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DNA Repair by Homologous Recombination, But Not by Nonhomologous End Joining, Is Elevated in Breast Cancer Cells[1,2]

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Abstract

Aberrant double-stranded break (DSB) repair leads to genomic instability, which is a hallmark of malignant cells. Double-stranded breaks are repaired by two pathways: homologous recombination (HR) and nonhomologous DNA end joining (NHEJ). It is not known whether these repair pathways are affected in sporadic breast tumors. Here, we examined the efficiency of HR and NHEJ repair in a panel of sporadic breast cancer cell lines and tested whether the efficiency of HR or NHEJ correlates with radioresistance. Homologous recombination and NHEJ in breast cancer cells were analyzed using *in vivo* fluorescent assays. Unexpectedly, our analysis revealed that the efficiency of HR is significantly *elevated* in breast cancer cells compared with normal mammary epithelial cells. In contrast, the efficiency of NHEJ in breast cancer cells is not different from normal cells. Overall, breast cancer cells were more sensitive to radiation than normal cells, but the levels of resistance did not correlate with either HR or NHEJ efficiency. Thus, we demonstrate that sporadic breast cancers are not associated with a deficiency in DSB repair, but rather with up-regulation of the HR pathway. Our finding of elevated HR in sporadic breast cancer cell lines suggests that therapies directed against the components of HR will be highly tumor-specific.

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Introduction

Genomic rearrangements such as translocations, deletions, and duplications are extremely frequent in cancer cells and, particularly, in breast cancer cells [1–4]. Genomic rearrangements are believed to result from the aberrant repair of DNA double-strand breaks (DSBs). These DSBs are repaired by two major pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ; reviewed in Helleday et al. [5]). Homologous recombination is conducted by proteins from the Rad52 epistasis group and is dependent on *BRCA1* and *BRCA2* breast cancer susceptibility genes (reviewed in San Filippo et al. [6]) and possibly on the members of the Fanconi Anemia pathway [7]. During HR-mediated repair of DSB, the sister chromatid is used as a template to copy the missing information into the broken locus. Because sister chromatids are identical to each other, DNA damage can be repaired faithfully with no genetic consequence. Nonhomologous DNA end joining is mediated by Ku70/Ku80, DNA-PKcs, Artemis nuclease, and the XRCC4/DNS-LigaseIV complex (reviewed in Lieber [8]). The NHEJ pathway fuses the two broken DNA ends with little or no sequence homology, leading to deletions or insertions of filler DNA.

Defects in HR or NHEJ factors may lead to radiosensitivity and a predisposition to cancer, as is the case with *BRCA1* and *BRCA2*, which are mutated in familial breast cancer [9]. It should be noted that BRCA proteins function in transcription, cell cycle control, and ubiquitination [10]. However, *BRCA* mutations account for only a small percentage of cancer cases and the status of DSB repair is less clear in sporadic breast cancers, which are not associated with an obvious DNA repair defect. On the contrary, it was suggested that an increase in DNA repair capacity may contribute to therapy resistance [11,12]. Thus, there are conflicting hypotheses as to whether DSB repair is upregulated

Abbreviations: DSB, double-stranded break; HR, homologous recombination; NHEJ, nonhomologous DNA end joining

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or downregulated in breast cancer. Overexpression of Rad51, a key enzyme in the HR pathway, has been detected in various cancer cells [13–23]. We previously showed that the *Rad51* promoter is strongly upregulated in several sporadic breast cancer cell lines and that *Rad51* promoter fused to the diphtheria toxin open reading frame (ORF) selectively targets cancer cells [23].

Here, we performed a systematic analysis of HR and NHEJ efficiency and radiosensitivity in a panel of sporadic breast cancer cell lines and in normal breast epithelial cells. Homologous recombination and NHEJ were measured using *in vivo* fluorescent assays in which cells were transfected with green fluorescent protein (GFP)–based reporter substrates from which functional GFP would only be expressed if successful DSB repair occurred. Our analysis revealed that HR efficiency is significantly increased in breast cancer cells, whereas NHEJ efficiency does not differ from that in normal breast cells. Survival after γ-irradiation did not correlate with either HR or NHEJ efficiency. Our results provide insight into the etiology of breast cancer: cancer cells upregulate HR, possibly to mitigate the replication-associated damage, and also under pressure to rearrange their genomes and evade host surveillance systems, but this increased HR does not lead to radioresistance. Another important implication of our study is that the HR pathway is a promising target for anticancer therapy.

Materials and Methods

Breast Cell Lines and Culture Conditions

The following breast cells were used: 1) normal human mammary epithelial cells HMEC1, HMEC2, HMEC3, and HMEC4; 2) cell lines derived from primary tumors HCC1954, HCC202, HCC70, and HCC2218; and 3) cell lines derived from metastatic tumors MCF-7, T47-D, MDA-MB-231, and MDA-MB-468. Human mammary epithelial cells were derived from four female donors: HMEC1, HMEC3, and HMEC4 donors were white, and HMEC2 donor was black. Human mammary epithelial cells were obtained from Clonetics, Walkersville, MD. All other cell lines were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in 5% CO_2 and 3% O_2 at 37°C. Human mammary epithelial cells were cultured in mammary epithelial growth medium (MEGM) BulletKit (CC-3150; Clonetics) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Breast cancer cell lines were cultured according to ATCC recommendations. HCC1954, HCC70, HCC202, and HCC2218 cells were cultured in RPMI 1640 medium (ATCC 30-2001) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. MCF-7 cells were grown in Eagle's minimal essential medium (ATCC 30-2003) supplemented with 0.01 mg/ml bovine insulin, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. T47-D cells were cultured in RPMI 1640 medium supplemented with 0.2 mg/ml bovine insulin, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Both MDA-MB-231 and MDA-MB-468 cell lines were grown in Leibovitz's L-15 medium (ATCC 30-2008) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Transfections

