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Mutations in the FHA-domain of ectopically expressed NBS1 lead to radiosensitization and to no increase in somatic mutation rates via a partial suppression of homologous recombination

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Ionizing radiation induces DNA double-strand breaks (DSBs). Mammalian cells repair DSBs through multiple pathways, and the repair pathway that is utilized may affect cellular radiation sensitivity. In this study, we examined effects on cellular radiosensitivity resulting from functional alterations in homologous recombination (HR). HR was inhibited by overexpression of the forkhead-associated (FHA) domain-mutated NBS1 (G27D/R28D: FHA-2D) protein in HeLa cells or in hamster cells carrying a human X-chromosome. Cells expressing FHA-2D presented partially (but significantly) HR-deficient phenotypes, which were assayed by the reduction of gene conversion frequencies measured with a reporter assay, a decrease in radiation-induced Mre11 foci formation, and hypersensitivity to camptothecin treatments. Interestingly, ectopic expression of FHA-2D did not increase the frequency of radiation-induced somatic mutations at the HPRT locus, suggesting that a partial reduction of HR efficiency has only a slight effect on genomic stability. The expression of FHA-2D rendered the exponentially growing cell population slightly (but significantly) more sensitive to ionizing radiation. This radiosensitization effect due to the expression of FHA-2D was enhanced when the cells were irradiated with split doses delivered at 24-h intervals. Furthermore, enhancement of radiation sensitivity by split dose irradiation was not seen in contact-inhibited G0/G1 populations, even though the cells expressed FHA-2D. These results suggest that the FHA domain of NBS1 might be an effective molecular target that can be used to induce radiosensitization using low molecular weight chemicals, and that partial inhibition of HR might improve the effectiveness of cancer radiotherapy.

Keywords: homologous recombination; NBS1; split dose irradiation; radiosensitization

INTRODUCTION

Cellular radiation sensitivity is altered by many factors such as the cell cycle distribution, dose rates, oxygen and free radical concentrations, and chemical components that can modify cellular DNA damage repair abilities. From the view of cancer radiotherapy, any chemicals that can sensitize tumor tissues would be expected to improve therapeutic effectiveness. Many radiosensitizers that function at a cellular level have already been investigated. These include apoptosis-enhancing natural chemical components [1], chemicals which increase DNA damage resulting from radiation [2, 3], kinase inhibitors for ATM/ATR/Chk1/Chk2 [3], and PARP inhibitors [3], although these agents are not tumor tissue-specific sensitizers.

The DNA double-strand break (DSB) repair pathway is a logical candidate to use for tissue-specific regulation of radiosensitization because the use of the DSB repair pathway following irradiation is cell cycle dependent [4]. There are multiple DSB repair pathways, but homologous recombination (HR) and non-homologous end joining (NHEJ) are

thought to be the major two repair pathways. NHEJ functions throughout the entire cell cycle, but HR activity is thought to be limited to the S and G2 phases because the pathway requires homologous DNA sequences on a sister chromatid to use as a template for repair [5]. Usually, tumor tissues have a large S–G2 phase population in their cell cycle distribution, suggesting that DSB repair via HR could be a more critical or important repair pathway in tumor tissue than in normal tissues. It was also reported that recovery from sublethal damage depends on the HR pathway [6], suggesting that modulation of HR repair ability can alter cellular sensitivity to split dose radiation. Therefore, the focus of the work described here was on a protein involved in the regulation of the HR repair pathway with the aim of increasing radiosensitization in exponentially growing tumor cells.

Several selective inhibitors of HR-related proteins have been screened and tested for their effectiveness in increasing radiosensitization. Mirin is one of the first reported drugs that are both protein-specific and an HR-specific inhibitor [7]. Mirin completely blocks MRE11 activity, which is a critical factor in DSB end processing, and almost completely suppresses HR events [7]. However, the authors also noted that mirin itself was quite toxic for vertebrate cells. In other words, complete suppression of HR may cause cellular lethality regardless of the tissue of origin (cancerous or normal). This is in agreement with the reported phenotypes of cells that completely lost the function of a key protein involved in HR. For example, gene knockout cells for RAD51, RAD50 and MRE11 are not viable [5]. Thus, any chemical agents that strongly inhibit HR events may be highly toxic, and as a result those drugs would likely be difficult to utilize in the clinical phases of cancer radiotherapy.

NBS1 is the protein responsible for Nijmegen breakage syndrome (NBS), in which patients display hypersensitivity to ionizing radiation. NBS1 functions as a key regulator in DSB repair responses such as the activation of ATM kinase, regulation of both the activity and nuclear localization of the MRE11/RAD50/NBS1 (MRN) complex, and the regulation of chromatin remodeling [5, 8]. By virtue of the nature of these functions, NBS1 is also an essential protein involved in HR [9]. We reported previously that the forkhead-associated (FHA) domain of NBS1 is a critical domain involved in nuclear foci formation of the MRN complex in response to radiation, an action that is required for proper HR pathway functioning [10]. This observation, and the fact that NBS patient cells express an NBS1 protein with a truncated N-terminus (which contains the FHA/BRCT domain) [11], suggested that the FHA domain of NBS1 might be a logical candidate in targeting radiosensitization through significant but partial inhibition of HR repair.

