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An Observed Effect of p53 Status on the Bystander Response to Radiation-Induced Cellular Photon Emission

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In this study, we investigated the potential influence of p53 on ultraviolet (UV) signal generation and response of bystander cells to the UV signals generated by beta-irradiated cells. Five cell lines of various p53 status (HaCaT, mutated; SW48, wild-type; HT29, mutated; HCT116$^{+/+}$, wild-type; HCT116$^{-/-}$, null) were irradiated with beta particles from tritium. Signal generation (photon emission at 340 ± 5 nm) was quantified from irradiated cells using a photo-multiplier tube. Bystander response (clonogenic survival) was assessed by placing reporter cell flasks directly superior to irradiated signal-emitting cells. All cell lines emitted significant quantities of UV after tritium exposure. The magnitudes of HaCaT and HT29 photon emission at 340 nm were similar to each other while they were significantly different from the stronger signals emitted from SW48, HCT116$^{+/+}$ and HCT116$^{-/-}$ cells. In regard to the bystander responses, HaCaT, HCT116$^{+/+}$ and SW48 cells demonstrated significant reductions in survival as a result of exposure to emission signals. HCT116$^{-/-}$ and HT29 cells did not exhibit any changes in survival and thus were considered to be lacking the mechanisms or functions required to elicit a response. The survival response was found not to correlate with the observed signal strength for all experimental permutations; this may be attributed to varying emission spectra from cell line to cell line or differences in response sensitivity. Overall, these results suggest that the UV-mediated bystander response is influenced by the p53 status of the cell line. Wild-type p53 cells (HCT116$^{+/+}$ and SW48) demonstrated significant responses to UV signals whereas the p53-null cell line (HCT116$^{-/-}$) lacked any response. The two mutated p53 cell lines exhibited contrasting responses, which may be explained by unique modulation of functions by different point mutations. The reduced response (cell death) exhibited by p53-mutated cells compared to p53 wild-type cells suggests a possible role of the assessed p53 mutations in radiation-induced cancer susceptibility and reduced efficacy of radiation-directed therapy.

INTRODUCTION

Biophoton emission is a phenomenon that has been reported extensively in the literature (*1–7*). Photon emission from biological matter can occur both spontaneously (*8*) and as a result of stressors such as visible light (*9*), ultraviolet (UV) light (*10, 11*) and chemicals (*4, 7*). The spectrum of observed emission extends from the UV range (*1, 6, 12*) to the visible wavelength range (*2, 4, 10*). Furthermore, in a recently published study, we showed that these electromagnetic photons emitted as a result of exposure to beta radiation, particularly UV radiation, may be a physical signal that mediates the bystander effect (*13*). Radiation-induced bystander effect (RIBE) has been widely studied but only in the context of media-borne signals via media transfer and co-culture techniques. To the best of our knowledge, the role of UV radiation as a physical signal for bystander communication is novel to the field of RIBE study. Although this is a novel idea in the ionizing radiation field, there is published evidence supporting intercellular communication of an electromagnetic nature between a virus-infected cell culture and bystander cultures (*12*). This evidence of intercellular communication by means of electromagnetic photons has prompted an investigation of the mechanisms driving signal generation and response.

In the current study, a potential role for p53 in mediating the UV-induced bystander effect was investigated. The p53 protein, also called tumor protein 53 (TP53) or tumor suppressor 53, is an intracellular protein that is crucial for regulating the cell cycle (*14–16*), initiating repair processes and promoting death pathways, such as apoptosis (*17, 18*), in response to DNA damage (*19*). The p53 activity is regulated by phosphorylation at multiple sites (*20*), whereby site-specific phosphorylation affects different aspects of function. The p53 protein becomes phosphorylated at Ser15 and 20 in response to DNA damage (*21*), which then functions to impair the binding of p53-negative regulator,

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MDM2, to p53 proteins (*21, 22*). Because MDM2 functions to ubiquinate and degrade p53 proteasomes (*23, 24*), the interference between MDM2 and p53 interaction by phosphorylation effectively promotes the activation of p53 and promotes p53 protein function. Phosphorylation at Ser46 is specific for influencing apoptosis induction (*25*) and phosphorylation at Ser392 affects binding of p53 to DNA (*26*) and transcriptional activation (*27*). Due to its crucial role in eliciting cellular responses to stress and genomic damage, it is referred to as the ''guardian of the genome''. The expression of p53 is mediated by the TP53 gene found on chromosome 17.

The idea that p53 has a role in the UV-induced bystander effect stems from a robust amount of data that shows the effect of p53 after media-borne bystander effect propagation. Research has demonstrated dependence of bystander signal generation by the p53 status of cells (*28, 29*). In a published study by He *et al*., HepG2 cells expressing wild-type p53 function exhibited a p53-dependent release of cytochrome-c in response to gamma radiation, thereby inducing micronuclei formation in wild-type Chang liver bystander cells (*28*). In contrast, the cells possessing mutated p53 or those that were p53 null did not exhibit a release of cytochrome-c after irradiation and micronuclei were not generated in bystander cells. Komarova *et al*. suggested a dependence of bystander signal generation upon p53, while reporting that bystander signal response was independent of p53 status (*29*). Using both the co-culture and media transfer techniques, Komarova and colleagues identified a p53-dependent release of growth-inhibitory factors from directly irradiated cells. In terms of bystander response, it was found that responses were exhibited even by p53-deficient cells.

There is also evidence to support that the dependence of the p53 media-borne bystander effects is not only limited to signal generation, but extends to the bystander response as well (*30, 31*). Mothersill *et al*. were able to demonstrate a lack of response by the p53-null cell line, HCT116$^{-/-}$ (*30*). In contrast, its p53 wild-type counterpart and the p53-mutated HPV-G cell line demonstrated responses to irradiated cell conditioned media transferred via media transfer technique. Tomita *et al*. also showed that wild-type p53 human non-small cell lung cancer cells exhibited great amounts of cell death at doses below 0.45 Gy, whereas p53-mutated cells of the same origin exhibited even greater cell death at doses below and also exceeding 0.45 Gy (*31*). Following evidence for a role of p53 in the media-borne radiation-induced bystander effect, it is the goal of the current study to investigate the potential influence of p53 on signal transduction and response in the context of the UV-mediated bystander system.

The link between UV-generated bystander effects and p53 is suggested based on the observation that UV radiation, characterized by wavelengths ranging from 100–400 nm, is carcinogenic to humans (*32*) and that p53 has been demonstrated as an important factor in protecting against UV-induced carcinogenesis (*33*). Jiang and colleagues reported UV-induced cancer susceptibility in mice possessing p53 gene heterozygosity and even greater susceptibility in those that possessed homozygous p53 gene knockouts. The aberrant and/or absent p53 functionality in these mice can explain the compromised ability for p53 to effectively activate protective processes such as programmed cell death (*34*) and cell cycle arrest, which subsequently facilitates DNA damage repair (*35*).

For this study, multiple cell lines possessing various p53 statuses were used to investigate UV signal generation and cellular response to those UV signals. A photosensitizer, lomefloxacin hydrochloride, was also utilized to amplify the effects of potentially weak, yet present, responses to UV photons.

MATERIALS AND METHODS

Cell Lines

Five cell lines possessing various p53 statuses were chosen for this study: HaCaT, HCT116$^{+/+}$ (p53 wild-type), HCT116$^{-/-}$ (p53 null), SW48, HT29. Immortalized, nontransformed human keratinocyte cell line, HaCaT, was chosen because of its proven generation of and response to UV-photon emission generated by beta-irradiated cells (*13*). The HaCaT cell line is p53 mutated, where it possesses point mutations His179Tyr, Asp281Gly, Arg282Trp on both of its alleles (*36*).

HCT116$^{+/+}$ and HCT116$^{-/-}$ cells, kindly provided by Dr. Robert Bristow (University Health Network, University of Toronto), are human colon carcinoma cell lines that possess wild-type and null p53 status, respectively. These cell lines were chosen since they are a suitable model system for studying the dependence of the UV-induced bystander signal and response on p53 status. Bunz *et al*. derived the HCT116$^{-/-}$ cell line by transfecting HCT116$^{+/+}$ cells with targeting vectors to facilitate the incorporation of an alternative codon in the place of the TP53 start codon (exon 2) (*37*). In this respect, the TP53 sequence is largely retained, yet protein production is disabled due to the lack of RNA transcript translation subsequent to start codon modification.

SW48 is a human colon carcinoma cell line that possesses wild-type p53. This cell line was chosen to investigate potential differences in signal generation and response in two different wild-type p53 cell lines. HT29 is a human colon carcinoma cell line with mutated p53. These cells possess a point mutation at codon 273 (Arg273His) (*38*).

Cell Culture

HaCaT, HCT116$^{+/+}$, SW48 and HT29 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS, 2 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulphate. HCT116$^{-/-}$ cells were cultured in McCoy's 5A modified medium supplemented with 10% FBS, 2 m*M* L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulphate. Reagents were obtained from Gibco®/Life Technologies (Grand Island, NY) unless otherwise specified. Cell cultures were tested and confirmed to be free of mycoplasma prior to experimentation (cat. no. rep-pt1; InVivoGen, San Diego, CA).

