The Response of $CD24^{-/low}/CD44^{+}$ Breast Cancer–Initiating Cells to Radiation

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Background: **If cancer arises and is maintained by a small population of cancer-initiating cells within every tumor, understanding how these cells react to cancer treatment will facilitate improvement of cancer treatment in the future. Cancer-initiating cells can now be prospectively isolated from breast cancer cell lines and tumor samples and propagated as mammospheres in vitro under serum-free conditions.** ***Methods:*** **$CD24^{-/low}/CD44^{+}$ cancer-initiating cells were isolated from MCF-7 and MDA-MB-231 breast cancer monolayer cultures and propagated as mammospheres. Their response to radiation was investigated by assaying clonogenic survival and by measuring reactive oxygen species (ROS) levels, phosphorylation of the replacement histone H2AX, CD44 levels, CD24 levels, and Notch-1 activation using flow cytometry. All statistical tests were two-sided.** ***Results:*** **Cancer-initiating cells were more resistant to radiation than cells grown as monolayer cultures (MCF-7: monolayer cultures, mean surviving fraction at 2 Gy [SF_{2Gy}] = 0.2, versus mammospheres, mean SF_{2Gy} = 0.46, difference = 0.26, 95% confidence interval [CI] = 0.05 to 0.47; *P* = .026; MDA-MB-231: monolayer cultures, mean SF_{2Gy} = 0.5, versus mammospheres, mean SF_{2Gy} = 0.69, difference = 0.19, 95% CI = −0.07 to 0.45; *P* = .09). Levels of ROS increased in both mammospheres and monolayer cultures after irradiation with a single dose of 10 Gy but were lower in mammospheres than in monolayer cultures (MCF-7 monolayer cultures: 0 Gy, mean = 1.0, versus 10 Gy, mean = 3.32, difference = 2.32, 95% CI = 0.67 to 3.98; *P* = .026; mammospheres: 0 Gy, mean = 0.58, versus 10 Gy, mean = 1.46, difference = 0.88, 95% CI = 0.20 to 1.56; *P* = .031); phosphorylation of H2AX increased in irradiated monolayer cultures, but no change was observed in mammospheres. Fractionated doses of irradiation increased activation of Notch-1 (untreated, mean = 10.7, versus treated, mean = 15.1, difference = 4.4, 95% CI = 2.7 to 6.1, *P* = .002) and the percentage of the cancer stem/initiating cells in the nonadherent cell population of MCF-7 monolayer cultures (untreated, mean = 3.52%, versus treated, mean = 7.5%, difference = 3.98%, 95% CI = 1.67% to 6.25%, *P* = .009).** ***Conclusions:*** **Breast cancer–initiating cells are a relatively radioresistant subpopulation of breast cancer cells and increase in numbers after short courses of fractionated irradiation. These findings offer a possible mechanism for the accelerated repopulation of tumor cells observed during gaps in radiotherapy. [J Natl Cancer Inst 2006;98:1777–85]**

One view of cancer is that it may arise from a single cell that has the ability to self-renew and thus to maintain the growth of a tumor, whereas the majority of its cellular progeny does not. There is increasing evidence that such a cell population exists and that these cells can be prospectively identified in brain tumors *(1)*, breast cancer *(2)*, prostate cancer *(3)*, and melanoma *(4)*. A considerable effort is going into determining unique properties of these cells with the assumption that this cell population, more than any other, will determine the outcome of cancer treatment. Because such cells might be expected to share properties with adult stem cells in normal tissues, they are often termed cancer stem cells *(5)*. However, in spite of old *(6)* and more recent *(1,2,7–9)* evidence that cancer stem cells exist, there is still a dearth of good phenotypic markers for such cells. In addition, many of the self-renewing cancer cell populations that are studied may also contain early progenitor cells that are derived from cancer stem cells but are also able to initiate and maintain tumor growth. Therefore, we join others *(2)* in preferring to use the term cancer-initiating cells.

In breast cancer, a population of $CD24^{-/low}/CD44^{+}$ cells has been isolated that is highly enriched for cancer-initiating cells *(2)*. This population is 1000 times more tumorigenic than cell populations that are depleted of $CD24^{-/low}/CD44^{+}$ cells, and injection of as few as 200 cells leads to tumor formation in SCID mice *(2)*. Breast cancer–initiating cells can be established from patients' surgical specimens or breast cancer cell lines and can be propagated in vitro as nonadherent mammospheres *(7)*.

Stem cell properties in normal tissues are tightly regulated by the Wnt, Shh, and Notch signaling pathways *(10,11)*. In addition, overexpression of Notch-1 was observed in breast cancer specimens, and the level of expression was associated with prognosis *(12)*. Activation of the Notch-1 pathway is initiated by the binding of Notch-1 ligands, e.g., Jagged-1, to the extracellular domain of Notch-1. This binding causes a conformational change in Notch-1 that allows the protease tumor necrosis factor alpha converting enzyme to cleave the extracellular domain of the molecule. Notch-1 is thereafter processed by γ-secretase–regulated intramembrane proteolysis, which allows the intracellular domain of Notch-1 (Notch-1 ICD) to translocate into the nucleus where it binds to and activates the transcriptional repressor CBF1. Activation of Notch-1 signaling leads to increased transcription of ErbB2 *(13)*, cyclin D1 *(14)*, CDK2 *(14)*, and Notch-4 *(15)*. ErbB2 is related to radiation resistance *(16)*, whereas cyclin D1 and CDK2 promote the transition from G1 to S phase of the cell cycle and thus promote proliferation. Notch-1 signaling promotes the self-renewal of mammary stem cells *(17)*, and there is strong evidence that Notch-1 is involved in the carcinogenesis of breast cancer *(17)*. In addition, Notch-1 maintains the malignant phenotype of Ras-transformed cells *(15)*, and overexpression of Notch induces mammary tumors in mice *(18)*.

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See "Notes" following "References."

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Radiation therapy (RT) is an integral part of the multimodal treatment concept for breast cancer. Its success depends on the complete elimination of all cancer stem cells. Radiation oncologists have been advocating the existence of stem cells in normal tissues and cancers for decades *(6)*. Accelerated repopulation—the increase in the rate of growth as a result of time between treatments *(19,20)*—is a cancer stem cell–related phenomenon that occurs during fractionated RT. During accelerated repopulation, each day of a treatment gap decreases the efficacy of RT by approximately 0.6 Gy, making it one of the major reasons for local failure of RT. Accelerated repopulation was first described for head and neck epithelial tumors *(21)*, but it also occurs in breast cancer even though it may be difficult to detect *(22–24)*.

