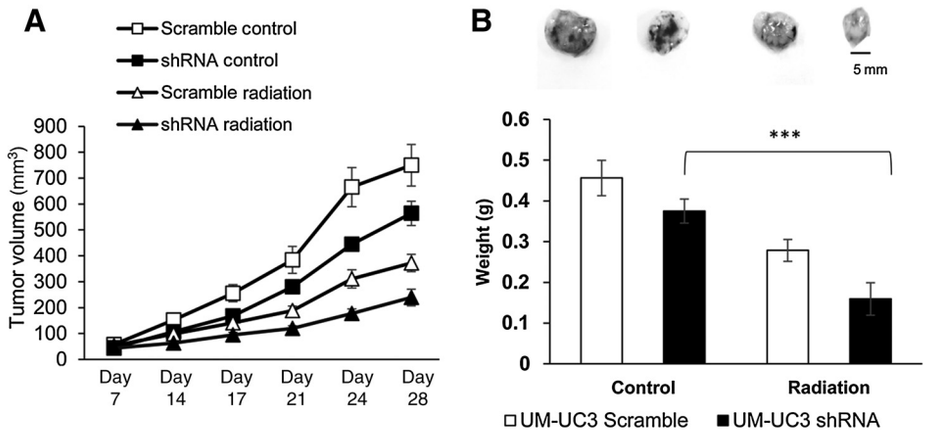

Figure 5.
HMGB1 loss delays bladder cancer growth *in vivo*. A, tumor growth curve showing the tumor volumes for the four different arms of the experiment at different measurement days. Day 7 is the pretreatment measure; during day 7 and 14, treatment was given to radiation group (no measurements).
B, quantification of tumor volume in the four groups with averages and representative pictures. ***, $P < 0.001$.

levels leads to heightened radiosensitivity and that mechanisms of DNA damage repair and autophagy play a significant role in this HMGB1-mediated radioresistance of bladder cancer cells.

Radiation-induced DNA damage consists majorly of DNA lesions like DSBs which can be repaired either by homologous recombination (HR) or nonhomologous end joining (NHEJ). During NHEJ, studies have pointed to the role of HMGB1 in targeting DNA-PKcs to DNA break ends for enhancing ligation (26, 27). This warranted an investigation into the extent of DSB damage postradiation in our HMGB1 knockdown cell lines, which was measured by the level of histone H2AX phosphorylation (γH2AX). It is well established that γH2AX is a crucial damage recognition step during DSB repair which decides whether the cell will undergo DNA repair, cell-cycle arrest, or apoptosis (28). We observed in our experiments that a loss of HMGB1 accumulates γH2AX in bladder cancer cells postradiation, suggesting an impairment in the DSB repair pathway of these cells. Where and how HMGB1 acts to regulate DNA DSB repair in bladder cancer cells is topic of further research. It is possible that it modulates the effect of other downstream repair proteins like DNA-PKcs, MLH1/MSH2 (29), or XPC/A (30) or facilitates chromatin remodeling through proteins like ACF and CHRAC (31). Strategies of radiosensitization that focus on HR pathways include targeting the MRN complex (Mre11/Rad50/NBS1) which is an important sensor of DNA damage and is responsible for resecting DNA strands in DSB repair (32). Inhibition of these proteins is hence linked to radiosensitization (33–35), whereas MRE11 expression itself has gained evidence for its predictability of radiation response in recent studies (36, 37). Alongside MRE11, ATM is also known to play a central role in DNA damage repair and cell-cycle checkpoints and has been thoroughly investigated in the field of radiosensitization (35). ATM inhibitors like KU-55933 and CP466722 have shown promising radiosensitization *in vitro* and in preclinical studies (38, 39). Recently, KU-55933 has also been implicated in DAB21P-defective bladder cancer radiosensitivity (40). It would be remarkable to see whether HMGB1 downregulation intensifies the KU-55933–caused radiosensitivity of bladder cancer cells, making this a potential project for future research.

Another mechanism that cancer cells use to develop resistance is induction of the "cell survival" autophagic process. Also, it is well established that HMGB1 and p53 mediate cancer cell survival through a balance of autophagy and apoptosis (23). Poststress, HMGB1 translocates from the nucleus to the cytoplasm and induces autophagy by binding to Beclin-1. Recent studies have also enumerated that during autophagy, HMGB1 promotes phosphorylation and activation of the ERK1/2 pathway (24), and HMGB1 binding to Beclin-1 relies upon the complex ULK1–mAtg13–FIP200 and this leads to the formation of the Beclin-1–PI3KC3 complex which facilitates autophagic progression (41). Moreover, the role of HMGB1 as a damage-associated molecular pattern (DAMP) molecule has special implications for regulation of cancer cell autophagy (42). These findings support our preliminary investigations of HMGB1-mediated autophagy in bladder cancer which depict that while HMGB1-mediated autophagy is activated upon radiation in bladder cancer cells, loss of HMGB1 leads to severe attenuation of this process. This conclusion merits further investigation into the detailed steps of HMGB1-mediated autophagy in bladder cancer, as only then we will be able to develop autophagy-targeted radiosensitization therapies for bladder cancer. Examining the BCL-2/Beclin-1 interaction as well as looking at upstream signaling pathways like MEK/ERK and MAPK would be important in identifying key players in this process (24, 43, 44). NF-κB signaling pathway is another important piece of this puzzle. Strong interactions have been established between the autophagy and NF-κB pathways (45). Moreover, NF-κB has been implicated as a downstream pathway of HMGB1 action in bladder cancer (46). The evaluation of if/how HMGB1 activates autophagy via NF-κB pathways in bladder cancer would give us insights into cancer cell metabolism and provide an opportunity for development of more radiosensitization techniques.