Cultures were incubated at 95% humidified air and 5% CO_2 at 37°C and received full volume media renewals every 2–3 days. Adherent monolayers were dissociated from flask substrates using a 1:1 solution of 0.25% trypsin and 1 m*M* ethylenediaminetetraacetic acid (EDTA). Cells were incubated with Trypsin-EDTA solution for 3–8 min,

depending on cell line. Neutralization was achieved by adding equal or greater volumes of culture media to the trypsinized stock.

Cells were seeded into 100 mm petri dishes at a density of 2,000 cells/cm^2 (157,080 total cells per 5 ml of cell culture media in a petri dish) for the purpose of photon quantification from directly irradiated cells. Cells were seeded into 25 cm^2 flasks at clonogenic densities (20 cells/cm^2, 500 cells per 5 ml of media in a flask) for the purpose of determining clonogenic survival for bystander cells or directly irradiated cells.

Direct Beta Irradiation

The beta emitter tritium (^{3}H) was used for cell culture irradiation. Tritium is a beta emitter that does not have gamma emission associated with its decay. Tritium electrons possess a maximum beta energy of 18.6 keV and average beta energy of 5.7 keV. The half-life of ^{3}H is 12.28 years, thus decay was considered negligible when dose calculations were determined.

Cells that were to be directly irradiated by beta-emitter tritium (^{3}H) were plated at a density of 2,000 cells/cm^2 in 100 mm petri dishes as described above, and appropriate volumes of tritiated water (PerkinElmer®, Boston, MA) were pipetted into the cell culture media 6 h after cell seeding to achieve the desired dose (specific activity: 1 μCi/μl). Cells were exposed to 85.7, 171.5 and 857.5 μCi of ^{3}H for 24 h to achieve total doses of 0.05, 0.1 and 0.5 Gy delivered by tritium, respectively. Because the half-life of tritium is 12.28 years, the radioactive source would have only decayed to 99.98% of its original activity over a 24 h period. For this reason, decay of the radioactive source was considered negligible and the dose rates were thus considered constant over the duration of exposure. The dose rates for the 85.7, 171.5 and 857.5 μCi sources were therefore 34.72, 69.44 and 347.2 μGy/min (0.347 mGy/min), respectively. A nonirradiated (sham) control was also included. Each of the five cell lines used was directly irradiated with ^{3}H. Directly irradiated cells were either used in bystander experiments as the source of the UV-induced bystander signal or used in photon quantification experiments or assessed directly for clonogenic survival after direct beta irradiation.

Photon Quantification

Photon emission was quantified individually from all five cell lines when each cell line was directly irradiated with three doses of ^{3}H. Photon quantification was accomplished using a Hamamatsu Photonics (Bridgewater, NJ) R7400P single-photon counting photomultiplier tube (PMT) fitted with an interference type band pass optical filter centered at 340 ± 5 nm (Edmund Optics Inc., Barrington, NJ). The total area on the PMT that was sensitive to light was 113.1 mm^2. The PMT was supplied with –800 V of high voltage. Photon counting took place in a light-tight aluminum box where the irradiated cells, cell culture media and tritium were contained within a 100 mm petri dish placed at the bottom of the light-tight box. Photon emission from each dish was conducted for a total of 3 min per measurement and was measured with the 0.95 mm lid of the petri dish in place. The PMT was positioned superior to the dish with the lens approximately 40 mm away from the cell monolayer and the photocathode approximately 105 mm away from the cell monolayer. Photon quantification was performed within 1 min after the addition of tritium into the culture media of cells destined to be directly irradiated. Because there was no method by which we could inject the tritium into the cell culture while maintaining a light-tight seal in our system, the tritium was added to the cells first, then the petri dish was transferred into the light-tight box, and the high-voltage supply for the PMT was then turned on prior to photon counting.

When spectral emission induced by cellular irradiation of tritium was studied in our laboratory, we found that tritium irradiation of cells induces an increase in photon emission intensity across a wide range of wavelengths from the UV into the visible range (unpublished data). We consider the measurements taken over the 10 nm wavelength range (340 ± 5 nm) in the current study to be a sensitive marker representative of photon emission across a wavelength range from 200 to 1,100 nm.

Bystander Exposure to Signals Generated by Directly Irradiated Cells

Bystander cells were plated into 25 cm^2 flasks at a clonogenic density of 500 cells per flask. These flasks were placed superior to directly irradiated cells contained in the 100 mm petri plates immediately after the addition of tritium into the directly irradiated cell culture (6 h after seeding cells). The distance between the directly irradiated cell monolayer and the bystander cell monolayer was approximately 15 mm. Within this separation, 3 mm was constituted by media, 0.95 mm plastic and 11.05 mm air. Each petri dish and bystander/reporter flask pair was placed into a light-tight dark box and incubated at 37°C for 24 h. After incubation, the bystander cells were removed from the path of the UV-emitting directly irradiated cells and were incubated for another 6–9 days away from the UV-field emitted from directly irradiated cells. HaCaT reporter cells were exposed to signals emitted from all five ^{3}H-irradiated cell lines. SW48 and HT29 cells were exposed to signals emitted from ^{3}H-irradiated SW48, HT29 and HaCaT cells. HCT116 $^{+/+}$ cells and HCT116$^{-/-}$ cells were exposed to signals emitted from ^{3}H-irradiated HCT116$^{+/+}$, HCT116$^{-/-}$ and HaCaT cells. ^{3}H-beta particles are very low in energy such that their range does not exceed 7 μm in tissue (*39*). For this reason, these beta particles did not reach the bystander cell culture and any effects observed in the bystander cells were presumed to be attributed to the signals emitted from directly irradiated cells.

A control experiment was also performed, in which all five cell lines were exposed to the photon signals emitted from ^{3}H-irradiated cell culture media (5 ml volume) in petri dishes. The irradiated media and petri dishes did not contain any cells. This experiment was conducted to determine whether any bystander responses observed in reporter cells were attributed to the receipt of signals from the irradiated culture media or plasticware alone.

Photosensitizer Treatment

Another set of experiments was performed in which the physical experimental setup was the same as previously described. However, the photosensitizer, lomefloxacin hydrochloride (Sigma-Aldrich®, St. Louis, MO), was added to the cell culture media of the bystander/reporter cells immediately before exposing these cells to the UV signals emitted from the directly irradiated cells. The rationale for treating the bystander/reporter cells with lomefloxacin was to determine whether the cell killing effects seen in the bystander cells could be exacerbated by the presence of a sensitizer, which has previously been proven to stabilize p53. The effect of lomefloxacin on p53 manifests as the upregulation of p53 transcriptional activity and the subsequent accumulation of p53 proteins in response to UVA irradiation (*40*). It is hypothesized that cells possessing wild-type p53 would exhibit an increased cell death, from lomefloxacin with UV treatment, above the levels observed subsequent to UV exposure alone. In contrast, if the cell death response in p53 wild-type bystander cells was not modified by the presence of lomefloxacin during UV exposure, it can be suggested that p53 is not an influential factor in the modulation of the UV-mediated bystander effect.

Lomefloxacin (0.5 ml of 200 μ*M*) was added to 4.5 ml of cell culture media to produce a final concentration of 20 μ*M* lomefloxacin in a total volume of 5 ml. Because the lomefloxacin was dissolved in 0.776 μl of 1 *M* sodium hydroxide (NaOH) and 499.224 μl of distilled water, the clonogenic survival and the pH of the cell culture media with lomefloxacin solution was tested to ensure that the solution itself was not cytotoxic; lack of cytotoxicity was confirmed by a test conducted in our laboratory, as previously reported (*13*).

The lomefloxacin was removed from the bystander cell culture 24 h after incubation by discarding the lomefloxacin-containing cell culture media, washing the cells three times with 5 ml warm PBS and then

replacing the cell culture media with 5 ml pre-warmed cell culture media free of lomefloxacin. A lomefloxacin-free control was also used, whereby a sterile solution of 0.776 µl of 1 *M* NaOH and 499.224 µl distilled water was added to the cell culture media in place of the lomefloxacin and was washed out 24 h later, following the same protocol as described. The survival of this control did not differ significantly from the cells that were not treated with water and did not undergo washing (plating efficiencies).

Clonogenic Survival Assay

Clonogenic survival assay was performed to determine the response of bystander cells to signals emitted from directly irradiated cells or to determine the response of cells directly exposed to tritium beta radiation. At 24 h prior to seeding of cells for the clonogenic assay, 80–90% confluent flasks were given full-volume media changes. Cell monolayers were detached from 75 cm^2 flasks using a 1:1 solution of 0.25% w/v trypsin and 1 m*M* EDTA. The trypsinization process was neutralized using an equal or greater volume of culture media. Cell stock concentration was determined using a Beckman Coulter Z2 particle count and size analyzer (Beckman Coulter LP, Mississauga, Canada), which had been calibrated using a hemocytometer. Flasks plated with cells at clonogenic densities (500 cells per flask) were incubated for a total of 7–10 days until cells formed colonies. Flasks were stained with carbol fuchsin and colonies containing at least 50 cells were counted according to the clonogenic survival assay developed by Puck and Marcus (*41*).