In this study, we investigated the radiation response of $CD24^{-/low}/CD44^{+}$ breast cancer–initiating cells, the population of cancer cells that are likely to be critical for success or failure of cancer therapy. We characterized the radiation sensitivity of these cells and the size of this cell population after clinical fractions of radiation and explored possible mechanisms for the failure of radiotherapy.

Methods

Cell Culture

MCF-7 and MDA-MB-231 breast cancer cells (American Type Culture Collection; Manassas, VA) were cultured in log-growth phase in modified Eagle medium (MEM) (supplemented with 0.1 mM nonessential amino acids and 1 mM sodium pyruvate; Cellgro, Kansas City, MO) and Dulbecco's modified Eagle medium (DMEM) (Cellgro), respectively, supplemented with 10% heat-inactivated fetal calf serum (FCS) and 0.01 mg/mL bovine insulin (Sigma, St Louis, MO) at 37 °C in a humidified atmosphere (5% CO_2). To obtain cancer-initiating cells and to propagate them as mammospheres, cells floating in the supernatant of 2-day-old cultures were collected by centrifugation for 5 minutes at 500*g*, washed in Hanks' buffered salt solution, and resuspended in phenol red–free DMEM–F12 (Cellgro) supplemented with 0.4% bovine serum albumin (BSA, Sigma), 5 μg/mL bovine insulin (Sigma), 20 ng/mL basic fibroblast growth factor 2 (bFGF, Sigma), and 10 ng/mL epidermal growth factor (EGF, Sigma) at a density of 1000 cells/mL. Growth factors were added to the mammosphere cultures every 3 days. To mimic mammosphere culture conditions in cells grown as monolayer cultures, cells were plated in MEM or DMEM media containing 10% FCS supplemented with 5 μg/mL bovine insulin, 20 ng/mL bFGF, and 10 ng/mL EGF.

Irradiation

For clonogenic assays, cells derived from monolayer cultures or 5-day-old mammospheres were enzymatically dissociated with trypsin–EDTA (monolayer cultures) or mechanically dissociated with a Pasteur pipette (mammospheres), both passed through a 40-μm sieve, and immediately irradiated (10^6 cells/mL) at room temperature with a ^{137}Cs laboratory irradiator (Mark I, JL Shephard, San Fernando, CA) at a dose rate of 4.95 Gy/minute for the time required to generate a dose curve of 0, 2, 4, 6, and 8 Gy. Corresponding controls were sham irradiated. Colony-forming assays were performed immediately after irradiation by plating cells into triplicate 100-mm culture dishes. After 28 days, cells were fixed with 75% ethanol and stained with 1% crystal violet, and colonies containing more than 50 cells were counted. To generate a radiation survival curve, the surviving fraction at each radiation dose was normalized to that of the sham-irradiated control, and curves were fitted using a linear–quadratic model (surviving fraction = $e^{(-\alpha\ dose-\beta\ dose^2)}$, in which α is the number of logs of cells killed per gray from the linear portion of the survival curve and β is the number of logs of cells killed per $[gray]^2$ from the quadratic component) *(25)*. Three independent experiments were performed.

To evaluate H2AX phosphorylation, single-cell suspensions were irradiated as above with 0, 2, or 10 Gy. Cells were harvested by centrifugation (500*g* for 5 minutes at 4 °C) at 5 and 60 minutes after irradiation.

To measure reactive oxygen species (ROS) accumulation, 100 000 cells were treated with 0, 2, or 10 Gy. Cells were immediately analyzed as described below.

To measure Notch-1 activation and Jagged-1 expression, cells were treated with single and fractionated doses of radiation. Cells (400 000 per dish) were plated onto 100-mm tissue culture dishes and allowed to grow for 24 hours. Cultures were then irradiated as monolayers at room temperature with 3 Gy daily for 5 consecutive days (days 2–6) or with a single dose of 10 Gy on day 6. Control cells were sham irradiated. Nonadherent and adherent cells were harvested 48 hours after the last irradiation (on day 8).

For primary mammosphere formation assays, cells were irradiated with 3 Gy daily for 5 consecutive days (days 2–6) or with a single dose of 10 Gy on day 6. Control cells were sham irradiated.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays to Measure Cell Proliferation

For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, MCF-7 cells in monolayer culture were irradiated; incubated for indicated times in MEM media supplemented with 10% FCS, 20 ng/mL bFGF, and 10 ng/mL EGF; washed twice with PBS; incubated with trypsin–EDTA; resuspended in MEM (containing 10% FCS); counted; and plated in 100 μL MEM (10% FCS) at 2000, 10 000, 15 000, and 20 000 cells per well into 96-well plates. After 7 days, 20 μL of MTT solution (5 mg/mL in PBS) was added to each well, and cells were incubated for 4 hours at 37 °C. Then 50 μL sodium dodecyl sulfate solution (20% sodium dodecyl sulfate, 0.01% HCl) was added to each well, and plates were incubated at 37 °C overnight. Absorbance was measured at 560 nm in a fluorescence plate reader (Spectrafluor, Tecan, San Jose, CA).

Flow Cytometry to Measure CD24, CD44, and Jagged-1 Expression; Notch-1 Activation; and H2AX Phosphorylation

CD24 and CD44 expression was analyzed in cells derived from monolayer cultures or in 5-day-old primary mammospheres following incubation in trypsin–EDTA or dissociation with a Pasteur pipette and passage through a 40-μm sieve. At least 10^5 cells were pelleted by centrifugation at 500*g* for 5 minutes at 4 °C, resuspended in 10 μL of monoclonal mouse anti-human CD24–fluorescein isothiocyanate (FITC) antibody (BD Pharmingen, San Jose, CA) and a monoclonal mouse anti-human CD44–phytoerythrin (PE) antibody (BD Pharmingen),

and incubated for 20 minutes at 4 °C. Ten independent experiments were performed.