In this study partial inhibition of DSB repair was tested by abrogating the NBS1 function involved in radiation sensitivity. It was found that expression of FHA domain-mutated NBS1 (FHA-2D) decreases HR efficiency but does not lead to a significant increase in somatic mutation frequencies. In addition, it was found that expression of FHA domain-mutated NBS1 sensitizes only exponentially growing cell populations, and that the effect became more pronounced if cells were exposed to split dose radiation.

MATERIALS AND METHODS

Cell culture and expression of mutant NBS1

HeLa cells, MRC5/SV cells (obtained from the RIKEN Cell Bank), or GM06318-10 cells [12] were used in this study. All the cell lines were cultured in D-MEM (GIBCO, Life Technologies) supplemented with 8% fetal bovine serum (HyClone) and 25 μg/ml gentamycin sulfate (SIGMA). For the establishment of ectopic NBS1-expressing cell lines, the cDNA for full-length wild-type or FHA-2D (G27D/R28D) mutants of NBS1 [10] were cloned into pcDNA3.1, and then the myc-his tagged expression cassettes were transferred into a pIREShyg vector (Clontech). Each of the myc-his tagged NBS1 (full-length wild-type or FHA-2D) expression vectors were transfected into cells by electroporation. At 48 h after transfection, cells were trypsinized and seeded in medium containing 200 μg/ml hygromycin B (Wako) and cultured until hygromycin-resistant colonies were formed. The colonies were picked and expression of myc-his tagged NBS1 was confirmed with immunoblot analysis.

Immunoblot analysis, immunofluorescent staining and immunoprecipitation

Immunoblot analysis was performed as previously described [13]. Briefly, cells were collected and incubated in RIPA lysis buffer containing a protease inhibitor cocktail (SIGMA) and 1 mM Sodium orthovanadate for 30 min on ice. Extracts were centrifuged for 30 min at 20 000*g* and the supernatant was recovered. Next, 30 μg protein samples were boiled in 25 μl of Laemmli buffer, and loaded onto 7% or 13% SDS polyacrylamide gels. After electrophoresis at 30 mA, proteins were electrotransferred onto PVDF membranes (Millipore) and probed with the appropriate primary antibody. Antibodies used were anti-myc-tag (clone 4A6, Millipore), anti-human NBS1 (GeneTex), and anti-beta-actin (Lab Vision). Primary antibodies were detected with HRP-conjugated anti-rabbit or anti-mouse IgG (GE Healthcare), and then visualized with an ECL plus chemiluminescence system (GE Healthcare). Fluorescence images were detected with an LAS3000 imaging system (Fuji Film).

For immunofluorescent staining for Mre11 foci, cells grown on a glass slide were irradiated with 10 Gy of X-rays and incubated for an appropriate time. The slides were then fixed with cold (−20°C) methanol for 20 min, rinsed with cold acetone for several seconds, and air dried. The slides were stained as described previously [10]. The primary antibody used was anti-hMRE11 (Novus Biologicals) and the secondary antibody was Alexa-488 conjugated anti-rabbit

IgG (Molecular Probe). For NBS1 staining of GM06318-10 cells, immunostaining was performed with anti-human NBS1 antibody (GeneTex). The excited green fluorescence from the Alexa-488 dye was visualized with a fluorescent microscope (Olympus).

Immunoprecipitation was performed with protein A sepharose (GE Healthcare) conjugated with anti-myc-tag antibody (Millipore) or rabbit IgG (SIGMA). Immunoprecipitants were analyzed with immunoblots with anti-human Rad50 antibody (GeneTex) or anti-human NBS1 antibody (GeneTex).

Homologous recombination assays

SCneo analysis [14] and analysis of the HR products were performed as described elsewhere [10]. After 2 weeks incubation, one G418-resistant colony was picked from each independent series of G418 treated dishes, and genomic DNA was extracted. The S2neo sequence in G418-resistant clones was amplified with PCR using a specific primer set [10] and Ex Taq DNA polymerase (TaKaRa). The amplified DNA was digested with *Nco*I or I-*Sce*I in order to identify two types of the HR products: short-tract gene conversion (STGC) or long-tract gene conversion/sister chromatid exchange (LTGC/SCE) [14].

Irradiation, survival assays, and cell cycle analysis

Cells were irradiated with 70 kVp X-rays using a soft X-ray generator (OM-B205, OHMiC, Japan) at a dose rate of 1.53 Gy/min. Camptothecin (CPT) treatment was performed by adding CPT to the medium and incubating the cells for 24 h. The irradiated or CPT-treated cells were trypsinized and an appropriate number of cells were plated in a 60-mm dish. After 14 d of incubation, colonies were fixed, stained with a Giemsa solution (Merck), and scored to calculate the surviving fraction. Pooled data from at least three independent experiments with two each of ectopic NBS1-expressing clones (clones #1 and #2 for wild type; clones #2 and #4 for FHA-2D) were used for further statistical analysis.