Validation of p53 Functionality Using Western Blot Analysis

To validate the p53 functionality of each of the five cell lines employed in the current study, p53 (53 kDa) and p21 (21 kDa) protein expression was investigated using Western blot analysis. Protein was extracted from HeLa cells (positive control cell line) and both irradiated (0.5 Gy ^{3}H) and nonirradiated HaCaT, SW48, HT29, HCT116$^{+/+}$ and HCT116$^{-/-}$ cells. Protein was extracted using 500 µl of lysis buffer per sample and the protein concentration was subsequently determined using a BCA protein assay kit (cat. no. 23335; Thermo Fisher Scientific™, Waltham, MA). Protein (20 µg) was added into each well. SDS PAGE was performed using 12% 15-well polyacrylamide gels (Thermo Scientific) run at 150 V for 70 min at room temperature. Proteins were transferred from the gels to nitrocellulose membranes at 10 V (or 0.15 A) for 90 min in ice-cold buffer. After electrotransfer, blots were blocked in 5% milk-TBST solution for 60 min at room temperature followed by incubation of each blot with rabbit polyclonal anti-p53 primary antibody (cat. no. 9282; Cell Signaling Technology®, Danvers, MA) at a dilution of 1:1,000 or with rabbit monoclonal anti-p21 primary antibody (cat. no. ab109520; Abcam®, Cambridge, MA) at a 1:1,000 dilution overnight at 4°C. Incubation with a 1:5,000 solution of donkey anti-rabbit secondary antibody (peroxidase-linked) was performed for 60 min at room temperature. Detection was performed immediately after a 5 min incubation of each blot with 0.5 ml of enhanced chemiluminescence substrate (cat. no. 32109; Thermo Scientific). Chemiluminescence was detected using a ChemiDoc™ MP (Bio-Rad® Laboratories Inc., Hercules, CA) under colorimetric and chemiluminescent settings to acquire images of both the visible protein ladder and the protein bands of interest. Subsequent to p53 or p21 detection, the blots were washed in TBS and TBS-T, stripped with Restore stripping buffer (cat. no. 21059; Thermo Scientific), blocked and subsequently incubated with rabbit anti-actin primary antibody (cat. no. A5060; Sigma-Aldrich) diluted in 5% milk-TBST (1:1,000) for 1 h at room temperature. Secondary antibody incubation and ECL treatment were repeated and blots were subsequently imaged again to visualize protein bands corresponding to the loading control, actin (42 kDa).

Statistical Analysis

Analysis of variance (ANOVA) testing was performed to determine the significance of differences in clonogenic survival when cell line and dose were independent variables and clonogenic survival was the dependent variable. Post hoc analyses were performed using Tukey's honestly significant difference test. Independent *t* tests were conducted to analyze differences between survival for a given radiation dose in the absence and presence of the photosensitizer, lomefloxacin. Significance was determined at the 95% confidence level. Linear regression analyses were performed to determine the relationship between UV-photon signal and bystander cell survival. ANOVA analyses were performed using SPSS® Statistics version 17.0 (Chicago, IL) and linear fits were conducted using GraphPad Prism 6 (LaJolla, CA). Western blot protein band density was assessed using ImageJ software (NIH, Bethesda, MD) to produce numerical values representative of relative density for each band. These values were then assessed for statistical differences using SPSS Statistics version 17.0.

RESULTS

340 nm Photon Emission by Various Cell Lines

Photon emission was quantified from ^{3}H-irradiated cell cultures using a single-photon counting PMT and a band pass filter centered at 340 ± 5 nm. Figure 1 shows that HCT116 $^{+/+}$, HCT116$^{-/-}$ and SW48 cells demonstrated greater photon emission at all doses compared to HaCaT and HT29 cells. Since both HaCaT and HT29 cells are p53 mutated, a prima facie assessment would suggest that p53 status could be a predictor of photon emission strength. If this simple explanation were the case, it might be expected that HCT116$^{-/-}$ cells, which lack p53 function, would also exhibit lower levels of photon emission. However, we observed that the emission magnitude of HCT116$^{-/-}$ is comparable to that of p53 wild-type cell lines, HCT116 $^{+/+}$ and SW48. The simple hypothesis that p53 function is directly linked to UV emission does not appear to be valid. All of the studied cell lines were able to emit photons in the UV range, regardless of p53 status, but specific p53 mutations may be linked to an altered mechanism or reduction for UV signal generation subsequent to beta irradiation.

Bystander Response of Cell Set 1: HaCaT, HT29 and SW48 Cells

The control experiments, in which reporter cells were exposed to the scintillation of ^{3}H-irradiated cell culture media and plasticware (in the absence of cells) conferred nonsignificant reductions in bystander cell survival (Supplementary Fig. S1; http://dx.doi.org/10.1667/RR14342.1.S1). Bystander cell survival data that are subsequently reported reflect values that have not been corrected for background levels of survival reduction, since the reductions induced by control groups were found to be negligible.

Each of the cell lines tested demonstrated various responses to the UV signals emitted from the beta-irradiated cells. As shown in Fig. 2, p53-mutated HT29 reporter cells,

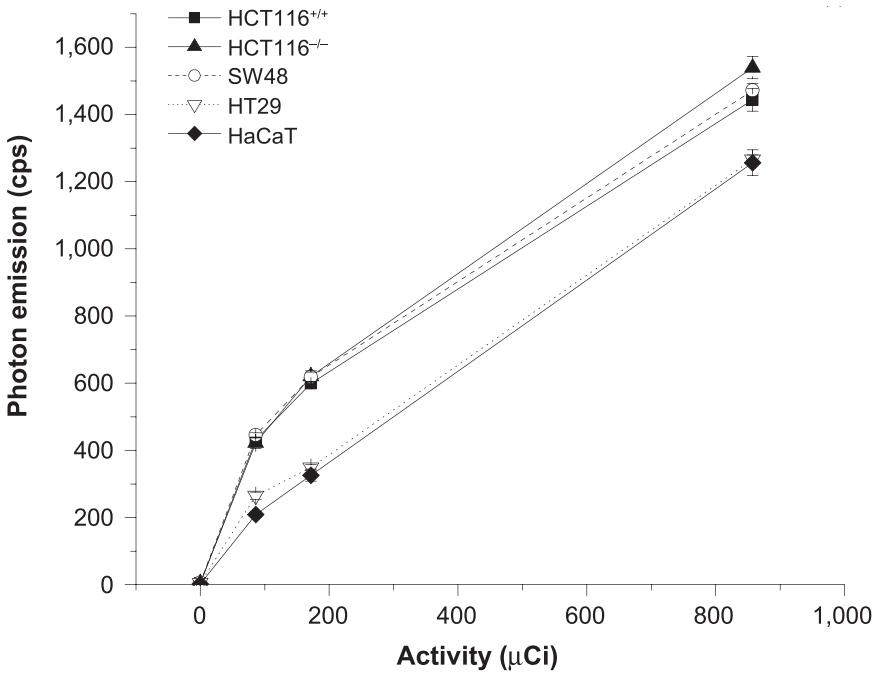

FIG. 1. Photon quantification at 340 ± 5 nm from HCT116$^{+/+}$, HCT116$^{-/-}$, SW48, HT29 and HaCaT cells that have been exposed to 85.7, 171.5 and 857.5 μCi ^{3}H beta particles. Errors represent the standard error of the mean (SEM), where n = 9 for three independent experiments.

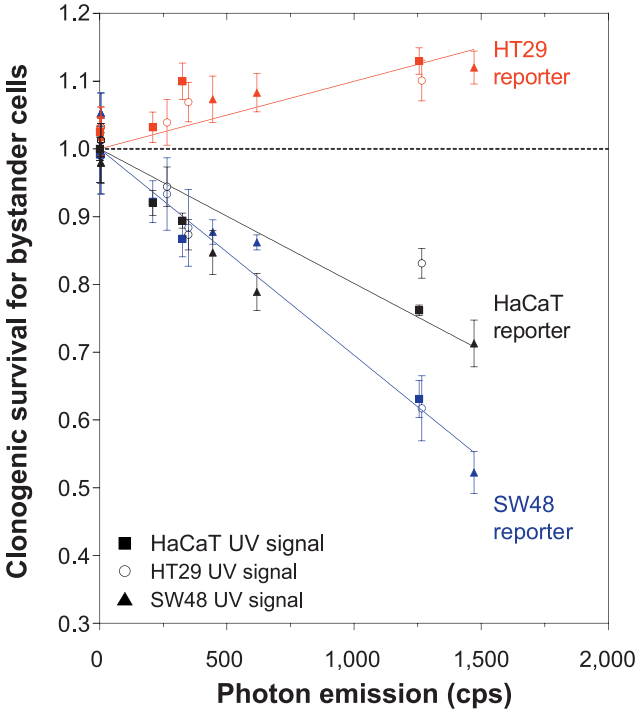

FIG. 2. HaCaT (black), HT29 (red) and SW48 (blue) reporter cell survival in response to signals from tritium-irradiated HaCaT (square), HT29 (open circle) and SW48 (triangle) cells. Error bars represent SEM where n = 3 for three independent experiments (total n = 9). Two-way ANOVA, post hoc: Tukey's HSD test.

represented in red, did not exhibit reductions in survival in response to UV signals of any of the three irradiated cell lines. Furthermore, the magnitudes of HT29 cell survival in response to the signals from each of the HaCaT, HT29 and SW48 cells were not significantly different from each other ($P > 0.781$).