Plasmids containing NHEJ or HR reporter cassettes were linearized by I-*Sce*I or *Hind*III restriction enzymes and purified using Qiagen Qiaex II purification kit (20021; Qiagen, Valencia, CA). Two days after splitting, exponentially growing cells were transfected with 0.5 μg of the NHEJ reporter construct, or 2 μg of the HR reporter constructs, and 0.1 μg of pDsRed-N1 as the internal control. The lower amount of the pDsRed-N1 was used to avoid interference from the bright DsRed fluorescence. Transfections were performed using the Amaxa Nucleofector (Walkersville, MD). Human mammary epithelial cells were transfected with HMEC Nucleofector kit (Amaxa VPA-1002) using program Y-001 (a transfection program is a proprietary set of electrical pulses preset by the manufacturer of the Nucleofector machine). All the other cell lines were transfected with Cell Line Nucleofector kit V (Amaxa VPA-1003). Cell lines HCC1954, HCC70, HCC202, and MCF-7 were transfected using P-020 program. Cell lines HCC2218, T47-D, MDA-MB-231, and MDA-MB-468 were transfected using programs T-030, X-005, X-003, and X-005, respectively. In the process of optimizing the transfection conditions, we used multiple Amaxa transfection protocols for each of the cell lines. Variations in transfection protocol changed the transfection efficiency, but the DNA repair efficiency expressed as GFP+/DsRed+ ratio was independent of the transfection conditions.

FACS Analysis

Cells were analyzed on the FACScalibur machine (BD Biosciences, San Jose, CA) using a green-versus-red fluorescent plot as described in Seluanov et al. [24]. Data were analyzed with the Cell Quest software (BD Biosciences).

Gamma Irradiation and Clonogenic Survival Assays

Cells were split 2 days before γ-irradiation. Cells were irradiated with increasing doses of γ-irradiation ranging from 0 to 6 Gy using a Gammacell 1000 irradiator (625 Ci ^{137}Cs from Atomic Energy of Canada, Ltd., Gaithersburg, MD). Serial dilutions of irradiated cells were plated immediately after irradiation (within 15 minutes). Cells were incubated for 7 to 10 days and stained with Commassie reagent (0.25% Commassie, 50% methanol, and 10% acetic acid) for 3 hours, after which the Commassie reagent was washed out with distilled water. Colonies containing at least 50 cells were counted. Survival was expressed as the relative plating efficiencies of the irradiated to control cells.

Statistical Analysis

The doses of γ-irradiation that kill 50% (LD_{50}) and 75% (LD_{75}) of the test population of experimental cells were estimated using a two-parameter logistic model to model the relationship between dose of γ-irradiation and the probability of cell survival. This was done separately for each of the 12 cell lines using data from three to four replicas of the dose-response experiment. Analyses were done using SAS version 9.0 (SAS Institute, Inc., Cary, NC).

Results

Homologous Recombination Is Increased in Breast Cancer Cells While NHEJ Is Not

Genomic instability, such as translocations and deletions, are a hallmark of cancer cells, including breast cancer cells [1–4]. Genomic rearrangements can arise as a result of the abnormal repair of DNA double-strand breaks by HR or NHEJ. To study whether HR and NHEJ are altered in breast cancer cells, we examined a panel of breast cell lines. As a normal control, we used primary human mammary epithelial cells HMEC1, HMEC2, HMEC3, and HMEC4. To examine breast cancer cells representing different stages of the disease, we chose four cell lines derived from primary breast tumors HCC1954,