To analyze cell cycle distribution during split dose intervals, cells just before the final dose were collected and fixed as described previously [15]. Fixed cells were stained with propidium iodide solution (20 μg/ml propidium iodide, 0.1 mg/ml RNase A, and 0.1% Triton X-100 in phosphate buffered saline) and analyzed with a Tali Image-Based Cytometer (Life Technologies).

Mutation assays

HPRT mutation assays using GM06318-10 cells were performed as described elsewhere [12, 15]. The GM06318-10 is a subcloned hamster cell line that carries a human X-chromosome and is hypersensitive towards mutation induction. Immediately after irradiation or after CPT treatment for 24 h, the surviving fraction was determined by using a portion of the cells, and the rest of the cells were divided into eight dishes and cultured for 9 d to allow expression of mutant phenotypes. The cells on each dish were trypsinized and inoculated into medium containing 5 μg/ml of 6-thioguanine (6-TG, Wako) at a density of 1×10^4 cells per 100-mm dish. After 14 d of incubation, cells were fixed with ethanol and stained with a Giemsa solution (Merck), and the induced mutation frequency was calculated from the number of 6-TG-resistant colonies as previously described [12, 15]. Pooled data from multiple independent experiments with multiple ectopic NBS1-expressing clones (clones #23 and #38 for wild-type; clones #14, #17 and #39 for FHA-2D) were used for further statistical analysis.

Statistical analysis

All experimental datapoints were obtained from at least three independent experiments, and statistical analysis was performed using the Student's *t*-test. For HR assays and mutation assays, pooled data obtained from two or three independent clones from a total of at least three independent experiments was used for statistical analysis.

RESULTS

Suppression of DNA damage response in the presence of ectopically expressed FHA-mutated NBS1

In this study, newly established cell lines were used that expressed wild-type or FHA-2D (G27D/R28D) mutants of myc-his tagged NBS1 (Fig. 1A). Although the expression level of FHA-2D, in both the HeLa and MRC5/SV cells, was relatively lower than that of intact NBS1 as reported previously [16], the established cell lines showed clear expression of ectopic NBS1 in addition to endogenous intact NBS1 (Fig. 1B and C). Using ectopic NBS1-expressing HeLa cells, MRE11 foci formation was analyzed following irradiation. The number of MRE11 foci observed was decreased in cells expressing FHA-2D NBS1 (Fig. 1D). This suppression of nuclear foci formation in the presence of FHA-2D NBS1 resulted in a decrease in the frequency of HR repair at a site-specific DSB (Fig. 2A), in agreement with our previous observation [10]. Suppression of HR caused by the expression of FHA-2D NBS1 was partial but statistically significant, and analysis of HR products revealed that the quality of gene conversion was not affected by ectopic NBS1 expression (Table 1). Thus, ectopic expression of FHA-2D NBS1 in cells expressing endogenous normal NBS1 results in a partial inhibition of DNA damage-induced foci formation and in decreased DSB repair via HR, but it does not affect the quality of HR events. The HR-decreased phenotype in FHA-2D cells was further confirmed by campthotecin (CPT) sensitivity because the HR repair pathway is essential in order to resolve the CPT-stabilized topoisomerase I cleavage complex [17]. As expected from the partial inhibition of HR, the FHA-2D cells displayed a CPT-hypersensitive phenotype (Fig. 2B).

