

## Quantitative, Noninvasive Imaging of Radiation-Induced DNA Double-Strand Breaks *In Vivo*

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### Abstract

DNA double-strand breaks (DSB) are a major form of DNA damage and a key mechanism through which radiotherapy and some chemotherapeutic agents kill cancer cells. Despite its importance, measuring DNA DSBs is still a tedious task that is normally carried out by gel electrophoresis or immunofluorescence staining. Here, we report a novel approach to image and quantify DSBs in live mammalian cells through bifragment luciferase reconstitution. N- and C-terminal fragments of firefly luciferase genes were fused with *H2AX* and *MDC1* genes, respectively. Our strategy was based on the established fact that at the sites of DSBs, H2AX protein is phosphorylated and physically associates with the MDC1 protein, thus bringing together N- and C-luciferase fragments and reconstituting luciferase activity. Our strategy allowed serial, noninvasive quantification of DSBs in cells irradiated with X-rays and <sup>56</sup>Fe ions. Furthermore, it allowed for the evaluation of DSBs noninvasively *in vivo* in irradiated tumors over 2 weeks. Surprisingly, we detected a second wave of DSB induction in irradiated tumor cells days after radiation exposure in addition to the initial rapid induction of DSBs. We conclude that our new split-luciferase-based method for imaging  $\gamma$ -H2AX–MDC1 interaction is a powerful new tool to study DSB repair kinetics *in vivo* with considerable advantage for experiments requiring observations over an extended period of time. *Cancer Res*; 71(12); 4130–7. ©2011 AACR.

### Introduction

DNA double-strand breaks (DSB) result from exposure to ionizing radiation such as cosmic galactic rays, medical X-rays, or terrestrial radon gas. In eukaryotes, it has been shown that DSBs are critical lesions that can lead to cell death (1). Misrepaired DSBs also have devastating consequences; they are a major cause of chromosome aberrations such as translocations, fusions, and rings (2, 3). As such, DSBs represent major underlying causes for various genetic diseases (4) and cancer (5). Because of such dire consequences, eukaryotic cells possess a robust system for detecting and repairing DSBs (4).

Eukaryotic cellular defense against DNA DSBs consists of sensing and repair factors. Upon initial generation of DSBs, the histone variant H2AX is rapidly converted into phosphorylated form ( $\gamma$ -H2AX) by DNA-PK, ATM, and/or ATR, which act

as sensors for DSBs (4).  $\gamma$ -H2AX then serves as an anchor to recruit additional factors through a series of highly coordinated molecular events to repair the damaged DNA. Some of these events include phosphorylation and activation of p53 (6, 7), which may lead to cellular apoptosis or cell-cycle arrest; recruitment of a large assortment of "effector" DNA repair proteins, such as Ku70, Ku80, and DNA ligase IV, XRCC4, which are involved in nonhomologous end joining (NHEJ) repair; and BRCA1, RAD51, and p53BP1, which are involved in homologous recombination repair (4, 8).

Despite the fact that a larger number of DSB repair factors and their roles have been identified, the dynamic interactions of these factors and how they respond to DNA damaging agents are not well understood *in vivo*, especially at the tissue level. At the cellular level, detailed studies of DSB kinetics have been conducted by use of immunostaining of  $\gamma$ -H2AX, identified by Rogakou and colleagues as a key marker for DSB in eukaryotic cells (9, 10). H2AX is rapidly phosphorylated (within minutes) at the site of DSB, forming visible foci-like structures surrounding DSBs. Each focus seems to correlate with a single DNA DSB. It has also been shown that at each focus  $\gamma$ -H2AX covers several megabases of DNA in mammalian cells (10). Furthermore,  $\gamma$ -H2AX needs to be dephosphorylated upon completion of DNA repair (11). Therefore, the total level of  $\gamma$ -H2AX in a cell is a kinetic balance of DSB formation, detection, and its successful repair. Detailed studies of  $\gamma$ -H2AX foci formation kinetics after exposure to DNA-damaging agents have been aided by an exogenously introduced H2AX–GFP fusion protein (12, 13), which allows for visualization of  $\gamma$ -H2AX through fluorescence microscopy detection of

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green fluorescent protein (GFP) foci. However, the sensitivity of such methods is poor and the studies were mostly conducted at the cellular level. At the tissue level, quantitative studies are still not available because of the lack of applicable methods. Lack of data at the tissue level has hampered efforts to achieve better understanding of DSB generation and repair kinetics in normal or pathologic tissues such as cancer.