To measure Jagged-1 expression and Notch-1 activation, cells were permeabilized with 4% formaldehyde and pelleted by centrifugation as above. Cells were then incubated with 0.25 μg of PE/Cy5-conjugated monoclonal mouse anti-human CD44 antibody, 10 μL of monoclonal mouse anti-human CD24–FITC antibody, and 200 μL of either polyclonal rabbit anti-human Jagged-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or polyclonal rabbit anti-human Notch-1-ICD antibody (Cell Signaling, Danvers, MA) that had been diluted 1 : 200 in PBS containing 2% BSA for 20 minutes at 4 °C. Cells were then washed with PBS/4% BSA and incubated with a secondary, PE-conjugated polyclonal goat anti-rabbit antibody (BD Pharmingen).

For analysis of H2AX phosphorylation, cells were centrifuged for 5 minutes at 500*g* and resuspended in 0.3 mL of PBS. To fix the cells, 0.7 mL of ethanol (99%) was added to the tube while vortexing, and samples were stored for 30 minutes at −20 °C. Cold Tris-buffered saline (TBS, pH 7.4, 1 mL) was added, and cells were pelleted by centrifugation at 500*g* and resuspended in 1 mL cold TST (TBS containing 4% FBS and 0.1% Triton X-100) for 10 minutes to permeabilize and rehydrate the cells. Cells were pelleted again and resuspended in 200 μL of monoclonal mouse anti-γH2AX–FITC antibody (Upstate, Charlottesville, VA) diluted 1 : 500 in TST, incubated on a shaker platform for 2 hours at room temperature, and washed twice in TST. Three independent experiments were performed.

Flow cytometry and cell sorting were performed on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). The CellQuest (Becton Dickinson) software package was used.

Reactive Oxygen Species Formation Assay

Cells derived from monolayer cultures or 5-day-old mammospheres were incubated with trypsin–EDTA or dissociated mechanically using a Pasteur pipette, respectively, resuspended in modified HBSS (10 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 2.7 mM glucose), passed through a 40-μm sieve, counted, and diluted to a final concentration of 10^6 cells/mL in 15-mL Falcon tubes (Becton Dickinson). Aminophenyl fluorescein (Cell Technology, Mountain View, CA) was added to a final concentration of 10 μM, and cells were incubated for 30 minutes in the dark and irradiated as indicated above. A total of 100 000 cells per well were plated into black 96-well plates, and fluorescence was measured in a fluorescence plate reader (Spectrafluor, Tecan; excitation: 480 nm, emission: 520 nm). Fluorescence was normalized to the fluorescence readings of untreated monolayer culture cells. Three independent experiments were performed, each in triplicate.

Primary Mammosphere Formation Assay

The ability of cells in the nonadherent population of monolayer cultures to initiate mammosphere formation after irradiation was assessed by harvesting, washing, and resuspending nonadherent cells in phenol red–free DMEM–F12 medium (supplemented with 0.4% BSA, 20 ng/mL bFGF, and 10 ng/mL EGF). Cells were then passed through a 40-μm sieve, counted, diluted, and plated into 96-well plates at clonal densities. Mammospheres were counted on day 5.

Statistical Methods

All data are represented as means and differences of the means with 95% confidence intervals (CIs). *P* values of .05 or less, calculated using a paired two-sided Student's *t* test, were considered to indicate statistically significant differences.

Results

Response of $CD24^{-/low}/CD44^+$ Breast Cancer–Initiating Cells to a Single Dose of Radiation

We established nonadherent mammosphere cultures from both MCF-7 and MDA-MB-231 breast cancer cells and analyzed the percentage of $CD24^{-/low}/CD44^+$ cells on day 5 by flow cytometry. In general, by day 5, MCF-7 (Fig. 1, A) and MDA-MB-231 (data not shown) mammospheres showed dramatically elevated percentages of $CD24^{-/low}/CD44^+$ cells.

The responses of cells from monolayers and $CD24^{-/low}/CD44^+$-enriched mammospheres (day 5) to radiation were compared by clonogenic assay. The plating efficiencies of MCF-7 cells derived from monolayer cultures and mammospheres without irradiation were similar (mean = 7.2%, 95% CI = 0.73 to 13.7, and mean = 11%, 95% CI = 8.8 to 13.2, respectively). However, cells derived from MCF-7 mammospheres were more radioresistant than cells derived from monolayer cultures (monolayer-derived cells: $\alpha = 0.79$, $\beta = 0.011$, mean surviving fraction at 2 Gy [SF_{2Gy}] = 0.2, versus mammospheres: $\alpha = 0.30$, $\beta = 0.044$, mean $SF_{2Gy} = 0.46$, difference = 0.26, 95% CI = 0.05 to 0.47; $P = .026$, n = 9; Fig. 1, B). Comparable results were found for mammospheres that were derived from MDA-MB-231 cells (monolayer-derived cells: $\alpha = 0.65$, $\beta = 0.0014$, mean $SF_{2Gy} = 0.5$, versus mammospheres: $\alpha = 0.31$, $\beta = 0.035$, mean $SF_{2Gy} = 0.69$, difference = 0.19, 95% CI = −0.07 to 0.45; $P = .09$, n = 6).

One hallmark of the recognition and repair of double-strand DNA breaks is phosphorylation of the replacement histone H2AX *(26)*. Single-cell suspensions from MCF-7 mammosphere and monolayer cultures were irradiated with 0, 2, or 10 Gy, and H2AX phosphorylation (γH2AX) was measured by flow cytometry at 5 and 60 minutes after irradiation (n = 2). MCF-7 cells derived from monolayer cultures showed a time-dependent increase of γH2AX after irradiation, whereas cells derived from primary mammospheres showed little change in γH2AX (Fig. 1, C). At 60 minutes, the increase in γH2AX for cells derived from monolayer cultures was dose dependent, with 10 Gy being more effective than 2 Gy (monolayer cultures: relative to control, 2 Gy, mean = 1.63-fold, not statistically significant, 10 Gy, mean = 3.2-fold, difference = 2.2, 95% CI = 1.46 to 2.92, $P = .006$, n = 3; mammospheres: relative to 0 Gy, 2 Gy, mean = 1.06-fold, not statistically significant, 10 Gy, mean = 1.13-fold, not statistically significant, n = 3); however, even the 10-Gy dose did not affect the phosphorylation of H2AX in mammospheres (Fig. 1, D).