In contrast, HaCaT reporter cells (Fig. 2, black markers), which also possess mutated p53, demonstrated marked reductions in survival with exposure to an increasing quantity of emitted UV photons. After fitting the HaCaT reporter cell data to a linear regression fit constrained to a (0.1) intercept, the r^2 value found was 0.55 ($P < 0.001$). Therefore, 55% of the HaCaT cell survival can be explained by the UVA photons emitted from the tritium-irradiated cells. The HaCaT bystander cells responded similarly to the UV signals from HaCaT and HT29 cells ($P < 0.986$). A significantly stronger response was exhibited when the HaCaT cells were exposed to the SW48 UV signals ($P < 0.001$).

The p53 wild-type cell line, SW48 (Fig. 2, blue markers), appears to be most sensitive to the UV signals emitted from the directly irradiated cells. The relationship between the SW48 cells' survival response and the UV signals emitted from tritium-irradiated cells was relatively strong ($r^2 = 0.6434$, $P < 0.001$). Over 64% of the cell survival exhibited by SW48 bystander cells can be attributed to the UVA photons. Therefore, it is suggested that SW48 cells are particularly responsive to the UVA photons emitted from irradiated cells. When assessing the response of the SW48 bystander cells to each of the HaCaT, HT29 and SW48 UV signals, it was found that their responses to each of the

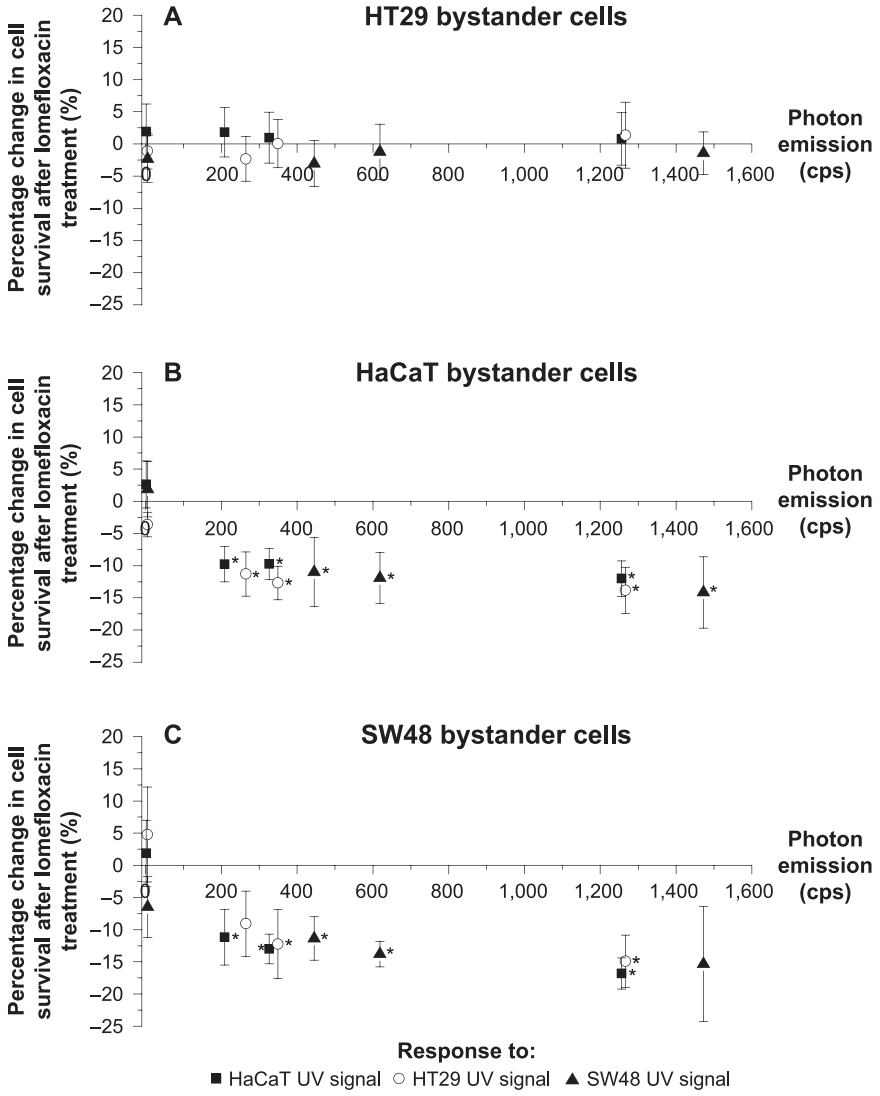

FIG. 3. Percentage change in HT29 (panel A), HaCaT (panel B) and SW48 (panel C) reporter cell survival when reporter cells were treated with lomefloxacin and concurrently exposed to cell-emitted UV signals, compared to bystander cell survival after UV exposure alone. Error bars represent SEM for n = 3 for three independent experiments (total n = 9). Independent *t* test: *Significant difference between nontreated and lomefloxacin-treated populations.

different sources of UV were not significantly different from each other ($P > 0.285$).

Cell Set 1: Photosensitizer Treatment

The previously mentioned bystander experiment was repeated with 20 μ*M* of the photosensitizer, lomefloxacin hydrochloride, supplemented into the cell culture media of the bystander cell population.

Lomefloxacin treatment of HT29 bystander cell cultures during exposure to cell-emitted UV signals did not confer any significant changes in bystander cell survival (Fig. 3A) ($P > 0.263$). After the addition of a photosensitizer, a further decrease in cell survival was the predicted response. Although unexpected, it is suggested that the actual response (maintenance of the survival at levels equal to nonirradiated controls) is indicative of the HT29 cell line's inability to respond to the UV-induced bystander signals. This lack of response may be attributed to aberrant or nonfunctional mechanisms required for bystander response.

The addition of lomefloxacin into HaCaT bystander cultures and subsequent UV exposure conferred significant reductions in survival ($P < 0.04$) (Fig. 3B). It is noted that exacerbated cell death was not exhibited when lomefloxacin-treated cells were not exposed to UV (0 cps). It can therefore be concluded that the photosensitizer alone (i.e., treatment with photosensitizer in the absence of photons) does not adversely affect bystander cell survival.

SW48 cells treated with lomefloxacin and exposed to bystander UV exhibited significant reductions in survival beyond those levels induced in the presence of bystander UV alone (Fig. 3C) ($P < 0.003$). This observation is

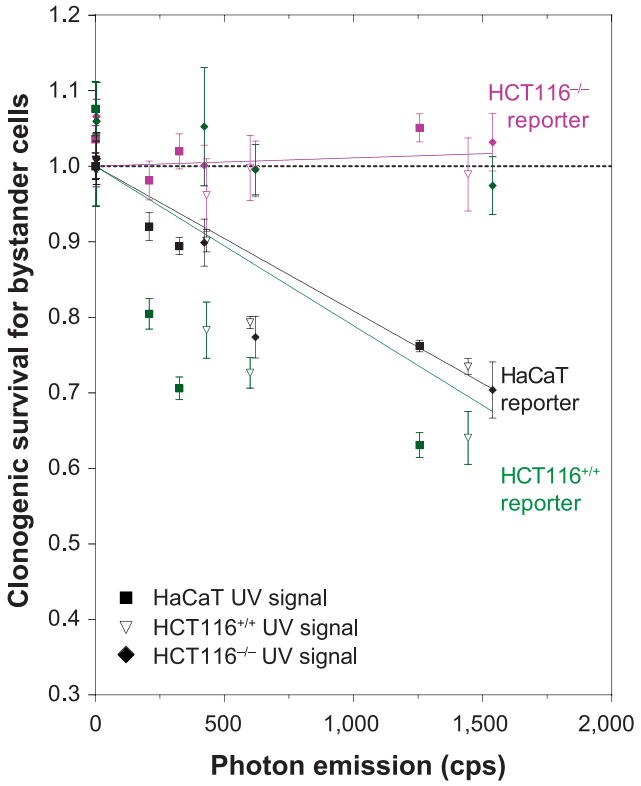

FIG. 4. HaCaT (black), HCT116$^{+/+}$ (green) and HCT116$^{-/-}$ (purple) reporter cell survival in response to signals from tritium-irradiated HaCaT (square), HCT116$^{+/+}$ (open inverted triangle) and HCT116$^{-/-}$ (diamond) cells. Error bars represent SEM for n = 3 for three independent experiments (total n = 9). Two-way ANOVA, post hoc: Tukey's HSD test.

evidence of a synergistic effect occurring between the photosensitizer and the bystander UVA photons. This effect can be defined as synergistic since the magnitude of effect generated in the presence of UV-photon exposure and 20 μ*M* lomefloxacin is much greater than the sum of the effects produced by lomefloxacin treatment alone (x = 0 cps) and that produced by UV-photon exposure alone (results shown in Fig. 2).

Bystander Response of Cell Set 2: HaCaT, HCT116$^{+/+}$ and HCT116$^{-/-}$ cells

Regardless of photon emission rate to which the HCT116$^{-/-}$ (p53-null) bystander cells were exposed, their survival was maintained around nonexposed control levels (100% survival) (Fig. 4, purple markers). The HCT116$^{-/-}$ cells were therefore unable to respond to any of the UV signals they received.

HaCaT bystander cells demonstrated decreasing cell survival with exposure to increasing UV-photon rates (Fig. 4, black makers). The r^2 value corresponding to a linear constrained fit to the HaCaT cell data was found to be 0.6507 ($P = 0.002$). Just over 65% of the HaCaT bystander survival can be explained by the UVA photons emitted from beta-irradiated cells. In terms of the individual responses of the HaCaT reporters to each of the sources of the UV-induced bystander signal, HaCaT response to signals from the two HCT cell lines was significantly stronger than the response elicited by the HaCaT signal ($P < 0.004$). This result is explained by the greater magnitude of photon emission exhibited by the two HCT cell lines compared to the HaCaT cell line.