In this report, we describe a novel system for noninvasive, quantitative imaging of  $\gamma$ -H2AX foci formation in mammalian cells and tissues. Our system is based on  $\gamma$ -H2AX–MDC1 interaction–mediated reconstitution of split luciferase fragments (14–16). One of the key proteins that physically associates with  $\gamma$ -H2AX is the protein mediator of DNA damage checkpoint, or MDC1. The main function of MDC1 was shown to be collaboration with  $\gamma$ -H2AX to recruit factors responsible for checkpoint activation or DNA damage repair (14, 15). More importantly, MDC1 was shown to associate with  $\gamma$ -H2AX in a phosphorylation-dependent manner (17). We show that this system works effectively both in cell culture and in tumor tissues. It allows for serial examination of  $\gamma$ -H2AX–MDC1 interaction over an extended period of time in the same cells or tissues, thereby providing significant insights into the kinetics of  $\gamma$ -H2AX foci formation after exposure to DSB generating agents. We show that  $\gamma$ -H2AX–MDC1 interaction could occur very early on after cellular exposure to radiation and after days, consistent with earlier discovery of persistent DNA damage and genetic instability in irradiated mammalian cells (18–22).

## Materials and Methods

### Cell lines and tissue culture

H322 cells were obtained from Dr. Paul Bunn and Barbara Helfrich of the University of Colorado Cancer Center (Aurora, CO). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum.

### Construction of the reporter plasmids

Please see details in Supplementary Data.

### Transduction of $\gamma$ -H2AX/MDC1-Luc2 reporter genes into tumor cells

Lentiviral vectors encoding the reporter genes were made following standard procedures (23). H322 lung cancer cells were then coinfecting with lentiviral vectors encoding the reporter genes. Clonal populations of stable transfectants with the binary reporter  $\gamma$ -H2AX/MDC1-Luc2 were derived through serial dilution. Please see Supplementary Data for details on establishing reporter-transduced cells.

### Imaging of $\gamma$ -H2AX/MDC1-luc2 reporter *in vitro*

Stably infected  $\gamma$ -H2AX/MDC1-Luc2 cells were seeded into 12-well plates at  $1 \times 10^5$  cells/well in RPMI 1640 medium supplemented with 10% fetal calf serum. When cells grew to 80% to 90% confluence, some plates were exposed to X-rays. At different time points after irradiation, culturing media were replaced by PBS solution containing luciferin (at 150  $\mu$ g/mL luciferin; Caliper Life Sciences). Reporter activities in the cells

were subsequently imaged with the IVIS200 optical imaging system (Caliper Life Sciences). Additional cells were plated in 12-well plates and irradiated with the same conditions so that cell numbers at different time points could be determined. To derive relative reporter activities, bioluminescence activities in each of the wells were normalized against the cell numbers and the control following the formula:

relative reporter activity

$$= (\text{bioluminescence}^{\text{IR}} / \text{cell number})$$

$$\div (\text{bioluminescence}^{\text{control}} / \text{cell number})$$

### Western blot analysis

Western blot analyses were done according to established procedures (24). Detailed antibody information is provided in the Supplementary Data.

### Radiation exposure to X-rays and $^{56}\text{Fe}$ ions

The activities of  $\gamma$ -H2AX/MDC1-luc2 reporter were evaluated after cellular exposure to X-rays and  $^{56}\text{Fe}$  ions. To conduct X-ray irradiation, an RS-2000 x-irradiator (Rad Source Technologies) was used. The dose rate used was 1 Gy/min. Doses given ranged from 2 to 6 Gy. To conduct irradiation with  $^{56}\text{Fe}$  ions, cells were transported to the NASA Space Radiation Laboratory at the Brookhaven National Laboratory (BNL; Brookhaven, Long Island, NY) in sealed T-25 flasks. The dose rate used was 0.5 Gy/min. Doses used were from 0.5 to 2.0 Gy. After irradiation, the cells were immediately shipped back to our laboratory in Denver. Immediately after receiving cells from the BNL (usually approximately 24 hours after radiation exposure at BNL), irradiated cells and mock-irradiated cells were seeded into 12-well plates at a density of  $1 \times 10^5$  cells/well in 1 mL of RPMI 1640 supplemented with 10% FBS. Subsequently, luciferase activities were serially imaged at different time points.

### Ionizing irradiation-induced nuclear foci by immunofluorescence microscopy

An established protocol was followed to examine  $\gamma$ -H2AX foci through immunofluorescence in irradiated cells (9). Please see details in the Supplementary Data.

### Imaging $\gamma$ -H2AX/MDC1-luc2 reporter *in vivo*

Please see details in the Supplementary Data.

### Statistical method

Student *t* test was used where necessary.  $P < 0.05$  was considered to be statistically significant.