The lack of γH2AX staining, and hence repair of DNA double-strand breaks in mammospheres, after irradiation could be due to a very rapid repair, failure of detection, or initially low induction of DNA double-strand breaks. Therefore, we next investigated whether irradiation induced the formation of ROS in MCF-7 monolayer cultures and mammospheres. Single-cell suspensions were irradiated with 0, 2, or 10 Gy. Cells derived from MCF-7

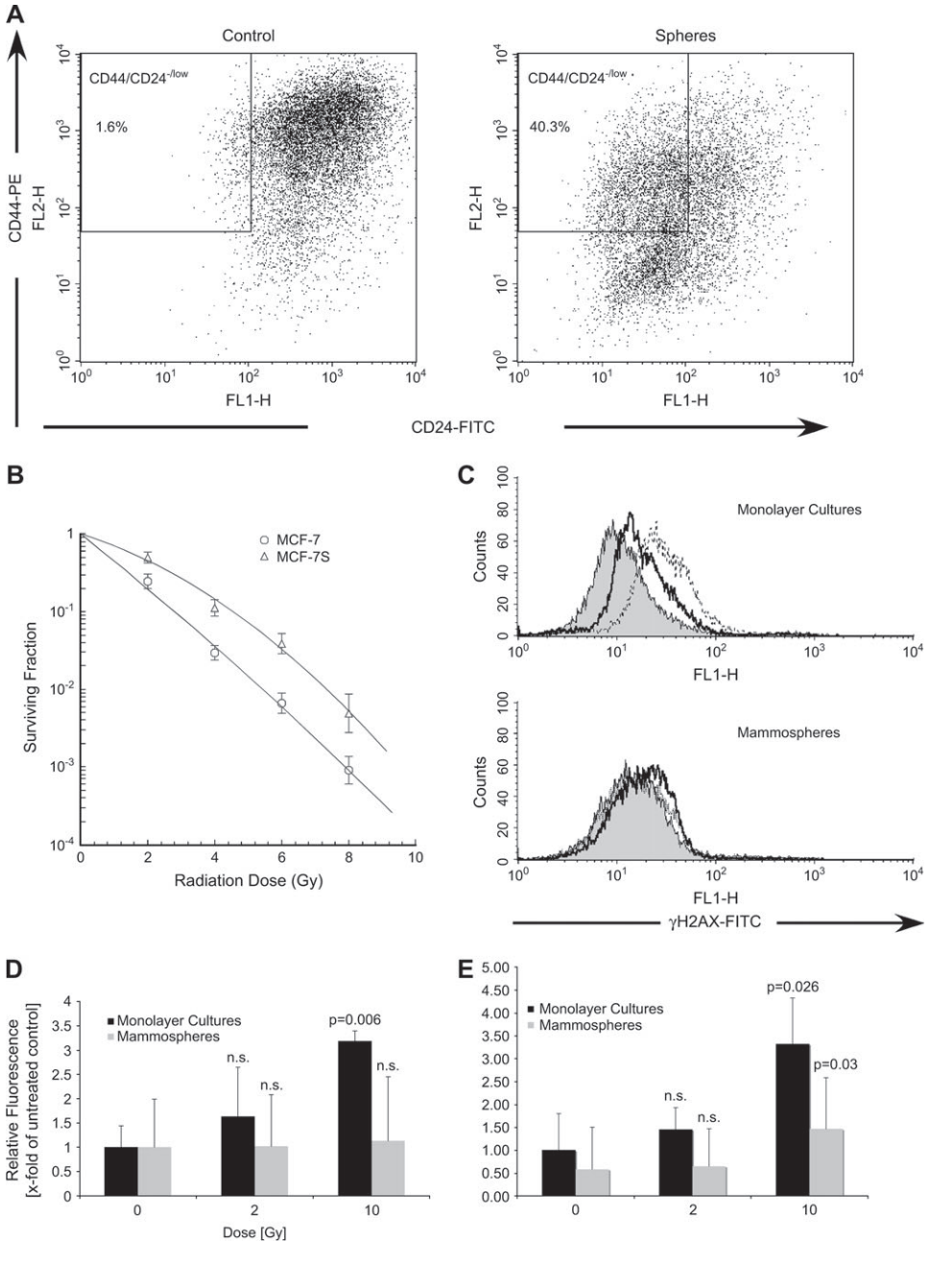

Fig. 1. Radiation response of cells in monolayer culture and mammospheres. **A**) Fluorescence-activated cell-sorting (FACS) analysis to measure CD44 and CD24 expression of cells derived from MCF-7 monolayer cultures (**left**) or primary mammospheres (**right**). Cells were incubated with trypsin–EDTA (monolayer cultures) or dissociated mechanically (mammospheres) and incubated with a fluorescein isothiocyanate (FITC)–conjugated monoclonal mouse anti-human CD24 and a phytoerythrin-conjugated monoclonal mouse anti-human CD44 antibody. After 20 minutes, cells were washed with phosphate-buffered saline and analyzed on a flow cytometer. Data from one of 10 experiments are shown. **B**) Clonogenic survival assay of cells derived from MCF-7 monolayer cultures (MCF-7) or 5-day-old mammospheres (MCF-7S). Cells were irradiated as single-cell suspensions and plated to allow colony formation. After 28 days, cells were fixed and stained with crystal violet, and colonies consisting of more than 50 cells were counted for each dose point. To determine surviving fractions, counts were normalized using the plating efficiency of the unirradiated corresponding control. Means and 95% confidence intervals are shown for three experiments, each performed in triplicate (n = 9). $P = .026$ difference at 2-Gy dose using a paired two-sided Student's t test. **C**) H2AX phosphorylation (γH2AX) was measured in MCF-7 cells derived from monolayer cultures or mammospheres using flow cytometry and a specific FITC-conjugated anti-γH2AX antibody. Single-cell suspensions were exposed to 0 Gy and harvested immediately (**filled histogram**) or to 2 Gy and harvested 5 minutes (**solid line**) or 60 minutes (**dashed line**) after exposure. Data from one of two independent experiments are shown. **D**) γH2AX levels in MCF-7 monolayer cultures (**black**) or mammospheres (**gray**) 60 minutes after exposure to 0, 2, or 10 Gy. Means and 95% confidence intervals of three independent experiments (n = 3) are shown. Relative fluorescence was calculated by normalizing all data to the fluorescence of corresponding unirradiated control cells. P values comparing data from experimental and corresponding control samples were determined using a paired two-sided Student's t test. n.s. = not statistically significant. **E**) Free radical formation in MCF-7 cells derived from monolayer cultures (**black**) and mammospheres (**gray**) after exposure to 0, 2, or 10 Gy as measured by flow cytometry. Cells were incubated with aminophenyl fluorescein at 10 μM concentration for 30 minutes to allow the fluorogenic dye entering into the cells. Cells were then irradiated, and fluorescence was immediately assessed in a fluorescence plate reader (excitation: 480 nm, emission: 520 nm). Relative fluorescence was calculated by normalizing all data to the fluorescence of unirradiated control cells. Cells were irradiated as single-cell suspensions. Means and 95% confidence intervals of three independent experiments performed in triplicate (n = 9) are shown. P values comparing data from experimental and corresponding control samples were determined using a paired two-sided Student's t test. n.s. = not statistically significant.