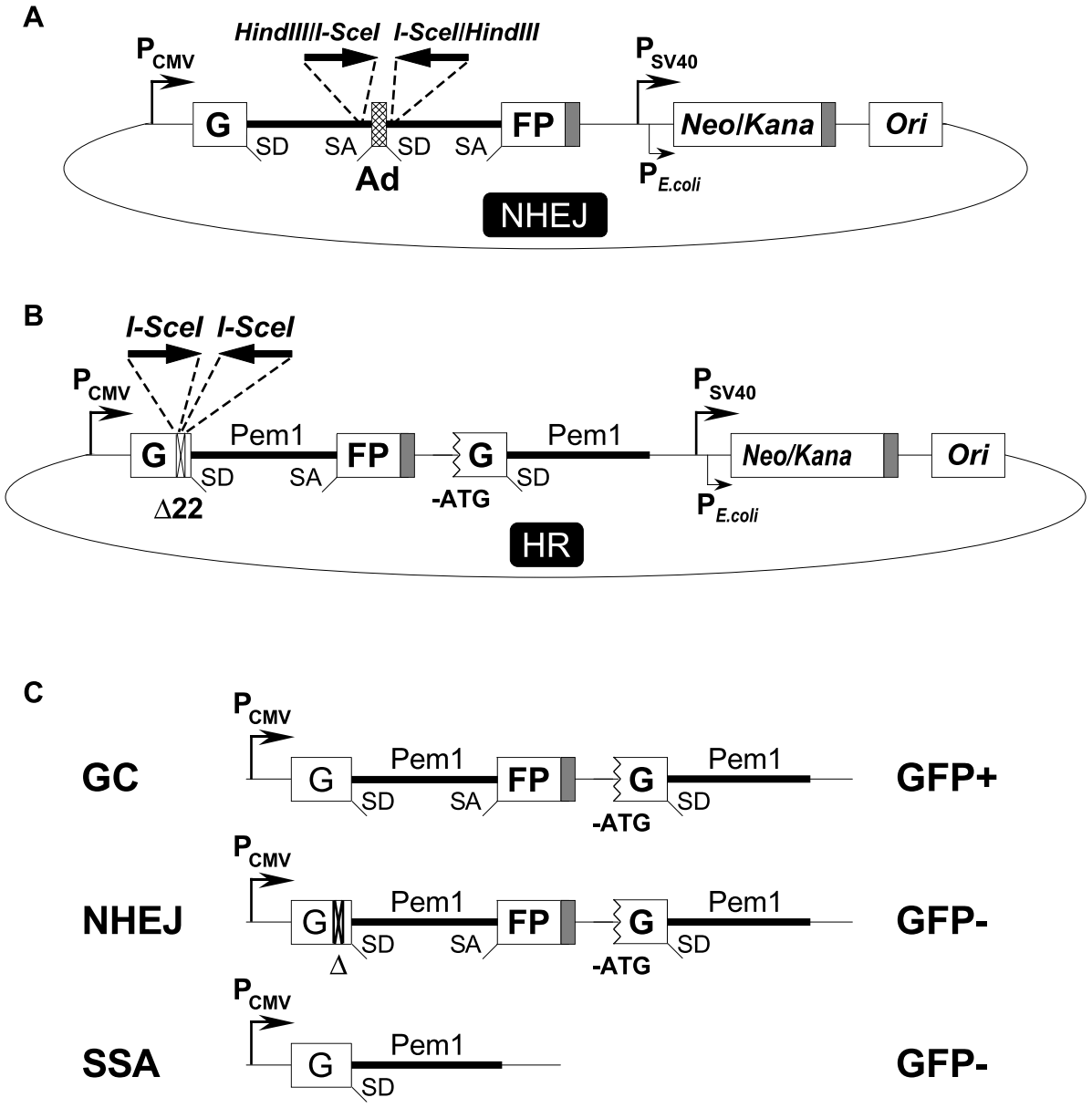

Figure 1. Reporter constructs for analysis of DSB repair. (A) Reporter plasmid for analysis of NHEJ. The reporter cassette consists of a *GFP* gene under a CMV promoter with an engineered intron from the rat *Pem1* gene, interrupted by an adenoviral exon (Ad). The adenoviral exon is flanked by I-*Sce*I recognition sites in inverted orientation for induction of DSBs. In this construct, the GFP gene is inactive; however, upon induction of a DSB and successful NHEJ, the construct becomes GFP+. *SA* indicates splice acceptor; *SA*, splice donor; *shaded squares*, polyadenylation sites. (B) Reporter plasmid for analysis of HR. The reporter cassette consists of two mutated copies of GFP-Pem1. In the first copy of GFP-Pem1, the first GFP exon contains a deletion of 22 nt and an insertion of two I-*Sce*I recognition sites in inverted orientation. The 22 nt deletion ensures that GFP cannot be reconstituted by a NHEJ event. The second copy of GFP-Pem1 lacks the ATG and the second exon of GFP. Upon induction of DSBs by I-*Sce*I, gene conversion events reconstitute an active *GFP* gene. (C) Repair products of the reporter plasmid for the analysis of HR shown in (B). *GC* indicates gene conversion; *SSA*, single-strand annealing. Only gene conversion leads to reconstitution of the GFP activity.

HCC202, HCC70, and HCC2218 and four cell lines from metastatic tumors MCF-7, T47-D, MDA-MB-231, and MDA-MB-468.

To analyze the efficiency of NHEJ and HR in a quantitative manner, we used fluorescent reporter constructs in which a functional *GFP* gene is reconstituted following an HR or NHEJ event (Figure 1) [24,25]. The NHEJ reporter (Figure 1*A*) consists of a *GFP* gene interrupted by an engineered intron (GFP-Pem1) and an adenoviral exon flanked by two *Hind*III and two inverted I-*Sce*I sites. The starting construct is GFP-negative because the adenoviral exon disrupts the GFP ORF. Digestion with *Hind*III removes the adenoviral exon and leaves compatible DNA ends. Nonhomologous DNA end joining of the *c*ompatible DNA ends (NHEJ-C) restores the functional *GFP* gene. Digestion of inverted I-*Sce*I sites leaves incompatible DNA ends. Similarly, NHEJ of incompatible ends (NHEJ-I) restores GFP activity. Because radiation and chemotherapy drugs cause unspecific damage to DNA, NHEJ-I may be more representative of the NHEJ of radiation-induced or chemically induced DNA damage. The functionality of the NHEJ cassette has been confirmed by plasmid rescue and sequencing [24]. The HR