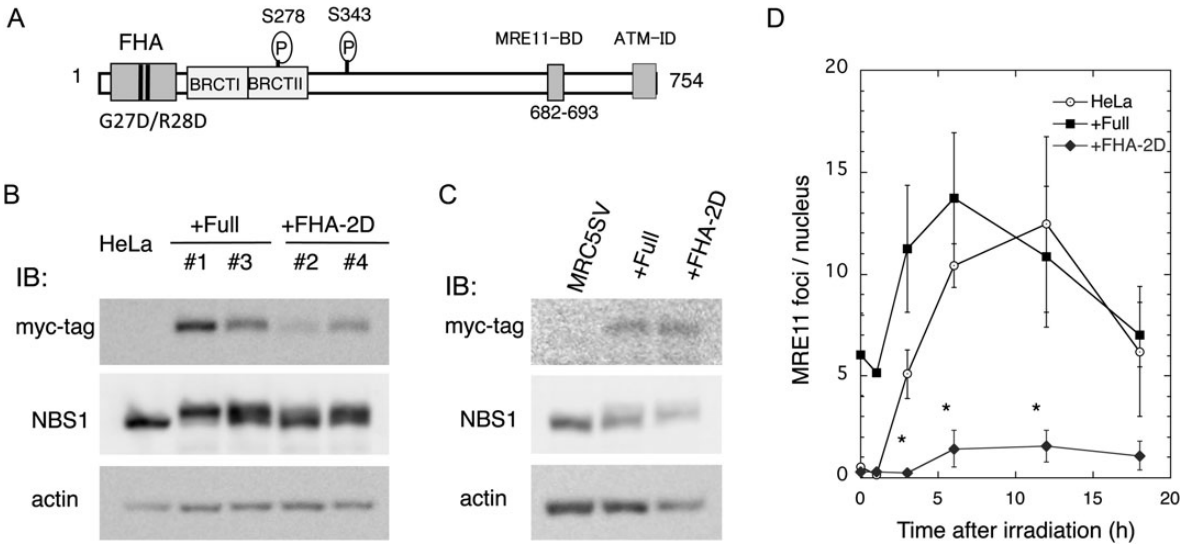

Fig. 1. (**A**) Schematic diagram of the FHA-2D mutant of NBS1. Amino acid residues Gly27 and Arg28 are substituted for Asp. BRCT, MRE11-BD and ATM-ID represent the BRCT domain, MRE11-binding domain, and ATM-interacting domain, respectively. S278 and S343 are phosphorylation sites by ATM/ATR kinases. (**B**) Ectopic expression of normal or mutant NBS1 in HeLa cells. Expression of myc-His-tagged hNBS1 was analyzed with immunoblots. Each lane represents an independent clone. (**C**) Ectopic expression of normal or mutant NBS1 in MRC5SV cells. (**D**) Time-course of MRE11 foci formation after 10-Gy irradiation of HeLa cells expressing myc-His-tagged NBS1. The designation '+ Full' indicates a full-length wild-type *NBS1* gene; '+ FHA-2D' represents the mutated form (G27D/R28D) of the *NBS1* gene. An asterisk indicates statistically significant ($P < 0.05$) by Student's *t*-test (Full vs FHA-2D).

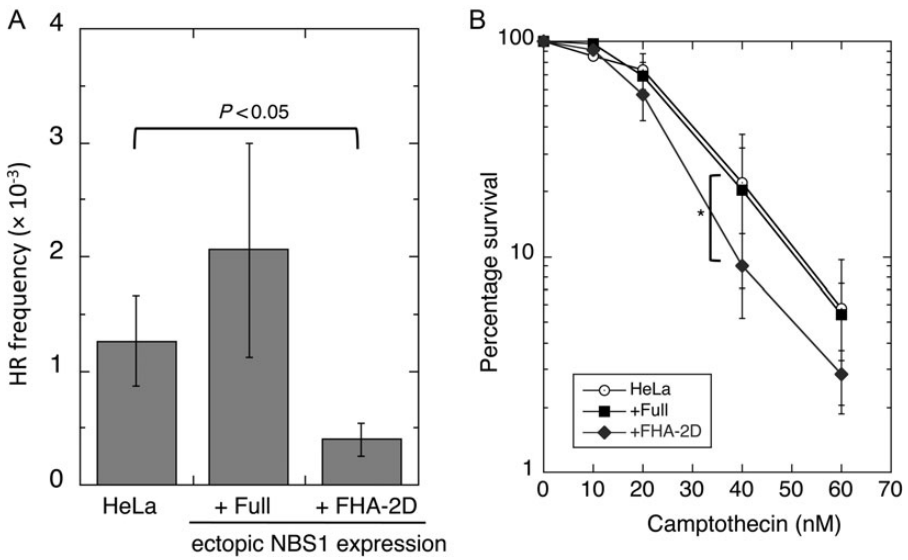

Fig. 2. Effects of ectopic expression of normal or mutant NBS1 on HR phenotypes. (**A**) HR frequency analyzed with an SCneo reporter. (**B**) Campthotecin (CPT) sensitivity of HeLa or ectopic NBS1-expressing cells. The designation '+ Full' indicates a full-length wild-type *NBS1* gene; +FHA-2D represents the mutated form of the *NBS1* gene. The CPT doses for 10% survival were 53 ± 8 nM for HeLa cells, 53.5 ± 13 nM for wild-type NBS1 cells, and 43.8 ± 4 nM for FHA-2D cells. An asterisk indicates statistically significant ($P < 0.05$) by Student's *t*-test.

Radiosensitization caused by the expression of mutant NBS1

Radiosensitization was examined in the presence of ectopic expression of the FHA-2D mutant of NBS1. Ectopic expression of wild-type NBS1 did not change the cellular radiosensitivity of HeLa cells and MRC5/SV cells (Fig. 3A, B and C). In contrast, ectopic expression of FHA-2D NBS1 slightly sensitized exponentially growing HeLa and

Table 1. Analysis of HR products

Cell line	No. of clones analyzed	STGC (%)	LTGC/SCE (%)
HeLa	26	22 (85)	4 (15)
HeLa + Full	49	38 (78)	11 (22)
HeLa + FHA-2D	33	27 (82)	6 (18)
MRC5/SV	40	33 (83)	7 (17)

Each individual clone was picked from a separate G418-treated dish in order to avoid picking duplicate clones arising from a single mutation event. STGC = short tract gene conversion, LTGC/SCE = long-tract gene conversion/sister chromatid exchange.