## Results

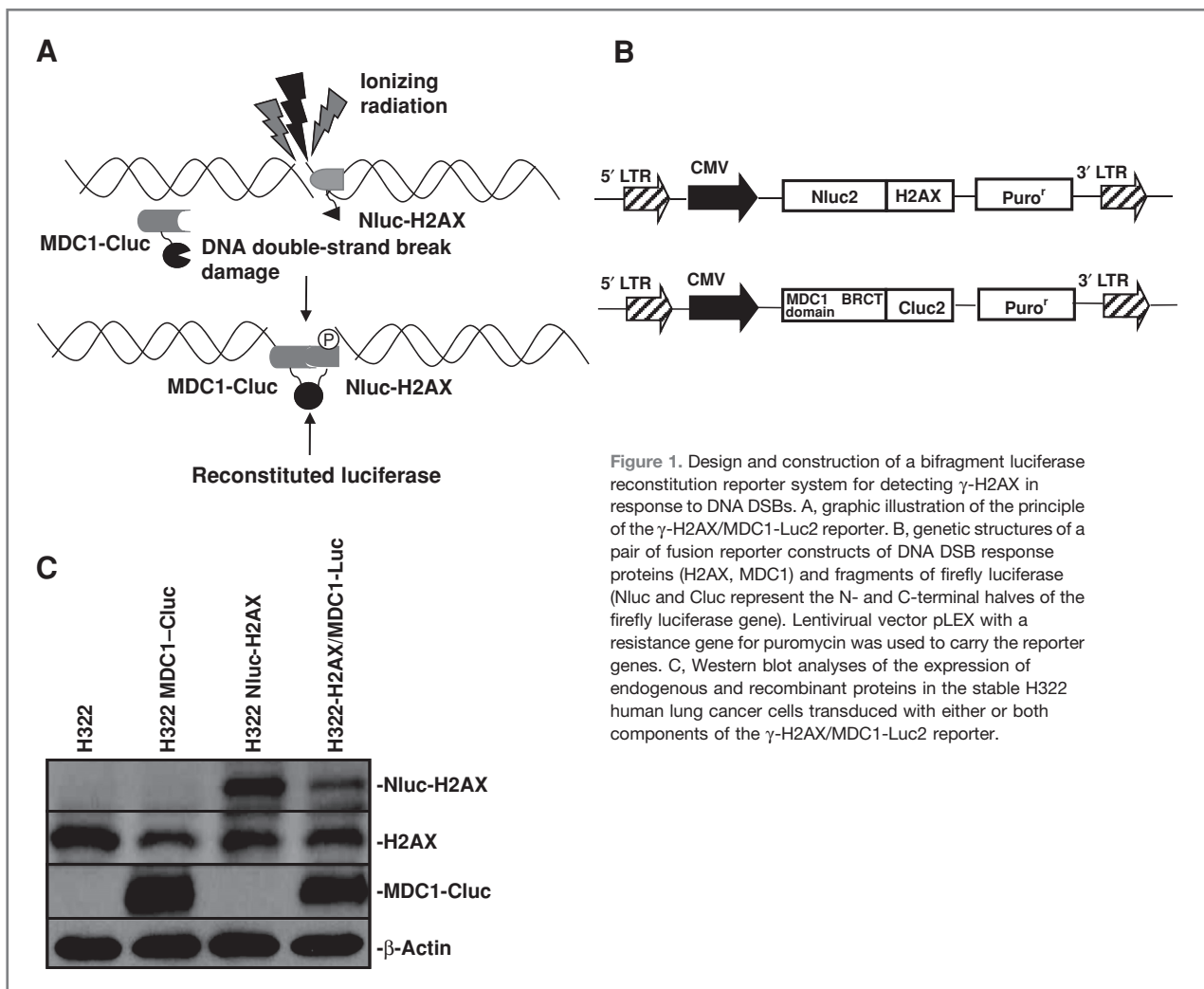
### Design and construction of a bifragment luciferase reconstitution system for noninvasive observation of DNA double-strand breaks in mammalian cells

To image DNA DSBs, we took advantage of the fact that the histone H2AX is rapidly phosphorylated at sites of DSBs and that phosphorylation of H2AX was accompanied by its

recruitment of an assortment of other proteins involved in DNA repair, checkpoint activation, and chromatin remodeling. We reasoned that we could visualize the phosphorylation of H2AX by imaging the physical interaction of  $\gamma$ -H2AX with MDC1, which occurs only when H2AX is phosphorylated (14, 15). To do this, we adopted a previously proven strategy of bifragment luciferase reconstitution (16). Two overlapping fragments of N- and C-terminal halves of firefly luciferase 2 (luc2) protein were fused with H2AX or MDC1. For  $\gamma$ -H2AX, fusion of luciferase occurred at its N terminus because N-terminal H2AX fusion with GFP had been successfully used in noninvasive detection of foci in cells suffering from DNA damage. In addition, the C terminus was left free because it was needed for its interaction with MDC1 (17). For MDC1, instead of using the full-length protein, we used the BRCA1 carboxy-terminal (BRCT) domain because it had been shown that this was the domain in the MDC1 protein that was exclusively responsible for interacting with phosphorylated Ser139 in the carboxy terminus of H2AX (Fig. 1A and B).

To image the interaction of  $\gamma$ -H2AX with MDC1 (BRCT domain), we reasoned that DNA damage such as DSBs would lead to the phosphorylation of H2AX and its interaction with MDC1, and such an interaction would bring together the N- and C-terminal halves of the luc2 enzyme, which would lead to the reconstitution of luc2 (Fig. 1A).