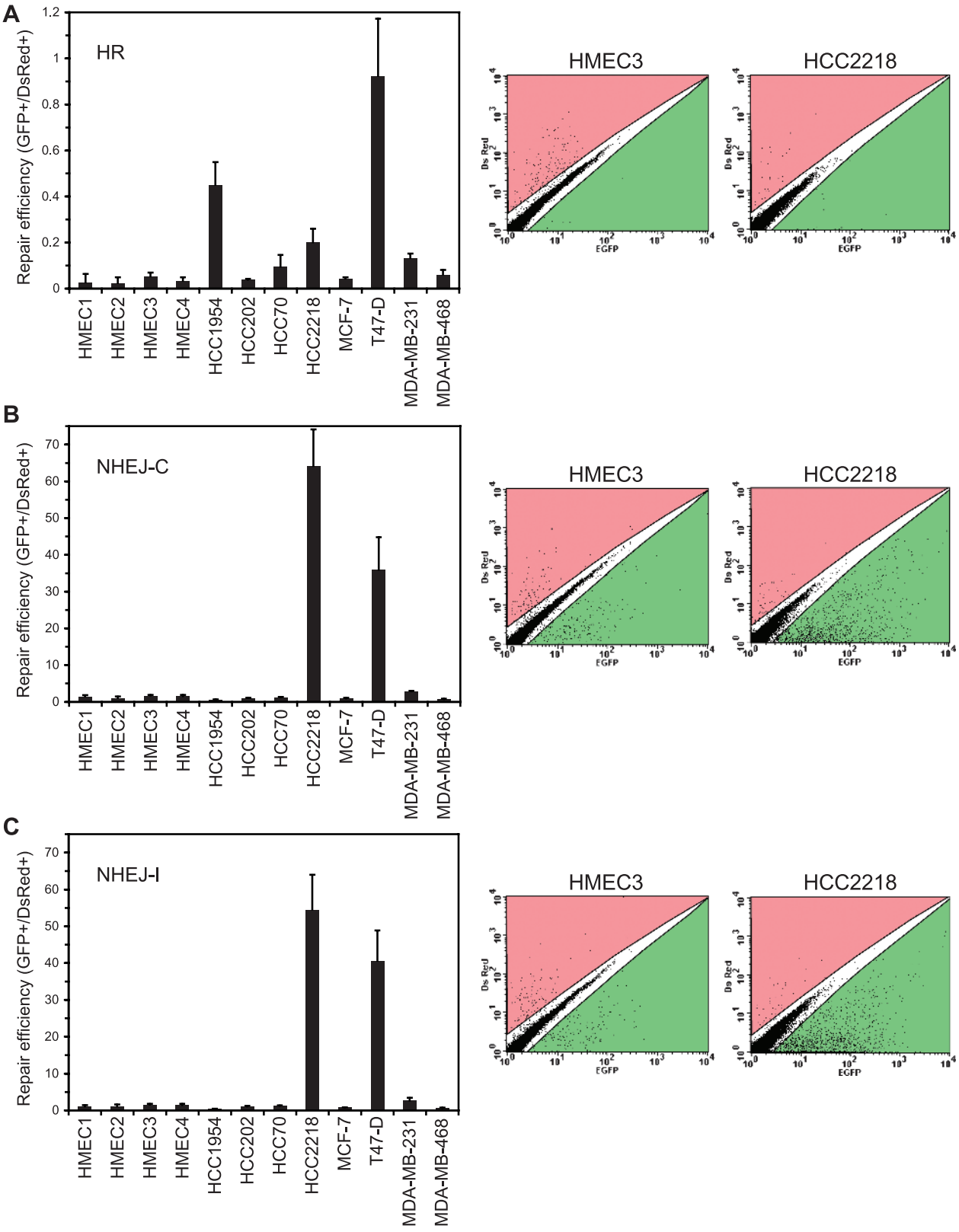

Figure 2. Analysis of HR and NHEJ in normal and malignant breast epithelial cells. Cells were cotransfected with 2 μg (HR) or 0.5 μg (NHEJ) linearized reporter plasmids (Figure 1) and 0.1 μg of the DsRed expression vector to normalize for the differences in transfection efficiency. Cells were analyzed on a green-versus-red fluorescence plot. The gating for the analysis of GFP+ and DsRed+ cells was set up using cells transfected with GFP or DsRed expression vectors and cells transfected with a negative control plasmid to exclude autofluorescent cells. The numbers of GFP+ and DsRed+ cells were determined by flow cytometry, and typical FACS traces are shown on the right. The ratio of GFP+ to DsRed+ cells was used as a measure of repair efficiency. (A) Efficiency of HR. (B) Efficiency of NHEJ of compatible DNA ends generated by digestion of the NHEJ reporter plasmid with *Hind*III. (C) Efficiency of NHEJ of incompatible DNA ends generated by digestion of the NHEJ reporter plasmid with I-*Sce*I. All experiments were repeated at least four times. Error bars, SD.