The response exhibited by HCT116$^{+/+}$ (p53 wild-type) cells proved to be variable and dependent on the cellular source of the UV photons (Fig. 4, green markers). The HCT116$^{+/+}$ cells responded well to signals from beta-irradiated HaCaT cells and HCT116$^{+/+}$ cells. However, The HCT116$^{+/+}$ bystander cells did not exhibit a significant response to the UV signal from HCT116$^{-/-}$ cells. The linear fit of the HCT116$^{+/+}$ data was weak ($r^2 = 0.2229$) and was not significant ($P = 0.382$). Therefore, it cannot be concluded that a linear relationship exists between the survival of HCT116$^{+/+}$ cells and UVA photon emission. The observed insensitivity to the HCT116$^{-/-}$ UVA signal may also suggest that HCT116$^{+/+}$ cells are not strongly sensitive to photon emission in the UVA wavelength range. It is possible that the responses elicited in HCT116$^{+/+}$ cells by the HCT116$^{+/+}$ and HaCaT signals are attributed to particular sensitivity of the HCT116$^{+/+}$ cells to photons that possess wavelength(s) alternative to those measured in the current study (340 ± 5 nm).

Cell Set 2: Photosensitizer Treatment

The addition of 20 μ*M* lomefloxacin into HCT116$^{-/-}$ bystander cell culture did not confer significant changes in survival ($P > 0.51$ at the 95% confidence level) in response to the UV signals from any of the HaCaT, HCT116$^{+/+}$ or HCT116$^{-/-}$ cells (Fig. 5A). Because an increase in cell killing is expected when a photosensitizer is present during photon exposure, it is suggested that HCT116$^{-/-}$ cells lack the functions or mechanisms required to elicit a response to the UV-induced bystander signals. This conclusion is the same as that which was suggested for the HT29 cell line.

Treatment of HaCaT bystander cells with lomefloxacin conferred significant reductions ($P < 0.005$) in HaCaT cell survival, beyond levels induced by UV photons alone (Fig. 5B). These results indicate the effectiveness of the photosensitizer and further prove the ability of the HaCaT cell line to respond to the cell-emitted UV signals.

Finally, the lack of response exhibited by the HCT116$^{+/+}$ cells to the HCT116$^{-/-}$ signal in the absence of the photosensitizer (Fig. 4) is the result that initially prompted the addition of a photosensitizer to each of the bystander cell cultures in the experiment. After the addition of lomefloxacin into the HCT116$^{+/+}$ bystander cell culture, a significant response to the HCT116$^{-/-}$ UV signal was indeed elicited when the signal intensity was greater than 600 cps ($P < 0.001$) (Fig. 5C). The photosensitizer's ability to induce a significant reduction in survival in response to the HCT116$^{-/-}$ signal confirmed the capacity of the HCT116$^{+/+}$

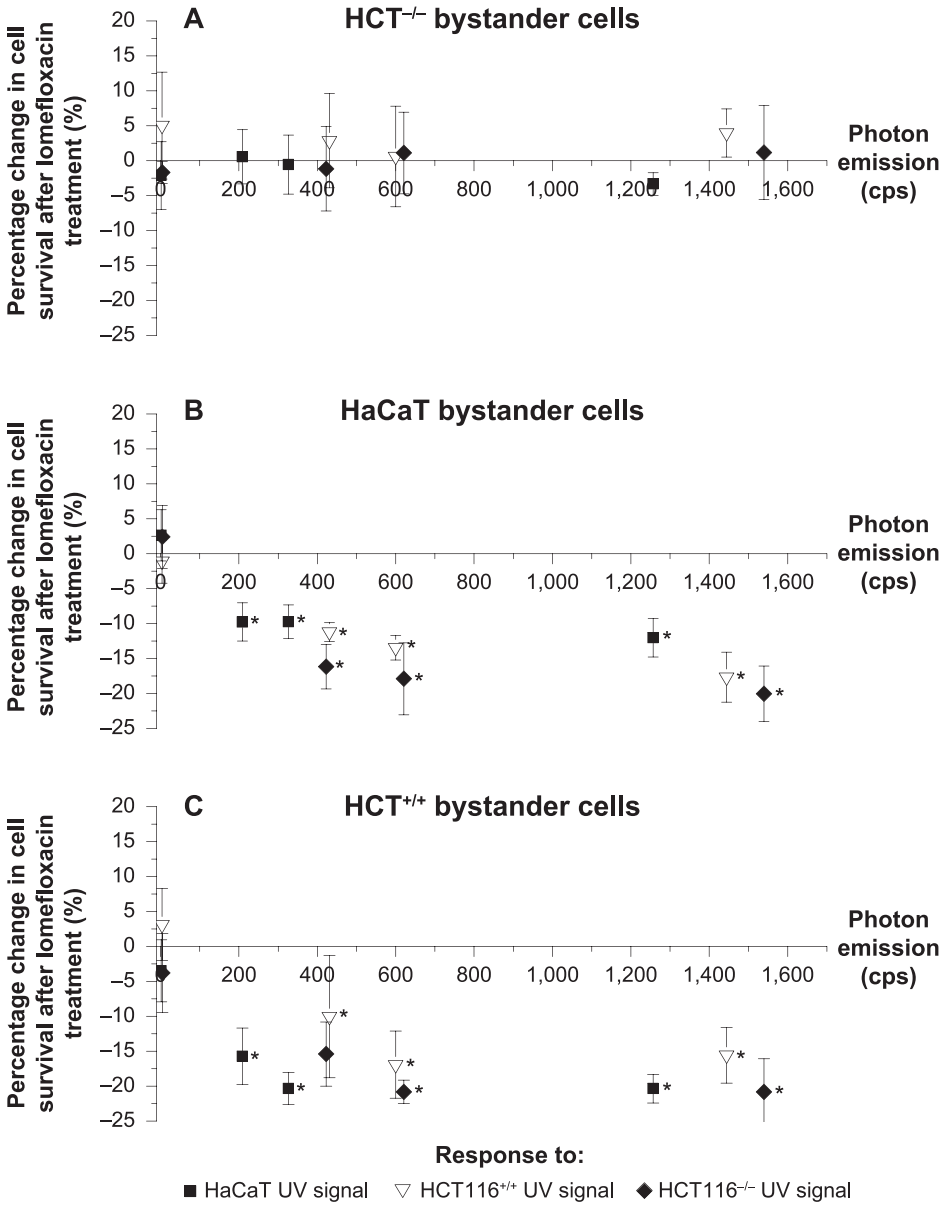

FIG. 5. Percentage change in HCT116$^{-/-}$ (panel A), HaCaT (panel B) and HCT116$^{+/+}$ (panel C) reporter cell survival when reporter cells were treated with lomefloxacin and concurrently exposed to cell-emitted UV signals, compared to bystander cell survival after UV exposure alone. Error bars represent SEM for n = 3 for three independent experiments (total n = 9). Independent *t* test: *Significant difference between nontreated and lomefloxacin-treated populations.

cell line to generate a bystander response to these UV signals. It is hypothesized that the result of this photosensitizer experiment would resemble a response similar to those of the HT29 and HCT116$^{-/-}$ cell lines if the HCT116$^{+/+}$ cells possessed an inability to respond to bystander signals. The HCT116$^{+/+}$ cell survival was indeed decreased by lomefloxacin and UV treatment. Therefore, it is suggested that the very weak response exhibited by HCT116$^{+/+}$ cells to the HCT116$^{-/-}$ signal was attributed to variable sensitivity to different photon wavelengths. A given cell line may be more strongly responsive to a given wavelength range than another cell line, and furthermore, different cell lines may emit photons of a given wavelength in different proportions.

Clonogenic Survival of Cells Directly Irradiated with Tritium Beta Particles

Direct tritium beta irradiation of cells elicited similar magnitudes of response among the HaCaT, HT29, HCT116$^{+/+}$ and HCT116$^{-/-}$ cell lines ($P > 0.357$) (Fig. 6A, C–E, respectively). In contrast, the SW48 cell line (Fig. 6B) demonstrated a greater magnitude of cell death compared to three other cell lines such that the SW48 cell line's survival was significantly different from that of the