On the basis of previous experience we designed a system in which H2AX and the BRCT domain of MDC1 were fused to either N terminus or C terminus of the firefly luciferase (Fig. 1B). In each of the 2 reporter proteins, a flexible linker (Gly<sub>3</sub>Ser)<sub>4</sub> was inserted between the luciferase domain and the interacting proteins ( $\gamma$ -H2AX or MDC1). We believed such a design would allow for maximal flexibilities of the luc2 domains and give them a better chance for reconstitution. See Supplementary Fig. S1 for detailed domain structures of the reporters. After successful engineering of the reporter genes, they were placed into lentiviral vectors and packaged into lentiviral particles. H322 lung cancer cells were infected with the vectors either alone or in



**Figure 1.** Design and construction of a bifragment luciferase reconstitution reporter system for detecting  $\gamma$ -H2AX in response to DNA DSBs. **A**, graphic illustration of the principle of the  $\gamma$ -H2AX/MDC1-Luc2 reporter. **B**, genetic structures of a pair of fusion reporter constructs of DNA DSB response proteins (H2AX, MDC1) and fragments of firefly luciferase (Nluc and Cluc represent the N- and C-terminal halves of the firefly luciferase gene). Lentiviral vector pLEX with a resistance gene for puromycin was used to carry the reporter genes. **C**, Western blot analyses of the expression of endogenous and recombinant proteins in the stable H322 human lung cancer cells transduced with either or both components of the  $\gamma$ -H2AX/MDC1-Luc2 reporter.

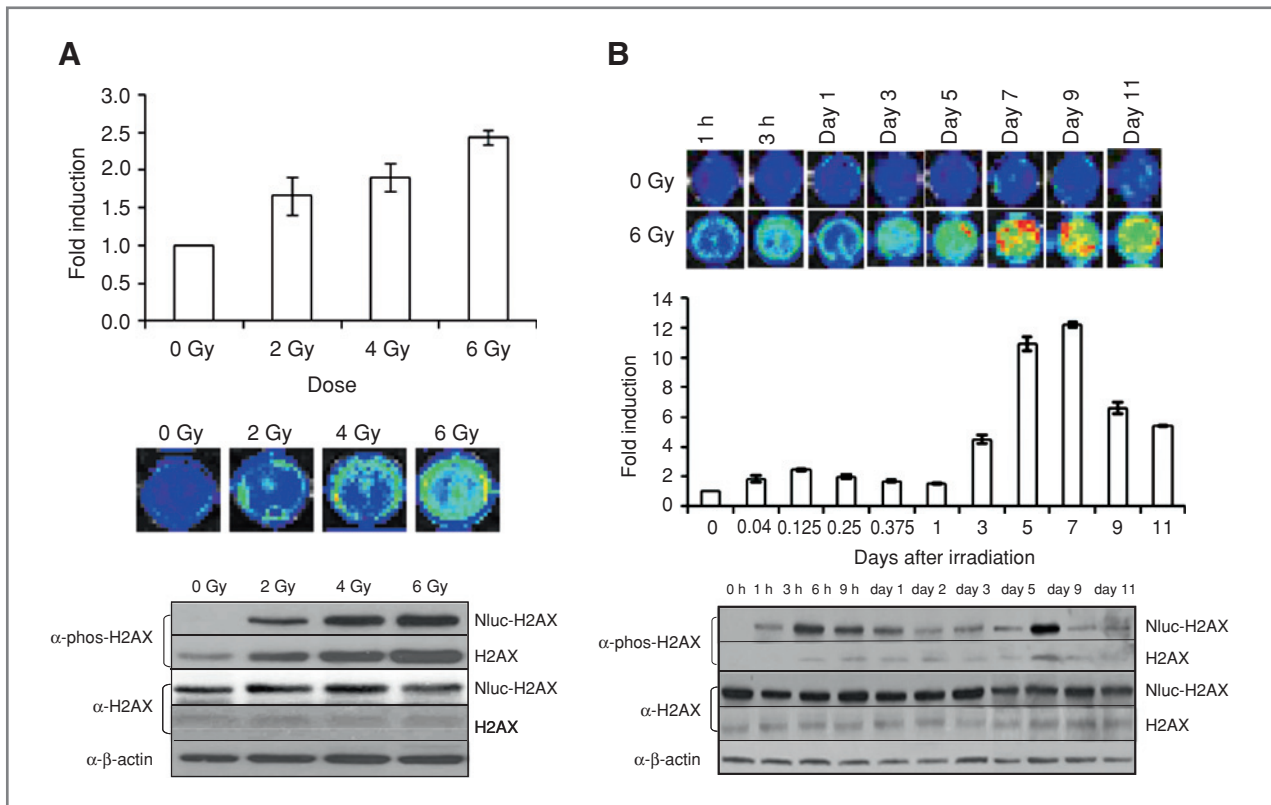
combination. Western blot analysis showed the presence of both endogenous and recombinant proteins (H2AX and MDC1) in infected cells (Fig. 1C).