Today, the main hurdle in bringing radiotherapy to the forefront of strategies being used for muscle-invasive bladder cancer is the development of radioresistance in cancer cells that leads to suboptimal control of disease. Chemotherapy, like gemcitabine and cisplatin, is currently in use with radiotherapy as a combination treatment strategy against aggressive bladder cancers. The trimodality therapy which included transurethral resection of bladder tumor (TURBT) in addition to chemo and radiotherapy has shown significant success as a bladder preservation technique (47). As we showed that HMGB1, in part, controls the subtle balance between death and survival in cells which are exposed to radiation and can predict radiation response, it would be interesting to study how HMGB1 modulation would impact the combination treatment. The details themselves are beyond the scope of this article, but as this investigation is currently in

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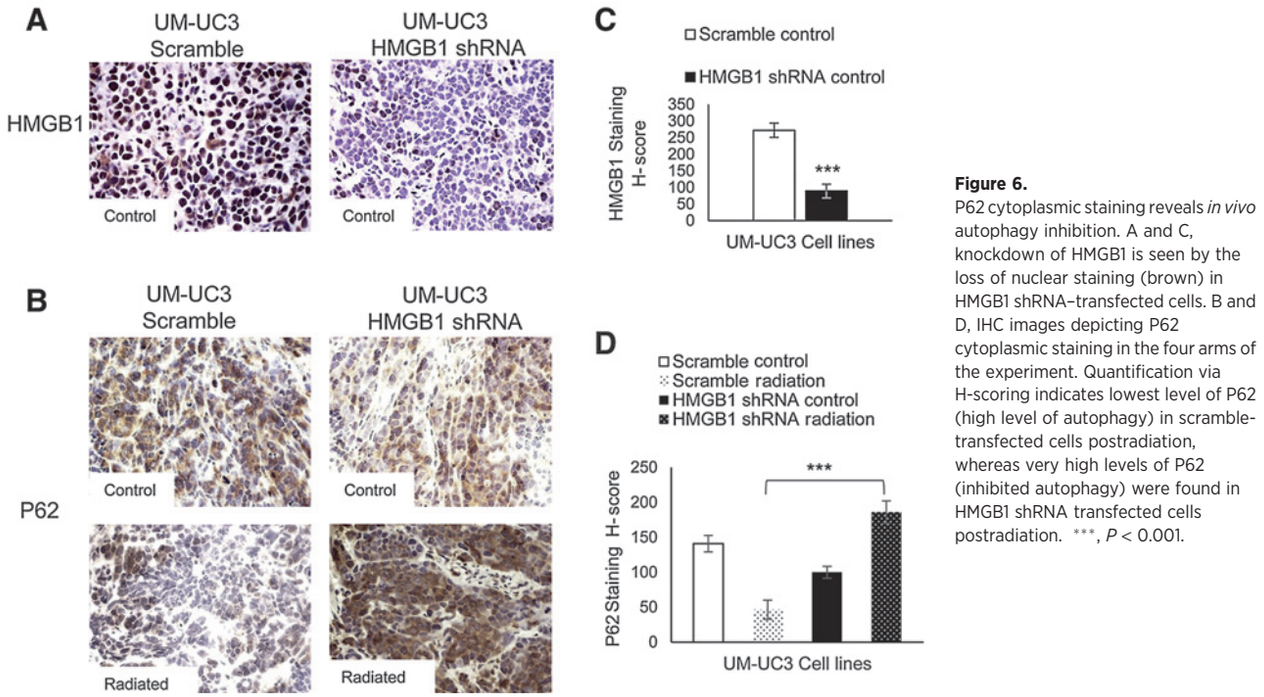

Figure 6.
P62 cytoplasmic staining reveals *in vivo* autophagy inhibition. A and C, knockdown of HMGB1 is seen by the loss of nuclear staining (brown) in HMGB1 shRNA–transfected cells. B and D, IHC images depicting P62 cytoplasmic staining in the four arms of the experiment. Quantification via H-scoring indicates lowest level of P62 (high level of autophagy) in scramble-transfected cells postradiation, whereas very high levels of P62 (inhibited autophagy) were found in HMGB1 shRNA transfected cells postradiation. ***, $P < 0.001$.

progress in our laboratory, we can say that HMGB1 knockdown and utilization of combination (gemcitabine and radiation) treatment will lead to promising radiosensitizing results.

In this article, we have presented that low HMGB1 expression in bladder cancer cells prevents efficient DNA repair, inhibits autophagy, and significantly ($P < 0.001$) radiosensitizes the bladder cancer cells *in vivo* and *in vitro*. This investigation on HMGB1 is a first of its kind in the context of radioresistance in muscle-invasive bladder cancer disease. Clinical correlation of HMGB1 expression was done by Yang and colleagues as well as Wang and colleagues, where they depicted that HMGB1 overexpression was significantly associated with tumor grade, stage, and progression via angiogenesis, and that HMGB1 was an independent prognostic factor for patients with bladder cancer (48, 49). Our findings validate these clinical correlations and collectively indicate that HMGB1 could be a great marker for predicting radiation response in muscle-invasive bladder cancer. We have investigated DNA damage repair and autophagy as pathways of HMGB1-mediated radioresistance, and future research will lead to radiosensitizing drugs/target genes in these pathways which would be beneficial for improving radiosensitivity of bladder cancer and optimizing radiotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Shrivastava, J.J. Mansure, M. Popovic, J. Seuntjens

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Shrivastava, J.J. Mansure, F. Cury, J. Seuntjens, W. Kassouf

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