monolayer cultures consistently showed dose-dependent formation of ROS (0 Gy, mean = 1.0, 2 Gy, mean = 1.45, difference = 0.45, not statistically significant; 10 Gy, mean = 3.32, difference = 2.32, 95% CI = 0.67 to 3.98; $P = .026$, n = 3; Fig. 1, E). Cells derived from primary mammospheres did so as well, but at levels that were approximately 50% of those formed by cells from monolayer cultures (0 Gy, mean = 0.58, 2 Gy, mean = 0.647, difference = 0.067, not statistically significant; 10 Gy, mean = 1.46, difference = 0.88, 95% CI = 0.20 to 1.56; $P = .031$, n = 3; Fig. 1, E).

Because EGF and bFGF, which were used to generate mammospheres, both decrease the radiation sensitivity of cells *(27,28)*, independent of the presence of $CD24^{-/low}/CD44^{+}$ cancer-initiating cells, MCF-7 and MDA-MB-231 cells were cultured as monolayers for 5 days in MEM or DMEM (10% FCS), supplemented with EGF and bFGF. As expected, cells derived from EGF- and bFGF-treated monolayer cultures exhibited increased radiation resistance that was similar to that of cells derived from primary mammospheres (Fig. 2, A and B), which could be ascribed to the increased percentage of $CD24^{-/low}/CD44^{+}$ cancer-initiating cells in the nonadherent fraction (i.e., supernatant) of monolayer cultures after cytokine treatment (Fig. 2, C). To further exclude an acute direct radioprotective effect of EGF and bFGF, we exposed MCF-7 monolayer cultures to growth medium supplemented with or without EGF and bFGF

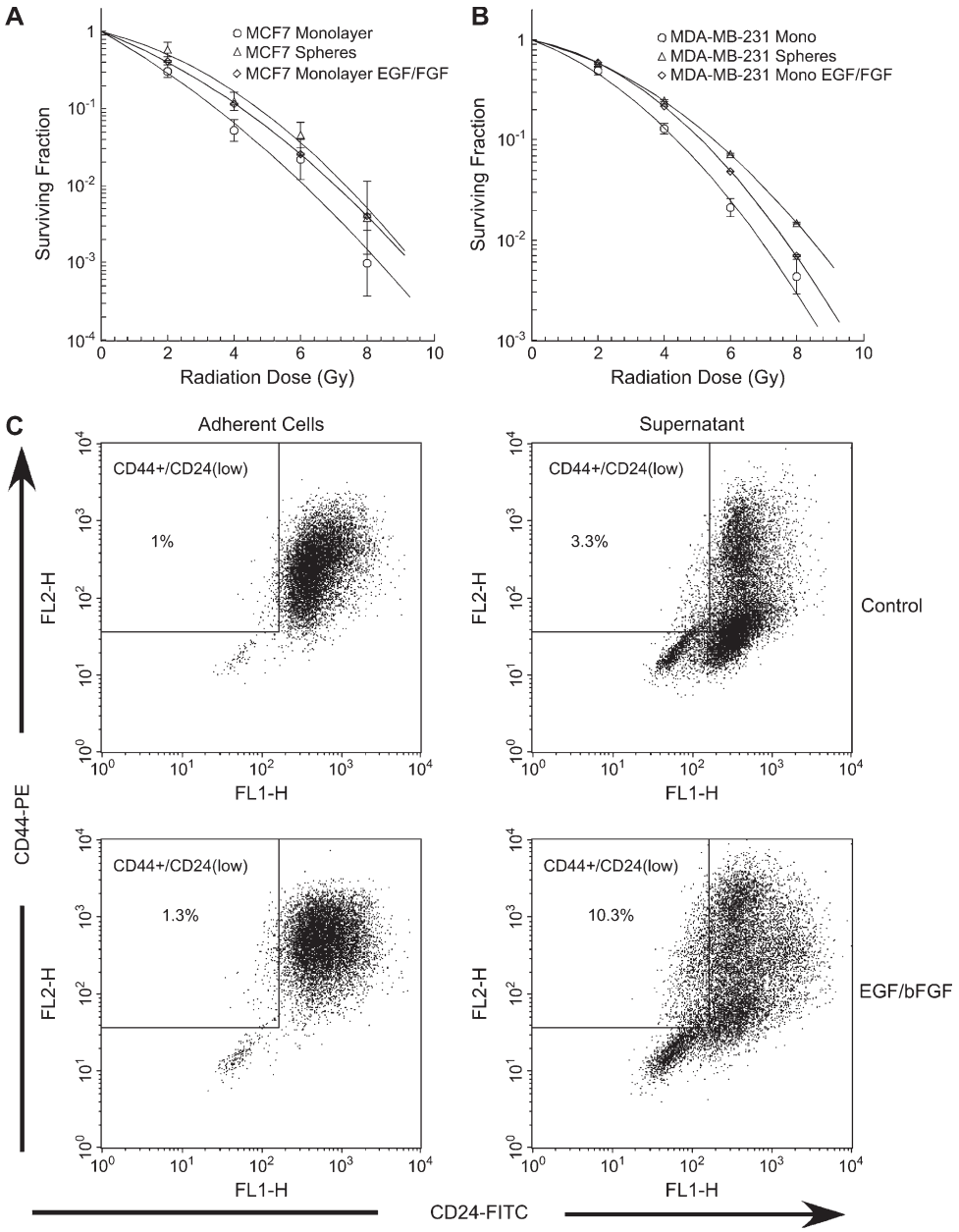

Fig. 2. Effects of epidermal growth factor (EGF)/basic fibroblast growth factor (bFGF) treatment on breast cancer cells. Clonogenic survival assays using **A**) MCF-7 and **B**) MDA-MB-231 monolayer cultures treated with EGF (10 ng/mL) and bFGF (20 ng/mL) for 5 days. Cells were irradiated as single-cell suspensions and plated to allow colony formation. After 28 days, cells were fixed and stained, and colonies consisting of more than 50 cells were counted for each dose point. To determine surviving fractions, counts were normalized using the plating efficiency of the unirradiated corresponding control. Means and 95% confidence intervals from three independent experiments are shown (n = 3). **C**) Fluorescence-activated cell-sorting (FACS) analysis of MCF-7 monolayer cultures treated with EGF and bFGF for 5 days. Cells were incubated with trypsin–EDTA and incubated with a fluorescein isothiocyanate–conjugated monoclonal mouse anti-human CD24 and a phytoerythrin-conjugated monoclonal mouse anti-human CD44 antibody. After 20 minutes, cells were washed with phosphate-buffered saline and analyzed on a flow cytometer. Data from one of six experiments are shown.