MRC5/SV cells to radiation exposure at a dose of 6 Gy (Fig. 3A, B and C). This radiosensitization effect became more significant when the radiation was delivered as a split dose (3 Gy × 2 or 2 Gy × 3) with 24-h intervals (Fig. 3B and C). However, confluent G0/G1 populations of MRC5/SV cells did not display enhanced radiosensitivity after exposures to split dose radiation (Fig. 3D). Although a slight effect on cell cycle distribution during split dose intervals was observed (Fig. 4), there was no difference between wild-type and FHA-2D-expressing cells. These results suggest that partial inhibition of HR clearly affects recovery from sublethal damage [18]. Because the HR pathway depends on the cell cycle phase, and may be minimally active in the G0/G1 phase, this observation is in agreement with a previous

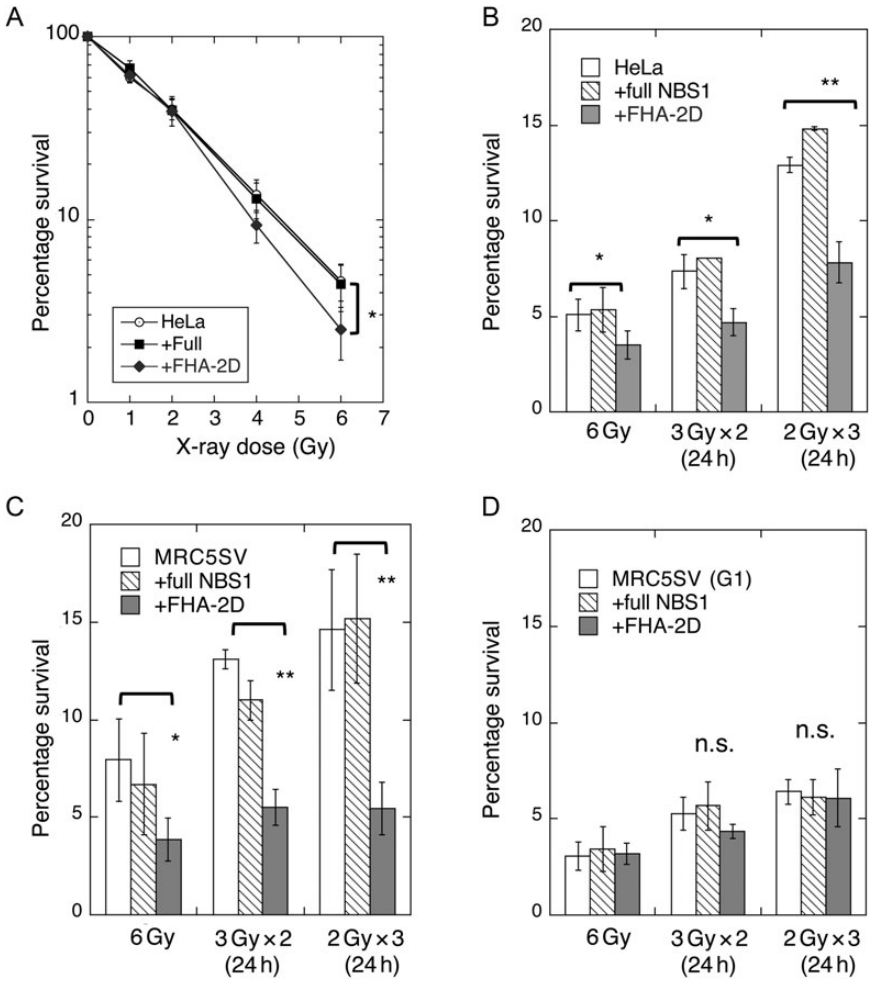

Fig. 3. Effects of ectopic expression of normal or mutant NBS1 on radiation sensitivity. (**A**) X-ray sensitivity of HeLa cells or ectopic NBS1-expressing cells. Exponentially growing cells were exposed to 1, 2, 4 or 6 Gy of X-rays. (**B**) Exponentially growing HeLa cells were exposed to 6 Gy of X-rays delivered as a single dose or split doses. (**C**) Exponentially growing MRC5SV cells were exposed to 6 Gy of X-rays with a single dose or split doses. (**D**) G0/G1 contact-inhibited MRC5SV cells were exposed to 6 Gy of X-rays as a single dose or split doses. Split doses were delivered with 24-h intervals between doses. The designation '+ Full' indicates a full-length wild-type *NBS1* gene; '+ FHA-2D' represents the mutated form of the *NBS1* gene. One asterisk or two asterisks indicate statistically significant ($P < 0.05$ or $P < 0.01$, respectively) by Student's *t*-test. n.s. = not significant.