### Validation of X-ray–induced reporter gene activation in tissue culture

To verify that our  $\gamma$ -H2AX/MDC1-luc2 reporter functions well in tissue culture, we exposed H322- $\gamma$ -H2AX/MDC1-luc2 cells with different doses of ionizing radiation and serially observed the activities of reporter activities by use of an IVIS200 imager. Robust reporter activation was observed in the irradiated cells (Fig. 2A). In addition, there was a clear dose-dependent response in reporter activities (Fig. 2A, top and middle). Reporter activities were consistent with Western blot analyses of phosphorylated H2AX or Nluc2-H2AX (Fig. 2A, bottom).

We next examined the time course of reporter activation in irradiated cells. Consistent with previous findings, ionizing radiation exposure caused rapid activation of reporters within 30 minutes of exposure. At 24 hours, activation dropped significantly, consistent with the earlier reports showing successful repair of DSBs in the irradiated cells. Surprisingly, after

24 hours, reporter activities again increased significantly, peaking approximately at day 5 and dropping back to background levels after day 12 (Fig. 2B). This happened despite significantly reduced cell numbers in the irradiated cell population (Supplementary Fig. S2). This second wave of DSBs were unexpected. Previous studies have shown ongoing radiation induced genetics instability in cells deficient in DNA-PKcs (22). We examined the hypothesis that the second wave of  $\gamma$ -H2AX–MDC1 interaction correlated with tumor cell apoptosis, during which strand breaks are routinely generated and  $\gamma$ -H2AX–MDC1 interaction could occur. Indeed, Western blot analysis of activated caspase 3 and poly(ADP-ribose) polymerase (PARP), which are well-recognized markers of apoptosis (25, 26), showed clear signs of apoptosis from days 3 to 9 (Supplementary Fig. S3). Furthermore, a comparative analysis of  $\gamma$ -H2AX foci and activated caspase 3 in H322 cells at different times after exposure to X-rays showed an early absence of activated caspase 3–irradiated H322 cells but a significant number of cells with activated caspase 3 (Supplementary Fig. S4). Moreover, many cells with activated caspase 3 activation showed strong  $\gamma$ -H2AX foci formation, consistent with the hypothesis that apoptotic cells do have elevated



**Figure 2.** Activation of  $\gamma$ -H2AX/MDC1-Luc2 reporter in X-ray-irradiated H322 lung cancer cells. **A**, radiation dose response of  $\gamma$ -H2AX/MDC1-Luc2 reporter after X-ray exposure. Top, the quantitative dose response of the reporter activation at 3 hours after radiation. Error bars, SD derived from 3 triplicate data points. Middle, representative images of reporter-transduced H322 cells (in 12-well plates) exposed to different doses of X-rays and then imaged in the IVIS200 instrument. Bottom, Western blot analyses of endogenous and recombinant H2AX protein expression. **B**, time course of  $\gamma$ -H2AX/MDC1-luc2 reporter activation after X-ray exposure. Top, representative IVIS200 optical images of reporter-transduced cells at different times after exposure to 6 Gy of X-rays. Middle, the time course of reporter activation after X-ray exposure. Error bars, SD derived from 3 triplicate data points. Bottom, Western blot analyses of X-ray–induced phosphorylation of endogenous and recombinant H2AX.

$\gamma$ -H2AX–MDC1 interaction levels. However, it is also clear that not all cells with strong  $\gamma$ -H2AX staining were positive for activated caspase 3 at later times (Supplementary Fig. S4), indicating that apoptosis is not the only reason for this second wave of  $\gamma$ -H2AX–MDC1 interaction. Indeed, among survivors following irradiation, many (up to 20%) of clonal cell populations showed elevated levels of  $\gamma$ -H2AX foci and  $\gamma$ -H2AX–MDC1 interaction (Supplementary Fig. S5). The exact mechanism for these elevated levels are unknown at this time. However, we speculate the second wave of  $\gamma$ -H2AX/MDC1 may be part of the "persistent genomic instability" that has been shown to present in irradiated mammalian cells (19–22).

To validate that the observed  $\gamma$ -H2AX/MDC1-luc2 reporter activities correlated with DSB formation, we carried out the standard irradiation-induced nuclear foci (IRIF) immunofluorescence analysis of  $\gamma$ -H2X foci in irradiated H322 cells. As shown in Supplementary Fig. S6, after 6 Gy of X-ray exposure, the percentage of cells with above background  $\gamma$ -H2AX foci correlated very well with data obtained with our  $\gamma$ -H2AX/MDC1-luc2 reporter (Fig. 2B).

Another question was whether our reporter system would function in normal cells as well as in tumor cells, which are immortal and in general easier to culture. Because the reporter constructs were encoded in lentiviral vectors, they should be easily established in any cells that are amenable for lentiviral infection. Indeed, the functionality of our reporter system was also confirmed in human fibroblast cell strain IMR90 (Supplementary Fig. S7). The main difference between IMR90 and that of H322 tumor cells was that scale of the second wave was much smaller in IMR90 cells. We speculate that this might be caused by the lack of radiation-induced apoptosis in primary human fibroblasts, which mainly exhibit a growth arrest phenotype (27).