for 0.5, 1, 2, 3, 4, or 6 hours after irradiation with 4 or 6 Gy (n = 2). Cells were then washed twice with PBS, incubated with trypsin–EDTA and plated into 96-well plates at 2000, 10 000, 15 000, or 20 000 cells per well. Although cytokine treatment increased the viability of the cells as assessed by MTT assays on day 7, we could not detect a radioprotective effect of the EGF/bFGF treatment (data not shown).

Response of $CD24^{-/low}/CD44^{+}$ Breast Cancer Cells to Fractionated Radiation

To determine whether the $CD24^{-/low}/CD44^{+}$-enriched cancer-initiating cells were truly more radioresistant than their non–$CD24^{-/low}/CD44^{+}$-enriched monolayer cell counterparts, or if they might even increase in numbers after clinical fractions of radiation (accelerated repopulation), monolayer cultures of MCF-7 cells were irradiated with either a single dose of 10 Gy on day 6 or five daily doses of 3 Gy on days 2–6. When unirradiated cells were analyzed for CD24 and CD44 expression on day 8, the size of the $CD24^{-/low}/CD44^{+}$ population of cells in the nonadherent fraction was, as expected, higher than that of the adherent cells (mean = 3.52% versus mean = 0.86%, difference = 2.66%, 95% CI = 0.63 to 4.7; n = 5, $P = .02$; Fig. 3, A and B). For monolayer cultures, the percentages of $CD24^{-/low}/CD44^{+}$ cells on day 8 in the adherent and nonadherent cell populations were not altered by a single dose of 10 Gy given on day 6. In addition, after five fractions of 3 Gy, the percentage of $CD24^{-/low}/CD44^{+}$ cells in the adherent cell population did not change (Fig. 3, A and B); however, the percentage of $CD24^{-/low}/CD44^{+}$ cells in the supernatant (nonadherent cells) increased (untreated, mean = 3.52%, versus treated, mean = 7.5%, difference = 3.98%, 95% CI = 1.67% to 6.25%; n = 5, $P = .009$; Fig. 3, A and B).

To further explore the biologic relevance of the increase of the proportion of $CD24^{-/low}/CD44^{+}$ cells after fractionated