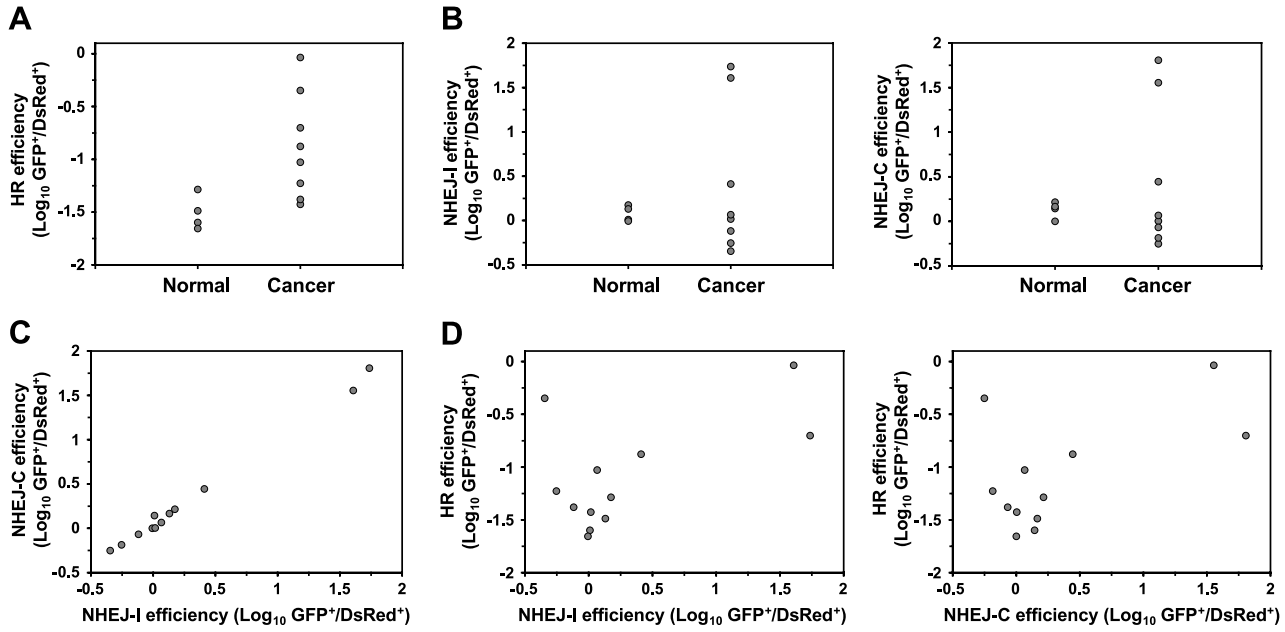

Figure 3. Homologous recombination but not NHEJ is elevated in breast cancer cells. (A) Relationship between HR efficiency and normal *versus* malignant cell status. Cancer cells display elevated HR ($P_{MWU} = 0.017$). (B) Relationship between NHEJ and normal *versus* malignant status. Nonhomologous DNA end joining efficiency does not differ between normal and cancer cells ($P_{MWU} > 0.999$ for NHEJ-I; $P_{MWU} = 0.734$ for NHEJ-C). (C) There is a strong correlation between NHEJ of compatible and NHEJ of incompatible ends ($r_s = 0.979$, $P = .001$). (D) We did not detect a significant correlation either between HR and NHEJ-I ($r_s = 0.329$, $P = .276$) or between HR and NHEJ-C ($r_s = 0.273$, $P = .366$).

reporter (Figure 1*B*) consists of two defective copies of GFP-Pem1, where the first copy contains two inverted recognition sites for I-*Sce*I. Digestion with I-*Sce*I followed by HR repair by gene conversion restores GFP activity (Figure 1*C*). The functionality of the HR reporter cassette has been confirmed by isolating the GFP+ cells by flow cytometry, plasmid rescue, and sequencing. In the recovered HR cassettes, *GFP* gene was reconstituted by a gene conversion event between the upstream internally deleted copy of the first *GFP* exon and the downstream promoterless copy of the first *GFP* exon.

To analyze HR, NHEJ-C, and NHEJ-I, we transfected cells with the HR reporter linearized by digestion with I-*Sce*I enzyme, or the NHEJ reporter linearized by *Hind*III or I-*Sce*I. The DSB reporter plasmids were cotransfected with a plasmid expressing DsRed to normalize for differences in transfection efficiency between the cell lines. After transfection cells were incubated for 72 hours to allow for the maximum expression of GFP and DsRed and were analyzed by flow cytometry. Cells were analyzed with a green-versus-red fluorescence plot as described by Seluanov et al. [24]. The gating for GFP+ and DsRed+ cells was determined in each experiment by using cells transfected with GFP, DsRed, or negative control plasmids. The ratio between GFP+ and DsRed+ cells was used as a measure of HR or NHEJ efficiency (Figure 2).

The HR efficiency detected in normal breast epithelial cells was low compared with the generally higher levels seen in breast cancer cells, with HCC1954 and T47-D cell lines showing extremely high HR (Figure 2*A*). We statistically evaluated whether the level of HR differs between normal and malignant cells (Figure 3*A*). The analysis showed that HR is significantly elevated in breast cancer cells (Mann-Whitney test, $P_{MWU} = 0.017$).

Human mammary epithelial cells are cultured in a proprietary medium, which differs from the medium used for cancer cell lines. To test whether the HMEC medium may have inhibitory effect on HR, we attempted growing the HCC70, HCC1954, MDA-MB-468, T47-D, MCF-7, and MDA-MB-231 cell lines in HMEC media. MDA-MB-468, T47-D, MCF-7, and MDA-MB-231 cell lines died in HMEC media; however, HCC70 and HCC1954 cells proliferated, enabling us to analyze HR. Under these conditions, the efficiency of HR in the cancer cells remained significantly higher than in the normal mammary epithelial cells (Figure W1). This result indicated that the observed differences in HR between the normal and malignant cells do not result from the differences in the growth media.

The efficiency of both NHEJ-I and NHEJ-C was much higher than the efficiency of HR, indicating that NHEJ is the preferred DSB repair pathway in both normal and cancer cells (Figure 2, *B* and *C*). There was, however, no correlation between the level of NHEJ and the normal *versus* malignant status ($P_{MWU} > 0.999$ for NHEJ-I; $P_{MWU} = 0.734$ for NHEJ-C; Figure 3*B*).

The two types of NHEJ showed strong correlation ($r_s = 0.979$, $P = .001$) indicating that the same machinery is involved in repair of compatible and incompatible DNA ends (Figure 3*C*). In addition, there was no significant correlation either between HR and NHEJ-I ($r_s = 0.329$, $P = .276$) or between HR and NHEJ-C ($r_s = 0.273$, $P = .366$; Figure 3*D*). Thus, the two pathways of DSB repair are independently controlled, and only HR is upregulated in breast cancer cells.