#### Activation of $\gamma$ -H2AX/MDC1-luc2 reporter in cells exposed to $^{56}\text{Fe}$ ions

$^{56}\text{Fe}$  ions are an important form of space radiation. Because of their high linear energy transfer,  $^{56}\text{Fe}$  ions are known for their powerful abilities to damage mammalian cellular DNA. Previous studies have indicated that exposure to  $^{56}\text{Fe}$  ions cause a significant induction  $\gamma$ -H2AX foci (28–32). In fact, because of the ability to blast through cells without their directions being altered, it is often possible to monitor the tracks of  $^{56}\text{Fe}$  ions through irradiated cells with analysis of  $\gamma$ -H2AX foci. Our results indicate that  $^{56}\text{Fe}$  ions induced significant activation of the  $\gamma$ -H2AX/MDC1-luc2 reporter in a dose-dependent manner (Fig. 3A). The induction was confirmed by Western blot analysis (Fig. 3B). Because cells had to be exposed at BNL and then shipped back to our laboratory for observation, we were not able to monitor reporter activation in the initial 24 hours. However, we were able to conduct serial monitoring of reporter activities in the irradiated cells after 24 hours. Similar to cells irradiated with X-rays, cells exposed to  $^{56}\text{Fe}$  ions showed significant increases in reporter activities that peaked approximately at day 5 and persisted beyond day 9 (Fig. 3C). These reporter-derived results were consistent with Western blot analysis of  $\gamma$ -H2AX (Fig. 3D). The

strong induction of the reporters was striking given that the number of irradiated cells was significantly lower than nonirradiated cells (Supplementary Fig. S8).

#### Radiotherapy-induced activation of $\gamma$ -H2AX/MDC1-luc2 reporter in xenograft tumor

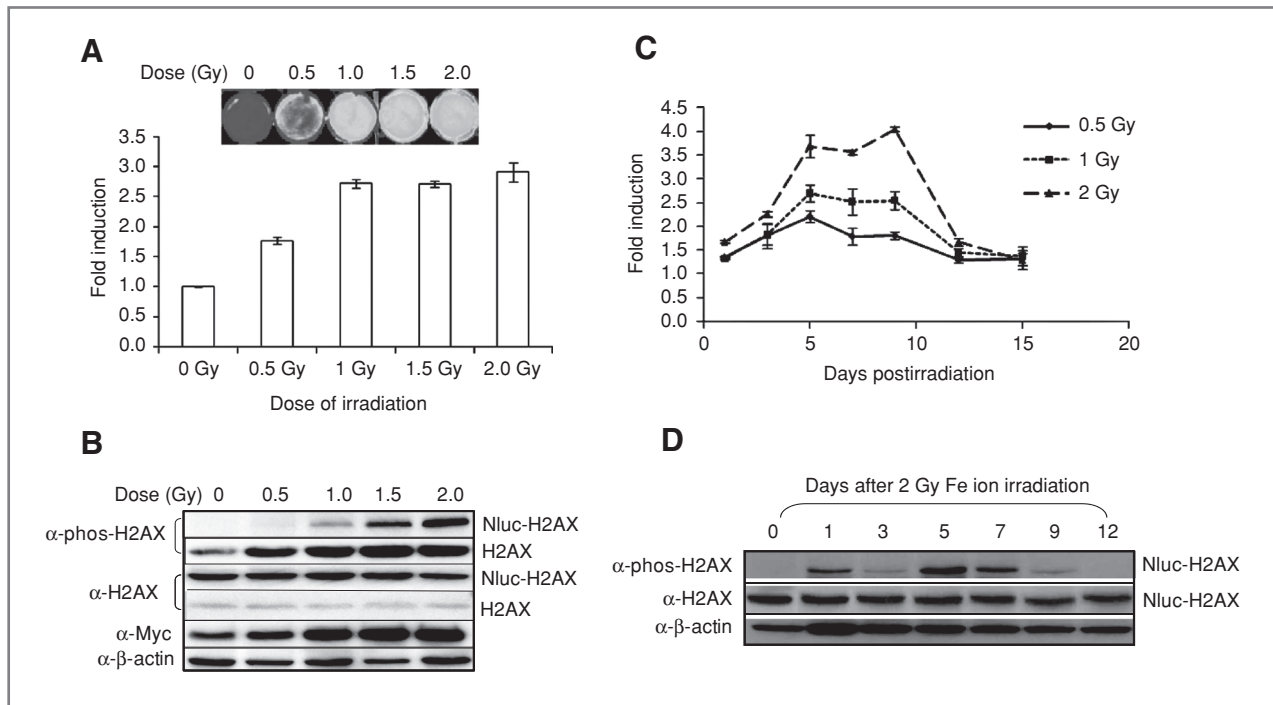
One of the main goals of our bioluminescence-based reporter system was to evaluate DSBs *in vivo* in intact tissues. To evaluate the functionality of the new reporter system in live animals, we established xenograft tumors with H322- $\gamma$ -H2AX/MDC1-luc2 in nude mice. When tumors reached 5 to 7 mm in diameter, they were irradiated with X-rays (1 dose of 6 Gys), and the activities of the reporter in control and irradiated tumors were monitored. Our results showed that ionizing radiation induced a very robust activation of  $\gamma$ -H2AX/MDC1-luc2 activities (~5-fold above background) within 3 hours of irradiation in the tumors (Fig. 4A and B). By 6 hours, reporter activities dropped down to close to background level. Consistent with our *in vitro* results, reporter activities slowly but steadily rose again, peaking approximately at day 4, when they reached 7- to 8-fold above background. They persisted at this level until approximately day 12, then dropped back to background levels by day 20 after radiotherapy (Fig. 4A and B).