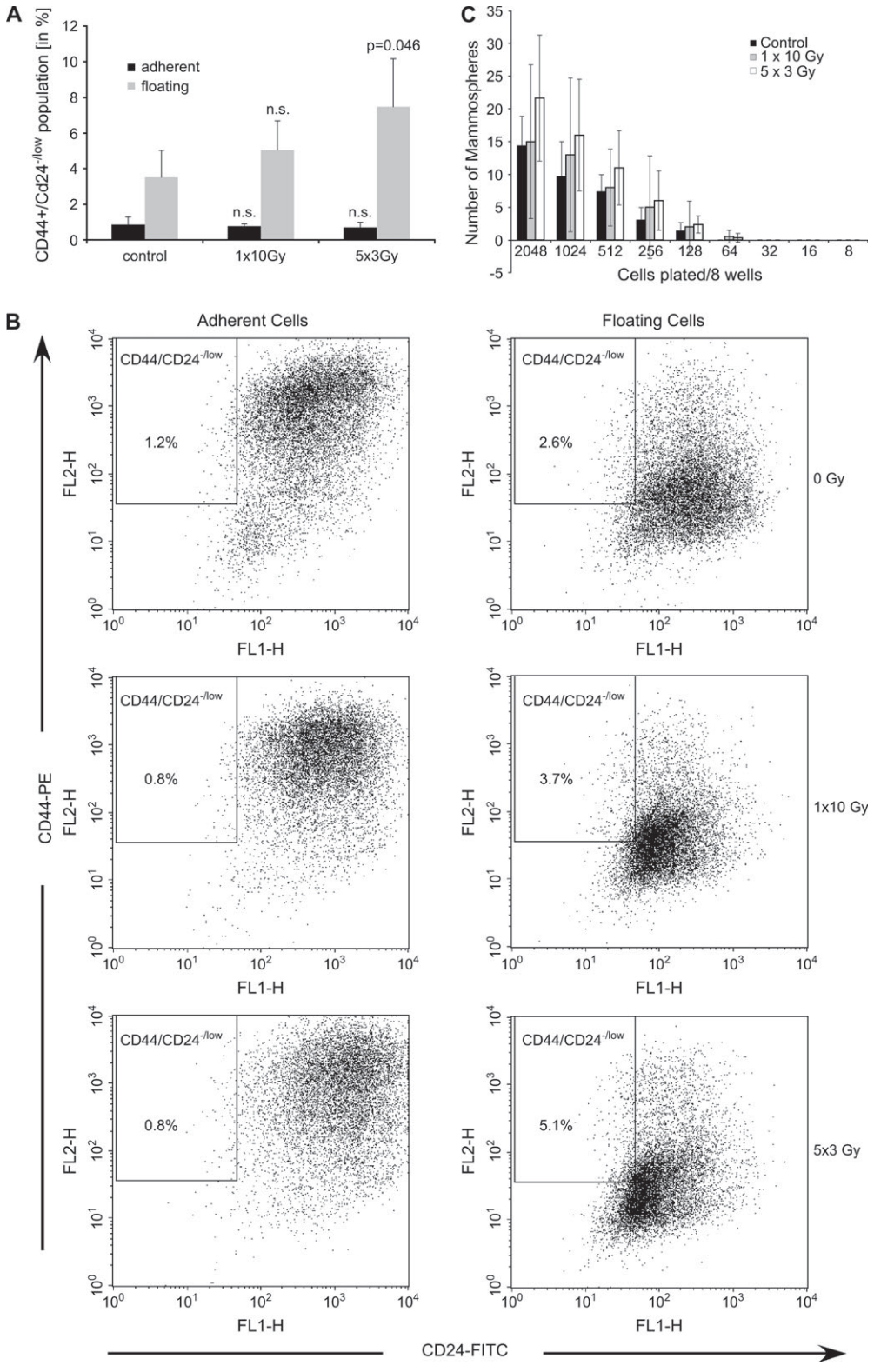

Fig. 3. Effect of fractionated irradiation on breast cancer cells. **A**) Fluorescence-activated cell-sorting (FACS) analysis was used to measure percentages of cells with $CD44^{+}/CD24^{-/low}$ in adherent (**black**) and nonadherent (floating, **gray**) monolayer MCF-7 cell cultures. Cells were treated with a single dose of 10 Gy or with five daily doses of 3 Gy. Cells were incubated with trypsin–EDTA (adherent cells) or harvested (floating cells) 48 hours after the last irradiation followed by incubation with a fluorescein isothiocyanate–conjugated monoclonal mouse anti-human CD24 and a phytoerythrin-conjugated monoclonal mouse anti-human CD44 antibody. After 20 minutes, cells were washed with phosphate-buffered saline and analyzed on a flow cytometer. Means and 95% confidence intervals are shown from four (10 Gy) and five (5 × 3 Gy) independent experiments. *P* values were determined using the two-sided Student's *t* test. **B**) Data from one experiment of each group of cells in (**A**). **C**) Primary mammosphere formation assay of nonadherent MCF-7 cells that were plated at increasing dilutions and treated with 0 Gy, a single dose of 10 Gy, or five daily doses of 3 Gy. Cells were plated at a starting density of 256 cells per well into 96-well plates in serum-free Dulbecco's modified Eagle medium–F12 media supplemented with bovine serum albumin, epidermal growth factor, and basic fibroblast growth factor and subsequently diluted (1 : 1). Mammosphere counts reflect the total number of spheres in eight wells for each dilution. Mammospheres were counted on day 5. Means and 95% confidence intervals of three independent experiments are shown.

irradiation, we performed a primary mammosphere formation assay, which allows estimation of the number of breast cancer cells that exhibit self-renewal capacity *(7)*. Primary mammosphere formation by nonadherent cells from cultures irradiated with a single dose of 10 Gy was similar to that of unirradiated control cultures. (Fig. 3, C). However, primary mammosphere formation capacity was increased in nonadherent populations from cultures that received five fractions of 3 Gy, although this increase did not reach statistical significance (Fig. 3, C).

Fractionated Irradiation and the Notch-1 Pathway

Self-renewal and lineage differentiation in normal mammary stem cells is regulated by the developmental Notch signal

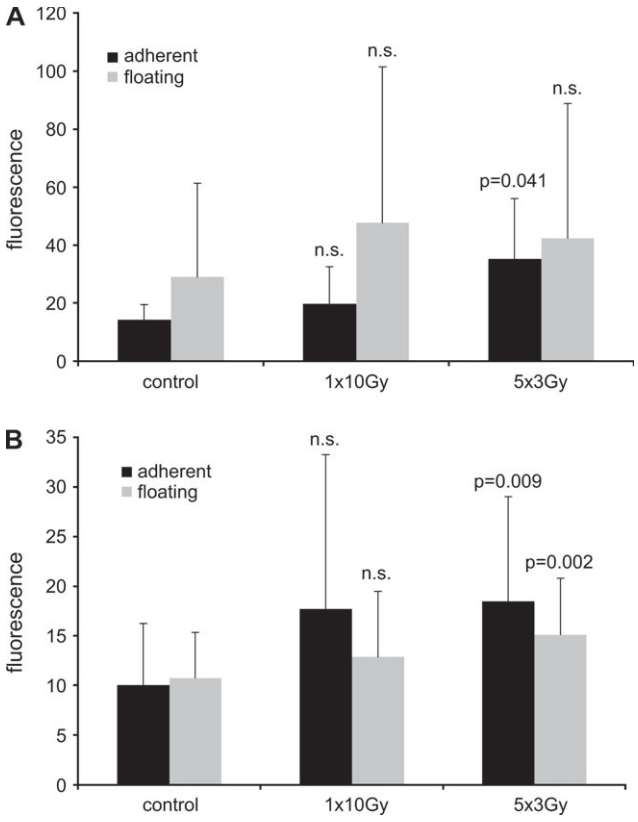

Fig. 4. Flow cytometry analysis of Jagged-1 expression and Notch-1 activation in adherent and nonadherent MCF-7 cells after irradiation. Monolayer cultures were exposed to five fractions of 5 Gy on days 2–6 or a single fraction of 10 Gy on day 6. Nonadherent (floating) cells were harvested 48 hours later, and adherent cells were incubated with trypsin–EDTA. Cells were fixed and stained with a fluorescein isothiocyanate–conjugated monoclonal mouse anti-human CD24, a phytoerythrin/Cy5-conjugated monoclonal mouse anti-human CD44 antibody, and either a polyclonal rabbit anti-human Jagged-1 antibody (**A**) or a polyclonal rabbit anti-human Notch-1-intracellular domain antibody (**B**). Means and 95% confidence intervals are shown from seven (adherent cells 0 and 5 × 3 Gy), five (floating cells 0 and 5 × 3 Gy), or three (1 × 10 Gy) independent experiments. *P* values were determined using the two-sided Student's *t* test.

transduction pathway *(29)*, which may also be involved in cancer stem cell self-renewal. As measured by flow cytometric analysis, adherent MCF-7 cells expressed detectable levels of Jagged-1 that increased after fractionated irradiation (5 × 3 Gy) on day 8 (untreated, mean = 14.2, versus treated, mean = 35.3, difference = 21.1, 95% CI = 1.23 to 41.0; n = 7, $P = .04$). Jagged-1 expression in nonadherent cells was slightly greater than that of adherent cells but did not statistically significantly change (Fig. 4, A). No increase in Jagged-1 expression was observed in either cell population after a single 10-Gy dose. Activated Notch-1, as measured by levels of intracellular Notch-1-ICD, increased after radiation in both adherent and nonadherent populations after five fractions of 3 Gy (adherent cells, mean = 10.0 versus mean = 18.5, difference = 8.48, 95% CI = 3.0 to 13.9; n = 7, $P = .009$, and nonadherent cells, mean = 10.7 versus mean = 15.1, difference = 4.4, 95% CI = 2.7 to 6.1; n = 5, $P = .002$) but not after a single dose of 10 Gy (Fig. 4, B). The increase in Jagged-1 expression and Notch activation (Notch-ICD) was not observed in the population of $CD24^{-/low}/CD44^{+}$ cells. However, the percentage of $CD24^{-/low}/CD44^{+}$ cells was low in the supernatant and even lower in the adherent population. Radiation increased the number of cells in this population by twofold in the supernatant, but the fraction was still less than 10% in both populations. Therefore, the histograms for Jagged-1 and Notch-ICD of the gated populations were statistically unreliable.