In summary, our analysis has revealed several important characteristics of DSB repair in breast cancer cells: 1) HR is significantly elevated in breast cancer cells compared with normal cells; 2) NHEJ is the major DSB repair pathway in both normal and malignant breast epithelial cells; 3) NHEJ efficiency does not differ significantly between normal and cancerous cells; 4) there is a strong correlation between the efficiency of NHEJ of compatible and incompatible DNA ends; and 5) there is no correlation between the efficiencies of HR and NHEJ.

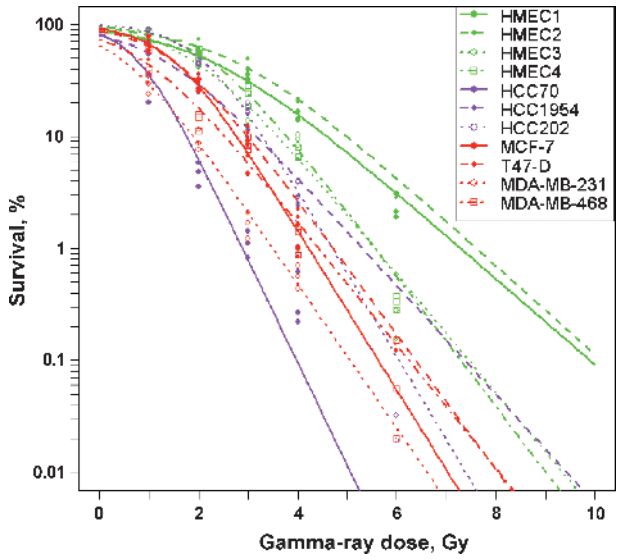

Figure 4. Survival curves of normal breast epithelial cells and breast cancer cells after γ-irradiation. Cells were irradiated with increasing doses of γ-irradiation. Survival was expressed as the relative plating efficiencies of irradiated *versus* control cells. Each treatment was repeated three to four times, and logistic regression using SAS version 9.0 was used to fit a mortality curve for each cell line. The HCC2218 cell line was not analyzed because these cells grow in suspension culture.

HR and NHEJ Efficiencies Do Not Correlate with Radiosensitivity

Increased efficiency of DSB repair may improve the ability of cells to deal with DNA damage induced by irradiation. Because radioresistance is a serious problem in breast cancer treatment, we set out to test whether an increase in HR or NHEJ contributes to this phenomenon. We determined LD_{50} and LD_{75} after γ-irradiation for normal and cancer cell lines (Figure 4 and Table 1). Normal cells were more resistant to γ-irradiation than cancer cells (LD_{50}, $P_{MWU} = 0.014$; LD_{75}, $P_{MWU} = 0.008$; Figure 5*A*). Neither NHEJ-I nor NHEJ-C efficiency correlated with radioresistance (NHEJ-I *vs* LD_{50}, $r_s = -0.082$, $P = .796$; NHEJ-I *vs* LD_{75}, $r_s = -0.027$, $P = .931$; NHEJ-C *vs* LD_{50}, $r_s = -0.009$, $P = .977$; NHEJ-C *vs* LD_{75}, $r_s = 0.073$, $P = .818$; Figure 5, *B* and *C*).

Homologous recombination efficiency showed a significant negative correlation with radioresistance (LD_{50}, $r_s = -0.836$, $P = .008$, LD_{75}, $r_s = -0.727$, $P = .022$; Figure 5*D*). This seemingly contradicts the common assumption that HR protects cells from radiation-induced death. However, this correlation may be explained by the contribution of normal cells, which have uniformly low HR efficiency and are also more resistant to radiation. Indeed, when normal cells are omitted from the analysis, the negative correlation between HR and survival disappears (LD_{50}, $r_s = -0.429$, $P = .294$; LD_{75}, $r_s = -0.107$, $P = .787$). Thus, when cancer cells are analyzed separately from normal cells, there is no significant correlation between HR and survival, indicating that increased HR in breast cancer cells does not lead to radioresistance.

Discussion

Our report shows that HR but not NHEJ is increased in breast cancer cells compared with normal breast epithelial cells. This result may have important implications for the development of anticancer therapies targeting the HR pathway. Breast cancer is frequently associated with chromosomal abnormalities [1–4], which could result from the abnormal function of either the HR or NHEJ pathways, with abnormal DSB repair meaning either deficient or elevated function. Our result argues that cancer cells show elevated HR, which probably leads to deregulated recombination and the chromosomal abnormalities frequently present in breast tumors.

What is the mechanism for increased HR in breast cancer cells? In our study, HMECs proliferated with the same rate or faster than breast cancer cells; thus, the high HR cannot be attributed to the higher fraction of proliferating cells among the cancer cells. Multiple reports detected increased levels of Rad51 protein or Rad51 paralogs in cancer cells [14–23]. Rad51 is a mammalian homolog of bacterial RecA, which plays a central role in HR (reviewed in Richardson [22]). Elevated levels of Rad51 may be responsible for the increased HR. Indeed, forced overexpression of Rad51 in rodent cells resulted in an increased frequency of HR and chromosomal instability [26–28]. In addition to the overexpression of Rad51, other proteins may stimulate HR. Overexpression of DNA polymerase β, found in some breast, prostate, and colon tumors, has been shown to stimulate HR in a Rad51-dependent manner [29]. Elevated and deregulated HR may be highly deleterious in many ways. The fidelity of HR may be compromised, or the checkpoint controls of recombination may be altered leading to loss of heterozygosity, translocations, and other rearrangements. These observations led to the hypothesis that elevated HR plays a role in carcinogenesis [30,31]. However, HR frequency has not been systematically examined in breast cancer. Our study provides the first analysis of HR in a panel of human breast tumor cells, which gives experimental support to the hypothesis that breast cancer cells have elevated HR.