#### Discussion

In eukaryotic cells, DNA DSBs occur due to mistakes in DNA replication, or after DNA damage from external sources. Because of the devastating consequences of DSBs, eukaryotic cells have evolved an elaborate system to detect and repair DNA damage. There is a high degree of interest in understanding the mechanisms and kinetics of DNA DSB repair because it is involved in many pathologic conditions such as cancer and rare genetic diseases such as ataxia telangiectasia, Bloom syndrome, and Werner syndrome (4). DNA repair is also involved in cancer treatment because many therapeutic agents such as radiation and some chemotherapy agents function because of their abilities to generate DSBs in cancer cells (33, 34).

Recent advances have led to significant insight into the mechanisms of eukaryotic DSB repair. However, one remaining deficiency has been the lack of methods to monitor DNA strand breaks *in vivo* in live tissue. Our  $\gamma$ -H2AX/MDC1-luc2 reporter is a significant step forward in this direction. Results shown in this study provide strong evidence that a bifragment luciferase reconstitution system functions well in detecting  $\gamma$ -H2AX–MDC1 interaction, which can serve as a surrogate for  $\gamma$ -H2AX foci formation and DSBs in DNA.

In comparison to previously available GFP-based systems for detecting  $\gamma$ -H2AX foci in live cells (12, 13) or immunofluorescence-based methods (9, 10), a luciferase-based reporter is significantly superior in several aspects. First, it allows for a quantitative determination of the total level of  $\gamma$ -H2AX in a whole-cell population in a noninvasive manner, which is impossible for a GFP-foci-based approach. Second, the sensitivity achieved by a bioluminescence approach is generally



**Figure 3.** Monitoring DNA DSBs in H322- $\gamma$ -H2AX/MDC1-Luc2 reporter cells exposed to  $^{56}\text{Fe}$  ions. **A**, dose response of H322- $\gamma$ -H2AX/MDC1-Luc2 cells exposed to  $^{56}\text{Fe}$  ions. Top, representative optical images of H322 cells (in 12-well plates) exposed to different doses of  $^{56}\text{Fe}$  ions 5 days after exposure. Bottom, quantitative measurement of reporter activities. Error bars, SD derived from 3 data points. **B**, Western blot analysis of phosphorylation status of endogenous and recombinant  $\gamma$ -H2AX in H322 cells treated with  $^{56}\text{Fe}$  ions at 5 days after radiation exposure. **C**, time course of reporter activation in H322- $\gamma$ -H2AX/MDC1-Luc2 cells exposed to different doses of  $^{56}\text{Fe}$  ions. **D**, Western blot analyses of  $\gamma$ -H2AX phosphorylation status in H322 cells exposed to 2 Gy of  $^{56}\text{Fe}$  ions at different time points postirradiation.

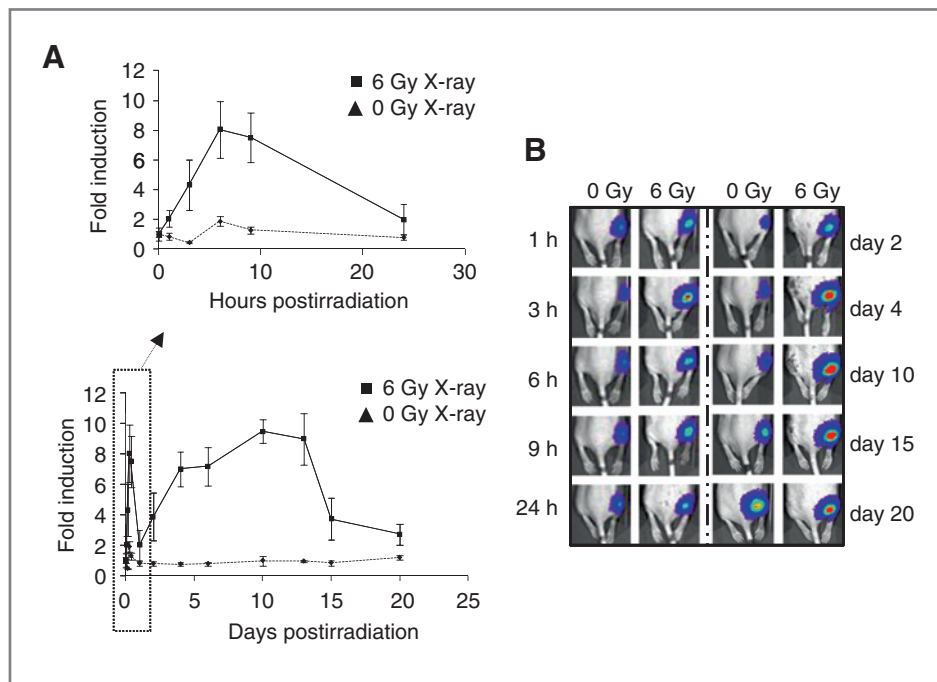
much higher than for a fluorescence-based approach because of the total lack of bioluminescence background versus the almost ubiquitous fluorescence background in mammalian cells/tissues. Third, it facilitates H2AX phosphorylation detection noninvasively and quantitatively in live animals in a serial manner, for an extended period of time. These desirable features make it possible now to ask questions that were previously impossible or difficult to answer.