Discussion

Using previously published techniques *(2,7)* and MCF-7 and MDA-MB-231 breast cancer cell lines, we isolated and propagated populations of cells that contain potential breast cancer stem cells and are tumor initiating *(2,7)*. We investigated the response of this cell population to both a single dose and a 5-day course of radiation and found that $CD24^{-/low}/CD44^{+}$-enriched cancer-initiating cells were more resistant to radiation than cells in monolayer culture. During a fractionated course of radiation, the number of breast cancer–initiating cells increased. This increase was accompanied by radiation-induced Jagged-1 expression and subsequent activation of Notch-1, suggesting that radiation activates this developmental pathway.

We observed that the plating efficiency of cells that were derived from mammospheres was similar to that of cells derived from monolayer cultures and was much higher than the frequency of primary mammosphere formation. One possible interpretation is that mammospheres are not exclusively formed by breast cancer stem cells but also contain early progenitor cells. Such a situation would support the use of the term breast cancer–initiating cells for this population rather than breast cancer stem cells. Alternatively, breast cancer stem cells could be the only population capable of forming mammospheres but could also give rise to non–stem cells within the mammosphere. Although non–stem cells would be incapable of mammosphere formation and thus self-renewal, they would still be considered as clonogenic in clonogenic survival assays.

We found that breast cancer–initiating cells were more radioresistant than non–breast cancer initiating cells. Interestingly, the radiation survival curve of cells derived from mammospheres had a shoulder that is characterized by a comparably higher resistance at lower and thus clinically more relevant doses of radiation. This shoulder indicates an enhanced capacity to repair potentially lethal damage. Resistance to apoptotic stimuli, including radiotherapy, was recently reported for nonproliferating $CD34^{+}$ chronic myeloid leukemia progenitor cells when compared with normal $CD34^{+}$ cells *(30)*.

Consistent with the increased radioresistance, treatment with ionizing radiation caused lower levels of ROS in cells derived from mammospheres compared with cells derived from monolayer cultures. This decrease in ROS levels indicated high intracellular levels of radical scavengers. Although cells in primary mammospheres may exhibit certain levels of hypoxia, as reported for spheroid cultures of tumor cells *(31)*, the absence of oxygen did not account for the observed effect in our experiments because cells derived both from monolayer cultures and mammospheres were irradiated as single-cell suspensions.

To our knowledge, this is the first study to directly investigate the radiation resistance of breast cancer–initiating cells. Three-dimensional in vitro culturing techniques for tumor cells have been used previously using nonselective serum-containing conditions that caused cells to aggregate. These tumor spheroids also exhibit resistance to radiation *(32)* and chemotherapeutic drugs

and have enhanced colony-forming efficiency *(33)*. The mechanisms leading to increased resistance and enhanced colony-forming efficiency were incompletely understood. However, given the size of these aggregates and the gradient of cytokine concentrations and nutrients from the periphery to the center of these spheroids, the previous techniques may have also selected for therapy-resistant cancer stem cells.

Our observation of increased radiation resistance in the cancer-initiating cell population most likely underestimates the resistance of breast cancer stem cells because the cycling population of progenitor cells present in the mammospheres are not necessarily as resistant as breast cancer stem cells. However, these results indicate that therapies that specifically target pathways that are deregulated in breast cancer stem cells may enhance the efficiency of radiotherapy in the future. In addition, these findings may have an impact on future design of predictive assays for drug or radiation sensitivity because the therapeutic response of cancer stem cells may not be reflected by the response of an unselected tumor cell population.

Using an in vitro system, we mimicked a week of clinical fractionated radiotherapy followed by a typical weekend gap of 2 days. This treatment increased the proportion of breast cancer–initiating cells. The observation that the increase in cell number was observed in the supernatant (i.e., nonadherent cells) of confluent monolayer cultures but not in adherent cells and the fact that these cells formed primary mammospheres at a higher rate than untreated cells indicate that the increase in the proportion of cancer-initiating cells was not caused by simple selection of a radioresistant subpopulation but by an absolute increase in the number of viable breast cancer–initiating cells with increased capacity for self-renewal.

Activation of the developmental Notch-1 signal transduction pathway promotes self-renewal of early progenitor cells derived from normal mammary stem cells *(29)*. In the present study, we investigated whether ionizing radiation interfered with the Notch-1 signaling pathway directly. Our observation that radiation induced Jagged-1 expression on the surface of cells from monolayer cultures and activated Notch-1 in cells in the supernatant is the first demonstration, to our knowledge, of an acute radiation effect on this developmental signaling pathway. Future studies will be necessary to define the population of cells in which this pathway is targeted by radiation.

The study has several limitations. Breast cancer stem cells have not yet been identified directly, although they can be enriched for and propagated in vitro with the techniques we used in this study. Still, thes e enriched populations are heterogeneous, and the results of our study may therefore actually underestimate the differences between breast cancer stem cells and non–stem cells. The number of different breast cancer cell lines used limits the conclusions for clinical radiotherapy that can be drawn from our study. Selection for a specific phenotype may have occurred during the establishment and maintenance of these lines, and thus, these cells may not accurately reflect the behavior of breast cancer cells in human tumors. Thus, our data need validation on mammospheres derived directly from patients' tumor specimens.

Taken together, our data indicate that breast cancer–initiating cells exhibit increased radiation resistance resulting from decreased ROS induction, followed by decreased double-strand break formation. Additionally, fractionated irradiation appeared to activate the Notch-1 developmental pathway, which may have caused the numbers of breast cancer–initiating cells to increase, offering a mechanism for accelerated repopulation during radiation therapy treatment gaps.

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NOTES

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