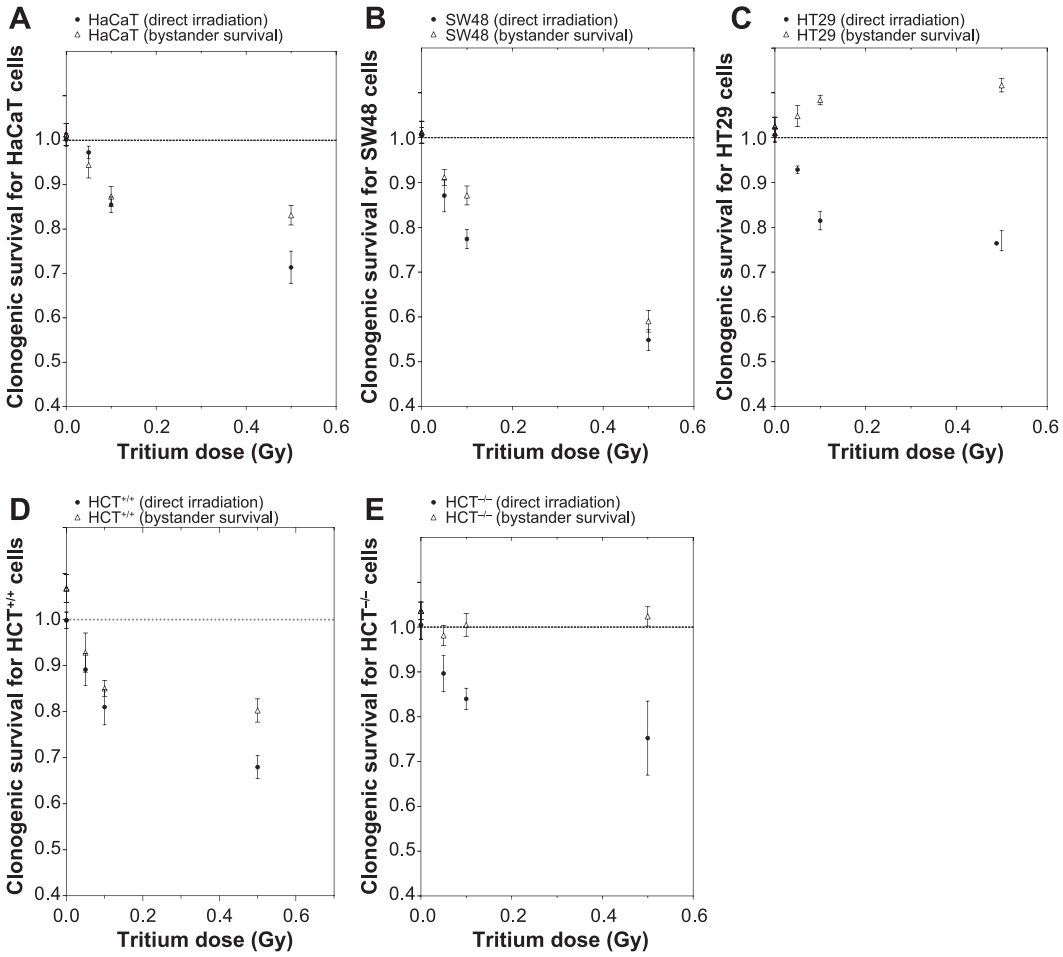

FIG. 6. Clonogenic surviving fraction for HaCaT (panel A), SW48 (panel B), HT29 (panel C), HCT116$^{+/+}$ (panel D) and HCT116$^{-/-}$ (panel E) cells in response to direct ^{3}H beta irradiation (circular markers). Bystander cell survival subsequent to exposure to UV photons emitted from beta-irradiated cells is also shown for comparison (open triangle markers). Error bars represent SEM for n = 3 for nine independent experiments (total n = 27).

HaCaT ($P = 0.001$), HT29 ($P = 0.004$) and HCT116$^{-/-}$ ($P = 0.010$) cell lines. Compared with the HCT116$^{+/+}$ cell line, the cell killing induced in SW48 cells was greater but not to a significant extent ($P = 0.250$). Thus, these results indicate that SW48 cells demonstrated the greatest radiosensitivity to direct beta irradiation among the five cell lines tested, the HCT116$^{+/+}$ cell line demonstrated intermediate sensitivity, and the HaCaT, HT29 and HCT116$^{-/-}$ cell lines demonstrated the least sensitivity to beta radiation.

The purpose of the direct irradiations with tritium beta particles was to determine the magnitude of effect elicited by the UV-biophoton signal relative to the effect of the beta radiation itself. When compared to the bystander cells which were exposed to UV biophotons, the directly irradiated cell populations demonstrated a greater degree of cell killing. The amount of cell killing induced by the direct beta irradiation was significantly greater than that induced by the UV-induced bystander signal in the HaCaT ($P = 0.008$), HT29 ($P < 0.0001$), HCT116$^{+/+}$ ($P = 0.003$) and HCT116$^{-/-}$ ($P < 0.0001$) cell lines. It is observed that, generally, the UV signals generate a lower amount of cell killing than the direct irradiation itself. Following this observation, it can be suggested that the UV signal quite possibly contributes to the cell death observed in the directly irradiated cell population if UV signals from one cell reach a neighboring cell contained within the same cell culture population. While the amount of cell killing induced in SW48 bystander cells was slightly lower than that induced by direct beta irradiation, this difference was not significant ($P = 0.081$). The nonconformity of the SW48 cell line to this general observation may be attributed to its greater radiosensitivity relative to the other cell lines used in this study.

Validation of p53 Functionality

Western blots were used to confirm the functional status of p53 in regard to its ability to prompt the generation of p21 and thus induce cell cycle arrest in response to ionizing radiation insult. Figure 7 shows the expression of p53 for each of the five cell lines employed under normal and

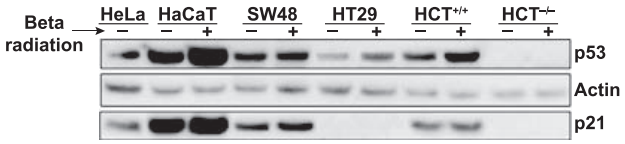

FIG. 7. Expression for p53 (53 kDa), actin (42 kDa) and p21 (21 kDa) proteins by HeLa (positive control), HaCaT, SW48, HT29, HCT116$^{+/+}$ and HCT116$^{-/-}$ cells exposed to either no radiation or 0.5 Gy ^{3}H beta radiation.

irradiated conditions. Of note, for three of the cell lines, p53 expression was significantly greater in irradiated cell populations compared to their nonirradiated counterparts (HaCaT $P < 0.0001$, HT29 $P = 0.002$, HCT116$^{+/+}$; $P = 0.001$). While SW48 cells did not demonstrate contrasting p53 protein expression between nonirradiated and irradiation populations, the levels of protein expression in SW48 cells could be considered high regardless of external stress relative to most of the other cell lines investigated. Despite the relatively great p53 protein expression of SW48 cells, HaCaT cells demonstrated the most p53 protein among all five of the cell lines assessed. In contrast, HCT116$^{-/-}$ cells did not express any p53; this finding was expected based on the cell line's p53-null status.

In addition to p53, p21 protein expression was also investigated so that it could be used as an indicator of p53's downstream function. P21 expression by HaCaT, SW48 and HCT116$^{+/+}$ cells was apparent for both nonirradiated and irradiated cell populations, whereas HT29 cells and HCT116$^{-/-}$ cells lacked p21 protein expression altogether (Fig. 7). Furthermore, the levels of p21 were significantly changed from nonirradiated to irradiated conditions (HaCaT $P = 0.032$, SW48 $P = 0.028$, HCT116$^{+/+}$; $P = 0.048$). It is suggested that in these cell lines, the binding of p53 to its corresponding DNA binding site successfully initiated the release of p21. In contrast with the three aforementioned cell lines, the HT29 and HCT116$^{-/-}$ cell lines did not express any p21 proteins. The observed lack of p21 expression by HCT116$^{-/-}$ was expected due to its absence of p53. We suggest that the lack of p21 expression in HT29 cells can be deduced to compromised propagation of the p53-mediated G_1 arrest pathway. This is suggested because upregulation of p53 is still initiated in HT29 cells after irradiation, thus the mechanisms responsible for generating p53 are still intact. However, the complete absence of p21 expression after irradiation would indicate a source of error in the pathway of communication between p53 and the processes downstream of p53 leading to p21 generation.

DISCUSSION

Relationship between p53 Status and UV Signal Generation

Photons quantified from all five ^{3}H-irradiated cell lines demonstrated significant emission regardless of p53 status. HCT116$^{+/+}$ (p53 wild-type), HCT116$^{-/-}$ (p53 null) and SW48 cells all demonstrated comparable magnitudes of emission and also exhibited a very similar pattern of emission to each other. In contrast, HaCaT and HT29 cell lines demonstrated emission that was weaker than the three former cell lines. However, emission was still significantly greater than background and nonirradiated controls. Preliminary analysis could suggest that the mutations associated with HaCaT and HT29 cell lines may contribute to the lower magnitudes of emission observed from these cells. This would suggest that a modification in p53 function would be linked to modulation of the generated UV signal in response to direct irradiation. This hypothesis, however, is not completely supported by the emission measurements taken from irradiated HCT116$^{-/-}$ cells. HCT116$^{-/-}$ cells lack p53 function, therefore if p53 function was an influential factor in UV signal generation, it would be expected that HCT116$^{-/-}$ cells would also demonstrate weak or even absent UV signals. Because HCT116$^{-/-}$ cells are actually able to generate a UV signal at levels comparable to the two wild-type cell lines used, it could be suggested that p53 functionality may not be a modulating factor in the generation of the UV-induced bystander signal, but rather that very specific p53 mutations alter the UV emission.