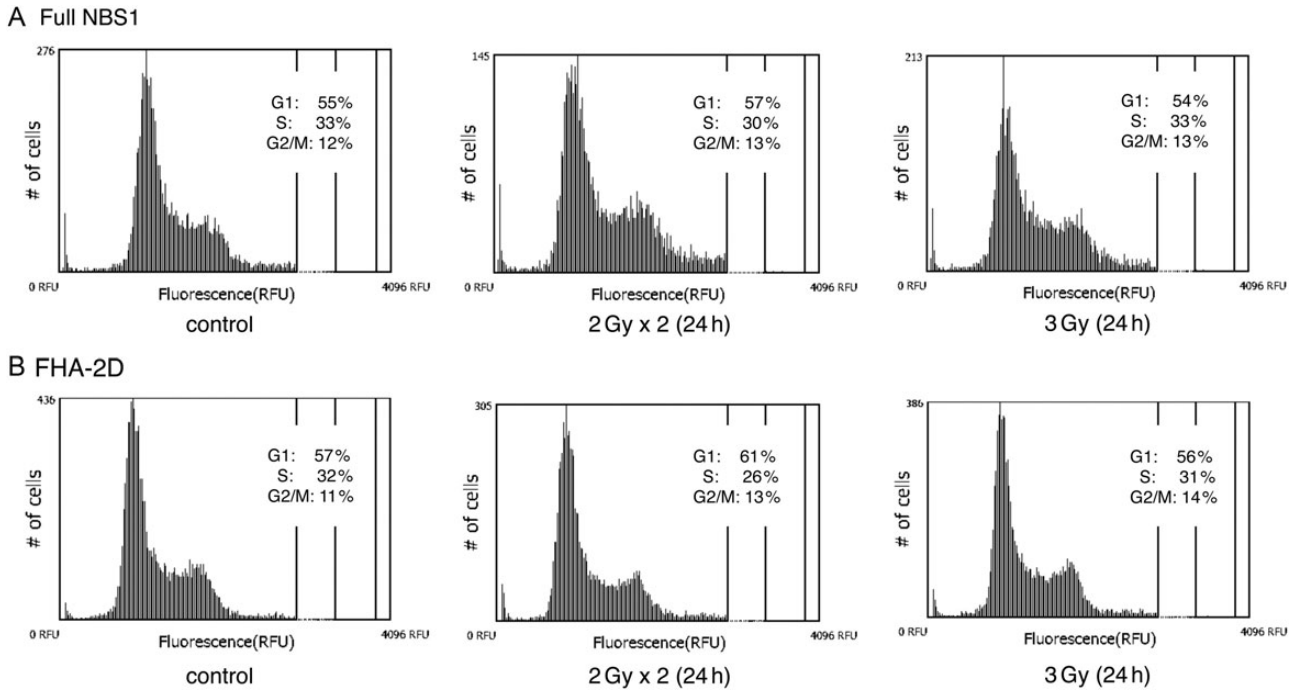

Fig. 4. Cell cycle distribution during split dose intervals in HeLa cells. Cells sampled just before the last dose in Fig. 3B were fixed and cell cycle distributions were analyzed. (**A**) HeLa cells expressing ectopic wild-type NBS1 (clone #3). (**B**) HeLa cells expressing the ectopic FHA-2D mutant form of NBS1 (clone #2).

report that recovery from sublethal damage depends on the HR pathway [6].

Radiation-induced somatic mutations in cells expressing mutated NBS1

There was a possibility that the HR-deficient phenotype observed in cells expressing FHA-2D NBS1 might also display increases in somatic mutation frequencies. Therefore, we next tested the effect of ectopic expression of normal or FHA-2D mutant NBS1 on somatic mutations by using GM06318-10 cells (Fig. 5A). GM06318-10 cells define a hypersensitive Hprt-deficient mutation assay system that exhibits an extremely high mutation frequency, even at radiation doses below 0.2 Gy, because Hprt-deficient mutants are viable even if they have lost a large portion of the human X-chromosome [12, 15]. The assay system would be expected to reveal a clear difference in mutation frequencies if a partial inhibition of HR induces any genetic instability. First, we confirmed that ectopically expressed human NBS1 could form an MRN complex with endogenous hamster Mre11/Rad50 by demonstrating that cgRad50 was immunoprecipitated with hNBS1 (Fig. 5B). It was also confirmed that expression of FHA-2D NBS1 abrogated nuclear foci formation by hamster MRN complexes following irradiation (Fig. 5C). These observations indicate that ectopic expression of hNBS1 causes similar HR reduction in hamster cells as it does in human cells. Surprisingly, we could not detect any significant difference in mutation frequencies between normal and FHA-2D mutant NBS1-expressing cells, although FHA-2D NBS1 expression resulted in a slight increase in spontaneous mutation frequencies (Fig. 6A and B). In addition, CPT-induced mutations, which may reflect the DNA repair efficiency by HR, did not increase, whereas CPT sensitivity was clearly enhanced by the expression of FHA-2D NBS1 (Fig. 6C and D). These results suggest that expression of the FHA-2D mutant NBS1 makes cells sensitive to killing by radiation or CPT, but it does not induce genetic instability.

DISCUSSION

In the present study the potential effects of partial inhibition of HR were tested by mutating the localization–regulatory domain of the NBS1 protein. The FHA domain of NBS1 was selected as a target that could potentially affect radiation sensitivity after exposures to split dose radiation. This appeared to be an appropriate model since radiation is delivered as a split dose during conventional cancer radiotherapy. Because HR is known to be a critical pathway for recovery from sublethal damage (also known as Elkind repair) [6], any agent that suppresses HR could theoretically be a good candidate to improve the effectiveness of cancer radiotherapy. Cell cycle dependency of the DNA repair pathway also supports this point of view. Because DSB repair by HR is maximal in the S–G2 phase, radiation sensitivity of exponentially growing