A recent report showed that overexpression of Rad51 in *BRCA1*-deficient DT40 cells rescued defects in proliferation, DNA damage survival, and HR [32]. It was hypothesized that because BRCA1 facilitates Rad51 subnuclear assembly, in its absence, an excess of Rad51 may circumvent the requirement for BRCA1. Our study included only sporadic breast cancer cell lines. Thus, up-regulation of HR is not limited to *BRCA1*-deficient tumors but frequently occurs in sporadic tumors as well. The signaling pathways leading to the up-regulation of HR may be different in *BRCA1*-deficient and sporadic tumors. For example, fusion tyrosine kinases, which result from chromosomal translocation and cause acute and chronic leukemias and non–Hodgkin lymphoma, stimulate the expression of Rad51 and Rad51 paralogs [18,33]. BCR/ABL was also shown to enhance Rad51 function by phosphorylating Rad51 on Tyr-315 [18]. Understanding the pathways leading to the up-regulation of HR in breast tumors may shed light on the mechanisms of cancer development.

Table 1. Sensitivity of Human Normal Mammary Epithelial Cells and Breast Cancer Cells to γ-Irradiation (Gy).

Cell Line	LD_{50} (95% CI)	LD_{75} (95% CI)
HMEC1	2.10 (1.95-2.24)	3.34 (3.18-3.53)
HMEC2	2.58 (2.37-2.78)	3.78 (3.54-4.08)
HMEC3	1.79 (1.67-1.91)	2.69 (2.57-2.83)
HMEC4	2.13 (2.03-2.24)	2.95 (2.84-3.09)
HCC70	0.72 (0.30-0.95)	1.24 (1.02-1.44)
HCC1954	1.20 (0.90-1.42)	2.18 (1.98-2.42)
HCC202	2.02 (1.92-2.11)	2.66 (2.56-2.77)
MCF-7	1.43 (1.32-1.53)	2.09 (1.99-2.21)
T47-D	1.40 (1.27-1.51)	2.19 (2.08-2.31)
MDA-MB-231	0.41 (0.11-0.62)	1.15 (0.98-1.29)
MDA-MB-468	0.79 (0.45-1.03)	1.66 (1.46-1.85)

CI indicates confidence interval.

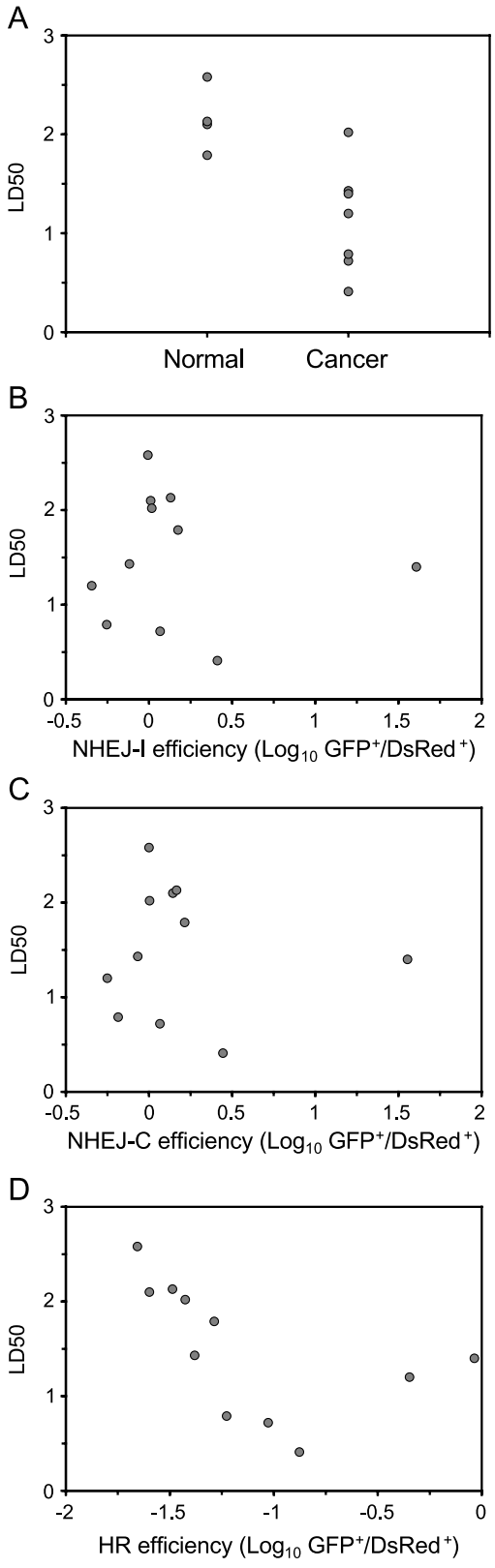

Figure 5. Relationship between survival after γ-irradiation (LD_{50}) and normal *versus* malignant cell status (A) and DSB repair efficiency (B, C, D). (A) Normal cell are more resistant to γ-irradiation ($P_{MWU} = 0.014$). (B, C) Neither NHEJ-I nor NHEJ-C efficiency correlated with radioresistance (NHEJ-I, $r_s = -0.082$, $P = .796$; NHEJ-C, $r_s = -0.009$, $P = .977$). (D) HR efficiency negatively correlates with radioresistance ($r_s = -0.836$, $P = .008$). However, this correlation disappears if normal cells are excluded (see text).