Given that all five tested cell lines demonstrated UV emission after beta irradiation, and further, because both HCT116 cell types demonstrated strikingly similar magnitudes of emission, an alternative or perhaps additional explanation is presented. Cell-specific characteristics, such as intracellular concentration of endogenous fluorophores, may be responsible for differences in UV signal magnitude. Endogenous fluorophores include aromatic amino acids tryptophan, tyrosine and phenylalanine, structural proteins such as collagen and elastin, porphyrins, and coenzymes NADH and FAD (*42*). Each of these endogenous fluorophores possess emission spectra at different wavelengths along the electromagnetic spectrum, ranging from the ultraviolet to the visible region. Therefore, it is not unreasonable to suggest that a given cell type may possess greater concentrations of a specific fluorophore than another cell type. Since all cells possess these endogenous fluorophores, this hypothesis also suggests that it is possible for any cell to generate UV signals when stimulated. Presently, the link between p53 status and the capacity for generating the UV-induced bystander signal is unclear. To elucidate the role of p53 in UV signal generation, further investigation involving p53 genomic modifications in the currently studied cell lines is recommended.

It is further noted that there may be a nonlinear relationship between tritium activity and photon emission for three of the five cell lines tested in these experiments: HCT116$^{+/+}$, HCT116$^{-/-}$and SW48. While photon emission continuously increases with increasing activity, the slope of photon emission dependent on activity becomes less steep after 85.7 μCi relative to the slope that exists between 0 and 85.7 μCi. In contrast, the other two cell lines tested, HT29 and HaCaT, are consistent with a linear response between

tritium activity and photon emission. It is possible that the discrepancy between the pattern of photon emission demonstrated by the three former and the two latter cell lines is due to a difference in the concentrations of different proteins or molecules within the cells themselves. That is, the three former cell lines may contain a greater concentration of molecules that are capable of absorbing photons emitted from autologous or adjacent cells, thus explaining the approach to an asymptote in photon detection above a given activity level. It is also possible that a small systematic offset occurred, whereby a time-dependent transition occurred at the 85.7 μCi point and the deviation from linearity occurred due to systematic errors in the manner in which the measurements were taken.

Relationship between p53 Status and UV-Induced Bystander Response

The UV-induced bystander responses discussed in this study suggest that p53 function is an influential factor in a given cell's ability to respond to any physical UV signals. Those cell lines possessing wild-type p53 (SW48, $HCT116^{+/+}$) demonstrated responses to all of the UV signals that they received, while the p53-null cell line tested ($HCT116^{-/-}$) lacked response capability, even when sensitized to the incoming UV photons by lomefloxacin. In this context, wild-type p53 would generate fully functional p53 proteins which are able to carry out all of the functions necessary to respond appropriately to stressors. The wild-type cells in our study were able to elicit, what we consider, a protective response to the UV signals emitted from directly irradiated cells. The p53 wild-type cells were able to undergo cell death before mutations or genomic instability could be propagated. In contrast, p53-null status confers a cell unable to produce and express p53 proteins (*37*). This deficiency, in turn, leads to the loss of some of the protective response mechanisms that a cell can employ against cellular damage; the result manifests as continued proliferation even in the presence of damage signals such as UV emission by irradiated cells. This effect of p53 nonfunctionality is reflected by the $HCT116^{-/-}$ bystander cells' inability to respond to any and all signals, even when exposed to the UV-induced bystander signals in concurrence with a photosensitizer. These results agree with those reported by Mothersill *et al*. in their experiments involving media-borne RIBE. Using the same HCT116 p53 wild-type and null cell lines, they found that $HCT116^{+/+}$ bystander cells were capable of responding to a soluble factor contained within irradiated cell-conditioned media (ICCM) while $HCT116^{-/-}$ bystander cells did not exhibit any changes in survival and were therefore deemed unable to respond (*30*). The similarity in the nature of response by a given cell line, regardless of the nature of the bystander signal (i.e., electromagnetic signal vs. molecular/soluble factor) suggests that bystander response kinetics are inherent to the characteristics of the cell line.

Using the results from the mutated p53 cell line (HT29 and HaCaT) responses to support the influence of p53 on the UV-mediated bystander effect is slightly more challenging. As noted above, both p53-mutated cell lines used in this study produced different responses even when exposed to the same given signals (UV signals from irradiated HaCaT, HT29 and SW48 cells). Despite the discrepancy between the responses of these two p53-mutated cell lines, a role for p53 in eliciting the bystander response is still possible due to the variable effects that different mutations can have on functions. Single p53 point mutations are able to alter the radiation response of a given cell or organism (*43–46*). Lee *et al*. studied the hematopoietic cells of transgenic mice with either an Arg193Pro mutation or an Ala135Val mutation (*43*). Their experiments showed that gamma-irradiated hematopoietic cells exhibited 35–57% greater radioresistance (clonogenic survival) than those cells extracted from mice possessing wild-type p53. Yount *et al*. (*44*) and Bristow *et al*. (*46*) also found that p53 mutation increased radioresistance (i.e., reduced cell killing). Further, Yount suggested that the reason for radioresistance in p53-mutated cells was due to evasion of G_1 cell cycle arrest by p53-mutated U-87MG cells; this contrasts with the reliable entrance into G_1 arrest after irradiation by wild-type p53 U-87MG cells (*44*). Contrasting with Lee *et al*. and Yount *et al*.'s findings, McIlwrath and colleagues showed an increased clonogenic sensitivity to gamma irradiation in p53 mutant transfectants (Val143Ala) compared to their wild-type equivalents (*45*). Since a single point mutation can have substantial effects on cellular response to ionizing radiation, it is logical to extend this evidence to lower-energy radiation such as that in the UV range.

It has been shown that functions and responses depend not only on the presence or absence of mutations, but also on the type of p53 mutation. We suggest that the HT29 and HaCaT results conferred in the current study reflect two different sets of p53 mutations that are capable of their own respective responses to a given stressor. HT29 cells possess a single point mutation where arginine is mutated to histidine on codon 273 (*38*). This particular mutation is known to lack traditional p53 activities such as sequence-specific DNA binding (*47*). Abolished DNA binding prevents the p53-dependent generation of the p21 protein (*48*), which then diminishes the probability of forming the p21-Cdk2 complex. The Western blot analyses of p53 and p21 expression in our experiments support the idea that the HT29 mutation compromises an intermediate component between p53 upregulation and p21 expression due to the lack of p21 protein expression after irradiation despite an observed upregulation of p53 in response to that same stressor. It is suggested that the observed lack of p21 is a product of p53's inability to bind to the p53 DNA binding domain as a result of conformational changes induced in the DNA binding site by the Arg273His mutation (*47*). Without inhibition of cyclin-dependent kinases by p21, G_1

cell cycle arrest cannot occur (*49–51*) and the opportunity to undergo repair or initiate cell death in response to damage is lost. The Arg273His mutation is characterized by continued proliferation even in response to damage or stress, although at the cost of genomic instability and exacerbated mutation frequency. This characteristic of the Arg273His mutation was confirmed by Barberi-Heyob *et al*. when the HT29 cell line was transfected with wild-type p53 to demonstrate improved sensitivity to photodynamic therapy, manifesting as an increase in apoptosis frequency (*52*). HT29 cells were also tested by Ryan *et al*. in their investigation of the RIBE using the media transfer technique (*53*). HT29 cells exposed to gamma-irradiated ICCM did not demonstrate reductions in survival and thus were considered insensitive to the media-borne bystander signal (*53*). The similarity between the HT29 cell line's response to media-borne and physical UV-induced bystander signals support the importance of inherent cell characteristics in the response to various bystander signals. The response of HT29 cells exposed to a primary source of UVA also agrees with the results conferred in the current study. Zacal and Rainbow studied multiple clonal variants of HT29 cells and reported that HT29 cells with decreased expression of mutant p53 tended to exhibit increased resistance to UVA radiation (*54*). Therefore, further investigation of the HT29 point mutation, Arg273His, is crucial to confirm its influence on cellular response to the UVA bystander signal.

It is also important to consider that p53 mutations do not always result in loss of function and that frequently, cells possessing mutations can still be active. Some mutations may still allow the p53 protein to respond to stress, regulate gene expression and interact with transcription factors. HaCaT cells possess three point mutations on both alleles: His179Tyr, Asp281Gly and Arg282Trp (*36*). The HaCaT mutation of particular interest is the aspartic acid-to-glycine mutation on codon 281. Several research groups have reported that this mutation is associated with a gain of function (*55, 56*). Specifically, this mutation elicits a functional gain manifesting as an alternative pathway for apoptosis (*57, 58*). The gain of function attributed to this particular mutation may explain the observed response to the UV-induced bystander signal exhibited by HaCaT cells possessing this particular p53 mutation. Although the extent of cell killing exhibited by HaCaT cells in response to the UV signal was weaker than that exhibited by the wild-type cell lines, it appears that the alternative apoptosis pathway is still an effective response mechanism. Further investigation will be required to confirm the proposed role of specific p53 mutations in the responses of these cells to UV-induced bystander signals. To address the influence of specific p53 mutations on the UV-induced bystander response elicited, a genomic modification experiment is proposed to induce specified point mutations.

Comparing the Observed UV-Induced Bystander Response with Typically Observed RIBE Responses

In much of the literature that describes the RIBE mediated by gap junction intercellular communication (GJIC) and via signal communication through the transfer of soluble factors (investigated using media transfer and co-culture techniques), there has been an observed saturation in the magnitude of the response elicited in bystander cells after reaching a dose falling within the low-dose range (*60–63*). The effect is characterized by an initial dependence of bystander cell effect on radiation dose followed by a critical dose point above which the bystander response persists and does not increase any further in magnitude. This saturation phenomenon, which becomes apparent beginning at 0.03 Gy (*63*) to just below 1 Gy (*60, 61*), has been consistently observed using various external-beam irradiation sources including alpha particle microbeams, X-ray microbeams and gamma-radiation sources.