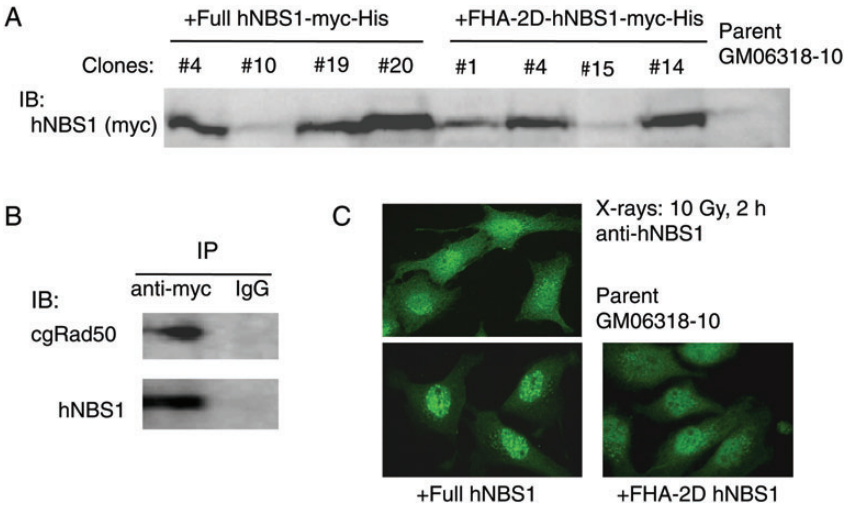

Fig. 5. Ectopic expression of normal or mutant hNBS1 in GM06318-10 cells. (**A**) Analysis with immunoblots. Lanes represent independent clones. (**B**) Interaction between hNBS1 and cgRad50. Cell extracts were immunoprecipitated with anti-myc-tag antibody, and the immunoprecipitants were analyzed with immunoblots with the indicated antibodies. (**C**) Typical views after immunofluorescent staining for hNBS1 at 1 h after 10-Gy irradiation. The designation '+ Full' indicates a full-length wild-type *NBS1* gene (clone #23); '+ FHA-2D' represents the mutated form of the *NBS1* gene (clone #14).

cancer cells should be more affected by inhibition of HR than that of G0/G1 cells in normal tissues. This should lead a selective sensitization of cancer cells, and as a result the total radiation dose required might be reduced. Previous research on radiosensitization with Gimeracil indicated that a slight but statistically significant increase in radiosensitivity was caused by suppression of HR [19]. Because many proteins are associated with the HR pathway, indirectly targeting the HR-related pathway might cause a partial suppression of this pathway. A proteasome inhibitor (such as MG132 and beta-lactin) [20] and tyrosine kinase inhibitors (such as imatinib, and erlotinib) [21, 22] were also suggested as possible radiosensitizers through their partial inhibition of HR. However, the mechanism of indirect HR inhibition used by those drugs is still unclear and there are possibilities that these indirect effectors may have side-effects capable of affecting genomic stability.

For direct and accurate molecular targeting of HR-related proteins by small chemical molecules, the choice of a target protein and its targeted domain would be critical because HR is essential for cell viability itself [5]. Mirin is the first reported MRE11 inhibitor that abolishes both HR efficiency and ATM activation. Mirin strongly suppresses DSB repair via HR and abolishes the ATM signaling pathway following DNA damage [7]. However, the authors also reported that mirin itself was quite toxic to the cells and that it could not be used for radiosensitization *in vivo*. It is also known that null mutant cells for key proteins in the HR pathway such as RAD51, RAD50 and MRE11 are lethal at the cellular level [5]. These reported observations indicate that agents that strongly inhibit HR may be too toxic to use in clinical trials or for further experiments *in vivo*.

In contrast to the complete inhibition of HR, cells which are partially deficient in HR are viable. This suggests that the toxicity of partial HR inhibitors may be lower than that of reported inhibitors that completely block HR. As a result, screening any potent partial inhibitor of HR could be a productive approach.

Recently, several chemicals have been reported as novel radiosensitizers. Groselj (2013) reported that panobinostat (PAN) has radiosensitizing effects in muscle-invasive bladder cancer cell lines [23]. They showed that PAN was more effective with Ku80-depleted cell lines than with RAD51-depleted cell lines, suggesting that PAN appears to target HR rather than nonhomologous end joining (NHEJ). A radiosensitizing effect was also reported for histone deacetylaze (HDAC) inhibitor PCI-24781 [24]. PCI-24781 induces significant reduction in transcription of DNA repair-related genes including *RAD51*. Because of the reduced expression of RAD51, HR efficiency is significantly reduced and the cells become radiosensitive.