Why do cancer cells upregulate HR? Does up-regulation of HR predispose cells to malignant transformation or does it appear later as an adaptation for the survival of malignant cells? Studies in *BRCA1*-deficient cells suggest that up-regulation of HR is an adaptation, which improves cell proliferation and resistance to DNA damage [32]. Initially, malignant transformation is associated with mutations such as the activation of oncogenes and the inactivation of cell cycle checkpoint apparatuses. Activated oncogenes lead to the firing of multiple replication origins. In the absence of cell cycle checkpoints, cells will continue proliferation, leading to high level of replication-related lesions. We speculate that, to counteract this replication, stress cancer cells may upregulate HR repair. Of the two DSB repair pathways, HR predominantly repairs replication-related DSBs, whereas NHEJ is active throughout the cell cycle. Therefore, this model provides an explanation as to why HR but not NHEJ is increased in cancer cells. An alternative scenario, where a mutator phenotype caused by elevated HR leads to malignant transformation, is also possible. In mammals, HR is strictly controlled, and it plays a relatively minor role in DSB repair when compared with NHEJ [34–36] because an inappropriate recombination within the highly repetitive mammalian genomes may lead to gross genomic rearrangements and cancer.

Gene expression profiling of melanoma samples found overexpression of genes involved in the repair of stalled replication forks in primary tumors with a bad prognosis [37]. These studies led to a hypothesis that the overexpression of DNA repair genes in primary tumors is associated with a higher metastatic potential [38]. Our study does not allow for differentiating between metastatic and primary tumors owing to the small sample size; however, it complements the gene expression data by demonstrating that the enzymatic activity of a DNA repair pathway involved in the repair of replication forks is elevated in cancer cells.

Breast cancer cells showed no significant changes in NHEJ. Most cell lines had the same frequency of NHEJ as the normal cells. However, two cancer cell lines had extremely high NHEJ (Figure 2, *B* and *C*). Intact NHEJ in most breast cancer cell lines is consistent with a previous report where NHEJ was analyzed *in vitro* [39]. We did not find a correlation between NHEJ and HR, which strengthens the idea that only HR but not NHEJ is altered in breast cancer.

A recent study of NHEJ in urothelial carcinoma cells showed that they performed NHEJ of compatible DNA ends more efficiently than normal urothelial cells and that the carcinoma cells also displayed a preferential use of microhomologies [40]. Our analysis of NHEJ-C and NHEJ-I did not reveal any differences in the processing of compatible and incompatible DNA ends between normal and malignant breast epithelial cells. Thus, different cancers may have differential effect on the DSB repair pathways.

Breast cancer cells were more sensitive to γ-irradiation than normal mammary epithelial cells, which perhaps reflects the susceptibility of breast cancer to radiotherapy. Surprisingly, we did not find a correlation between HR efficiency and resistance to γ-irradiation among the cancer cells lines. Even the cell lines with the highest levels of HR were more sensitive to radiation than the normal breast epithelial cells. Homologous recombination plays a relatively minor role in mammalian DSB repair and its function is limited to the S/G_2 phases [34,35]. Therefore, increased HR may help cancer cells deal with endogenous DNA damage that arises during DNA replication but is not effective enough to protect the cells from exogenous DNA damage applied to unsynchronized cells.

The most important implication of our findings is that HR is a promising target for anticancer therapy. The inhibition of NHEJ components was shown to sensitize cancer cells to therapy [41–45]. However,

inhibition of NHEJ is toxic to normal cells and may not provide the desired selectivity. Our results suggest that the inhibition of HR will be selective against breast tumor cells. Inhibitors of HR proteins can be used in combination with radiotherapy or chemotherapy to sensitize the cells [46–48]. An even more attractive possibility would be to use anti-HR agents alone, avoiding the toxicity of DNA-damaging agents. If breast cancer cells require efficient HR for survival, inhibition of HR may selectively kill the cancer cells in the absence of an exogenous DNA-damaging agent. Such a strategy has been applied to selectively kill *BRCA2*-deficient cells using poly-ADP-ribose-polymerase inhibitors [49,50]. Our results hold promise that similar approaches can be developed to treat sporadic breast cancers. Another powerful approach would be to use elevated HR to transcriptionally target cancer cells. We recently showed that the *Rad51* promoter is on average 840-fold more active in cancer cells than in normal cells and that the fusion of *Rad51* promoter with the diphtheria toxin gene selectively kills cancer cells [23]. Transcriptionally targeted therapies taking advantage of upregulated HR gene expression would allow effective elimination of cancerous cells with no toxicity to normal tissue.

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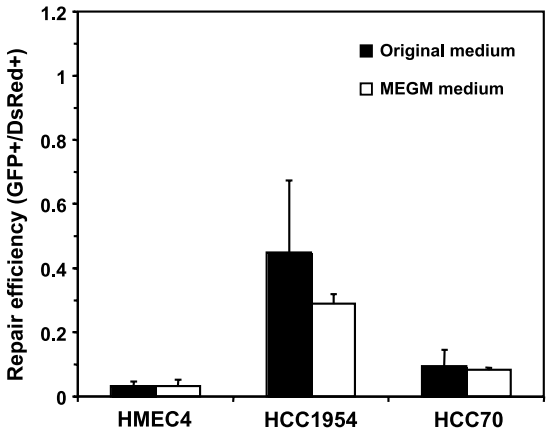

Figure W1. Comparison of HR efficiency in different growth media. Black bars represent HR efficiency in cells cultured in the recommended medium (see Materials and Methods), also shown in Figure 2. White bars represent HR efficiency in cells cultured in the MEGM recommended for HMEC (Clonetics). Homologous recombination efficiency was not significantly different between the two growth conditions (HCC1954, $P = .215$; HCC70, $P = .741$) indicating that MEGM does not have a suppressive effect on HR.