The most significant advantage of our  $\gamma$ -H2AX-MDC1 reporter system over currently available methods is the ability of our system to monitor DNA DSB generation in live tissue *in vivo* during and after cancer treatment, as we show in Fig. 4. Our method enables study of DSB DNA repair kinetics in the same animals over an extended period of time, which has not been possible before. Furthermore, the application of our method is not limited to tumor tissues, as we have shown; it could also be used to study normal mouse tissues if transgenic mouse strains with the reporters are established. With such mouse strains, it should also be possible to study the effects of various DNA-damaging carcinogenic agents or chemopreventive agents living in the same animals over an extended period of time.

An intriguing finding from our new method was a second wave of DSB generation in cells exposed to X-rays, both *in vitro* and *in vivo*. Previously, this has only been shown with  $\gamma$ -H2AX immunostaining, and only in NHEJ repair-deficient cells,

presumably because in most cases only earlier time points were examined. Using our new reporter system, this phenomenon became clear and reproducible. These results are consistent with the earlier published phenomenon of persistent genetic instability after cellular exposure to ionizing radiation or other stress (18–22).

We showed that cellular apoptosis contributes to a significant degree to the second wave of  $\gamma$ -H2AX-MDC1 interaction. However, it is clearly not the only contributor; there were some cells with no apoptotic caspase activation but quite clear  $\gamma$ -H2AX foci, long after initial radiation exposure. In this context, it is also interesting to note that normal fibroblast cells, despite exhibiting similar kinetics, showed much less DSB damage when exposed to the same radiation dose. The reason for this is not clear at present, but it is a worthwhile question to pursue in future studies. Another interesting question related to apoptosis-induced  $\gamma$ -H2AX foci is its implication for cancer therapy. If all cells with activated caspases die, then it is of no consequence. However, it is entirely possible that some of the cells with activated caspases do not die. These cells presumably will have suffered from multiple instances of DNA damage caused by apoptotic nucleases. Therefore, they could suffer mutations at multiple loci in their genome, and thus could evolve into more malignant phenotypes. If this is true, then activated caspases could be an important source of genomic instability during



**Figure 4.** Radiotherapy-induced activation of  $\gamma$ -H2AX/MDC1-Luc2 reporter in H322 xenograft tumors. A, time course of reporter activation in H322- $\gamma$ -H2AX/MDC1-Luc2 xenograft tumors established in nude mice. Tumors with diameters of 5 to 7 mm were irradiated with 6 Gy of X-rays and observed for reporter activation. Reporter activation in the first 24 hours after radiotherapy was plotted separately to show detailed kinetics. Error bars, SEM;  $n = 5$ . Reporter activities were corrected to tumor volume. B, representative optical images of reporter activation in irradiated and control tumors at different time points after radiotherapy.

cytotoxic radiotherapy. Induction of genetic instability would add 2 other important, nonapoptotic functions of caspases recently discovered in our laboratories: the "phoenix rising" pathway for tissue regeneration (35), and a facilitative role for caspase in the derivation of induced pluripotent stem cells (36).

Finally, although we have provided a substantial amount of evidence, further validation of our technology may be necessary to use it widely for DSB research. For example, it is important to make sure that the activities of our reporter truly reflect the interaction kinetics of endogenous  $\gamma$ -H2AX-MDC1 within radiation-induced foci. One would also need to ensure that additional, exogenous levels of  $\gamma$ -H2AX do not induce false-positive reporter activities, although we believe the likelihood is low given the robust data we have already obtained.

In summary, we have designed and validated a novel reporter system to study H2AX phosphorylation based on a bifragment luciferase reconstitution system. Our data show that this is a powerful approach that can be used to study the

kinetics of DNA DSB repair in mammalian cells in tissue culture or in live animals. Such a reporter should find application in both fundamental studies of DNA repair mechanisms and in therapeutic development and evaluation.

#### Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

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