HR-deficient mutants often show a mutator phenotype. For example, Hintz *et al.* (2006) reported that Rad51D mutant cells exhibit a 12-fold increase in the rate of spontaneous Hprt-deficient mutations [25]. If a drug partially inhibits HR and causes an increase in somatic mutation frequencies, the combination of radiation and the drug can result in an increased risk of secondary tumorigenesis. It should be noted that the ectopic expression of FHA-2D NBS1 resulted in a slight increase in sensitivity to DNA-damaging agents, but there was no apparent increase in the frequency of HPRT-deficient mutations (Fig. 6). This suggests that functional abolition of the FHA domain of NBS1 renders the

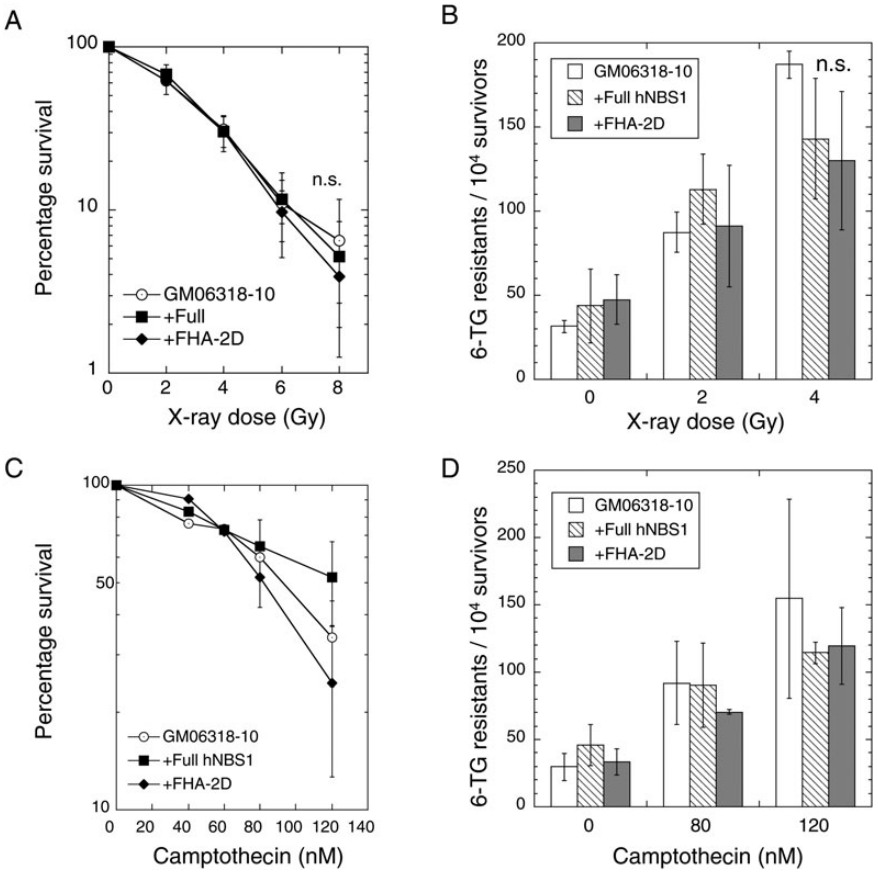

Fig. 6. Sensitivity and induced mutation frequencies after exposure to X-rays or campthotecin (CPT) in GM06318-10 cells. (**A** and **B**) X-ray sensitivity and induced Hprt-deficient mutation frequencies after exposure to X-rays (single exposure). (**C** and **D**) CPT sensitivity and induced mutation frequencies after exposures to a CPT treatment. Cells were treated with CPT for 1 h. The designation '+ Full' indicates a full-length wild-type *NBS1* gene; '+ FHA-2D' represents the mutated form of the *NBS1* gene. n.s. = not significant.

cells sensitive to radiation without any increases in genomic instability. Although the mechanism by which FHA-2D did not increase mutation frequency remains unclear, our results suggest that a certain amount of HR capability could be enough to suppress the mutatator phenotype.

NBS1 is a multifunctional protein that regulates not only DSB repair through HR, but also DNA damage responses such as the induction of apoptosis [13], the activation of ATM/ATR kinases [26], telomere maintenance [27, 28], chromatin remodeling [7], and translesion synthesis [29]. The critical domain in the NBS1 protein differs for each function or pathway. The critical domains of NBS1 for the HR pathway are the MRE11-binding domain and the FHA/BRCT domains for phospho-dependent localization of the MRN complex [10]. Among these domains, the MRE11-binding domain is more critical for HR efficiency than the FHA/BRCT domains. This also suggests that point mutations in the FHA domain, or any chemicals that abolish the FHA domain, can affect the NBS1 function in HR regulation with minimal effects on other functions.

NBS1 has been considered as a target for radiosensitization because of its multiple functions during DSB damage responses. Knockdown of NBS1 sensitizes cells regardless of p53 status [30, 31]. This also supports efforts focusing on the functional domain of NBS1 as a target for radiosensitization. The present results suggest that a partial inhibition of HR by ectopic expression of FHA-2D NBS1 does not affect cell viability and genomic stability, and that it renders proliferating cells slightly sensitive to a single radiation exposure, but renders them significantly sensitive to split dose radiation exposures. The phenotype observed in FHA-2D NBS1-expressing cells should be advantageous for cancer radiotherapy in which radiation is delivered as a split dose. Thus the FHA domain of the NBS1 protein is a potential molecular target for radiosensitization through the partial inhibition of HR. This suggests that small molecules that bind to the FHA domain of NBS1 and reduce MRN foci formation following irradiation could be good candidates for effective sensitizers in cancer radiotherapy.

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