In contrast to the existing bystander literature, the results conferred in the current investigation involving the assessment of UV as a bystander signal lack a demonstration of RIBE saturation. Despite the assessment of doses up to 0.5 Gy in the current study, it has been observed that the saturation effect in bystander populations possesses a large threshold range; thus, even investigation of doses up to 0.5 Gy may not be sufficient to observe the saturation. It is possible that saturation in response to UV biophotons occurs at a dose greater than 0.5 Gy. However, considering the constraints associated with radioactivity use limits, we were unable to investigate the effects that manifest at doses exceeding 0.5 Gy ^{3}H.

An alternative reconciliation for this contrasting result may be explained by the manner in which bystander signals are communicated via gap junctions and the media transfer technique. Because these two means of communication are motivated by biological signals, they can be restricted by limitations inherent to the biological systems themselves, whereas the manner in which the UV-induced bystander signal (a physical signal) is communicated can evade such limitations and result in a response that more closely reflects dose dependence. As for the assessment of bystander effects in microbeam-based experiments, the observed saturation in the bystander effect is expected because the quantity of cells that receive direct traversal by radiation are purposely limited [e.g., it is common to purposely limit directly irradiated nuclei to 1% of the cell population (*60*)]. Therefore, even subsequent to the application of higher doses, the same quantity of cells will be directly irradiated, and the effect of increasing the dose can simply be described by a greater number of particle traversals per nucleus, but not a greater number of directly traversed cells. In this respect, the effect exhibited in the nonirradiated population would not be expected to increase beyond a certain level with increasing dose because the directly irradiated cells can only communicate via GJIC with those

cells that are in direct contact with themselves. When all of the nonirradiated cells possessing gap junction connections with directly irradiated cells exhibit bystander responses, an increase in the volume of molecules exchanged between directly irradiated cells and their adjacent nonirradiated cells presumably would not result in an observable exacerbation in effect.

Similar to GJIC-mediated bystander effects, the bystander effects communicated by soluble factors can also be expected to exhibit a lack of dose dependence. RIBE saturation is possible in this bystander mode because some of the soluble factors thought to be involved in the effect [e.g., cytokines (*64*)] require binding to receptors to activate associated functions. Therefore, it is reasonable to suggest that if a bystander cell possesses only a finite number of receptors specific to a given signaling ligand, the magnitude of the observed bystander response is limited by the availability of appropriate receptors, regardless of the abundance of signaling factors released into the media.

The current experiments, relying on UV-driven signaling, have proven to elicit bystander responses that appear to be dose dependent. This electromagnetic bystander signal can be seen as radiation generated secondary to a primary irradiation event. Therefore, the action of these electromagnetic bystander signals more closely resembles that of a direct radiation source as opposed to the action of a molecule or cytokine released by the cell. When considered from that perspective, the observed dose dependence exhibited by bystander cells subsequent to UV signal receipt is not at all surprising.

Potential Interplay between UV-Mediated and Soluble Factor-Mediated Bystander Effects

When bystander effects are assessed using media transfer techniques, it is apparent that the saturation response predominates. However, when the RIBE is assessed under experimental conditions that allow only for the transduction of the UV-induced bystander signal, the response increases with dose. Furthermore, these experimental conditions result in magnitudes of cell killing that are quite significant, extending to levels even greater than those observed when using the media transfer technique. The apparent lack of interplay between these two bystander mechanisms may be perceived as such because bystander cells receiving irradiated cell culture media via media transfer are never in close proximity of the directly irradiated cells during direct irradiation; thus, they will not be subjected to any UV signals emitted from the cells as a result of direct irradiation. Subsequently, the strength of their bystander response is assumed to be attributed only to the soluble factors transferred after irradiation and not to the UV signal that has been seen to generate dose-dependent responses.

Despite the perceived absence of interplay among these seemingly separate bystander mechanisms, it is possible that they share a common etiology in the form of exosome involvement. Recently published research has shown the involvement of exosomes in the mediation of the RIBE (*65–67*). Following the confirmation of the role of exosomes in the RIBE, our research group hypothesized that exosomes could be a missing link between the UV-mediated and soluble-factor-mediated bystander effects. In our experiments, we investigated the possibility that the UV signal emitted from directly irradiated cells initiates exosome release in those cells affected by the UV signal. In the media transfer environment, this hypothesis would subsequently indicate that the UV signal generated by directly irradiated cells would affect neighboring cells in the same culture such that exosome release would be prompted. Those exosomes would be capable of propagating bystander effects in a nonirradiated population when extracted with the irradiated cell culture media. Conversely, for those experiments using the UV-induced bystander setup, those bystander cells exposed to the cell-emitted UV signals would presumably release exosomes that are capable of eliciting damage signals in a new population of cells when the exosome fraction from the UV-exposed bystander cells were extracted and put onto those new cells. Preliminary work done in our laboratory, in which we investigated the potential for exosome release by cell-emitted UV signals, indicated that the exosome fractions extracted from UV-exposed bystander cells are capable of reducing clonogenic survival when added to nonirradiated and non-UV-exposed reporter cells. However, this exosome fraction was able to initiate membrane depolarization in reporter cells (unpublished). While the cause for this discrepancy has yet to be investigated, these preliminary results show promise toward elucidating a potential link between the UV-mediated bystander response and those well-established bystander effects. Furthermore, these findings provide insight into how the physical detection of photons could translate into a biological response.

Range of the Effects of UV-Biophoton Signals

Given the previous discussion regarding a potential relationship between UV-induced bystander signals and soluble factors, it is plausible that the effects of a UV-induced bystander signal could be disseminated systemically via the circulation of biologically triggered release of soluble factors throughout the body. However, we can also look at the range of UV photons from a purely physical perspective for an ''average'' tissue. The actual range in tissue of a particular UV wavelength will, of course, depend on the tissue. The optical properties of tissue in the UV range depend on water content, as well as other factors including fat, blood, bilirubin and melanin content. As the composition varies, the range in tissue can vary. It is noted that the range of a 200 nm photon in an ''average'' tissue is approximately 0.01 mm while that of a 340 nm photon is closer to 0.1 mm. Since a typical mammalian cell is, on average, 10 microns in diameter, the bystander effect

elicited by UV-photon emission *in vivo* has the potential of reaching up to 10 cells that surround the cell if UV signals of UVA (340 nm) wavelength are involved. For shorter wavelength UV photons, such as those in the UVC wavelength range (100–200 nm), it is possible that these photons can reach and induce effects in cells located within 0.01 mm of the cell of signal origin (1 cell width away from the UV-emitting cell). In an *in vivo* environment, it can be expected that the UV-induced bystander effect can affect cells in close proximity to those being directly irradiated. A practical example, which validates the possibility of UV action on nonirradiated cells lying within the appropriate UV traversal range of directly irradiated cells, is demonstrated by Fernandez-Palomo in a study investigating the width of damage induced by tracks of microbeam radiation. The width of damage, measured via immunolabeling of γ-H2AX and induced by microbeam tracks in rat brains, demonstrated expansion of 25 μm microbeam tracks to approximately 50 μm (*68*). The expansion of the tracks by approximately 12.5 μm or 0.0125 mm on either side of the directly irradiated track of cells indicates a spread of damage that is within the range achievable by the traversal of UV tracks emitted from directly irradiated cells. After critical analysis, it is not impractical to hypothesize the attribution of this observed effect to the action of UV photons emitted from directly irradiated cells.

On the other hand, the relatively limited range of UV-photon traversal also indicates that there should not be an expectation that UV emission is directly responsible for expression of bystander effects in regions very distant from a given site of origin *in vivo* (i.e., effects of an abscopal nature), since the UV photons do not appear to be capable of traversing the great ranges required to directly induce effects in distant sites. It is still presumed that abscopal effects observed after direct irradiation of a targeted site are attributed to the release of clastogenic factors into the circulatory system after a radiation event, thereby generating effects in sites distant from the primary irradiation site. Thus, it is possible that UV-induced bystander signals could indirectly induce bystander responses at sites distant from the site of UV release via the circulation of soluble factors (such as exosomes). However, the direct effects of the UV-induced bystander signals are limited to regions immediately surrounding the site of UV-photon emission due to their relatively limited range within tissue.

Possible Link between UV Signal Response and Li-Fraumeni Syndrome

In a translational context, p53 mutations and their relationship to an altered UV-induced bystander response may be a significant factor in contributing to the development of pathologies linked to radiation-induced cancer sensitivity, such as Li-Fraumeni syndrome (LFS) or Li-Fraumeni-like syndrome (LFL). Both are cancer predisposition syndromes presenting as the development of sarcomas, breast cancers, brain tumors or adrenocortical carcinomas manifesting before the age of 45 (*59*). LFS and LFL are distinguished by the presence or absence of a familial history of cancer, respectively (*69, 70*), and these patients are also particularly susceptible to radiation-induced cancer (*71*). We suggest that the compromised response of p53-mutated cells to radiation-induced UV-induced bystander signals may be a contributing factor to the mechanism by which ionizing radiation increases this susceptibility. Because the UV-induced bystander signals emitted from directly irradiated cells have been proven to induce cell death in neighboring cells (*13*), a weak response or absent response to these signals could result in damage persistence and thus may allow for clonal expansion of nonlethal aberrations, subsequently potentiating carcinogenic progression. It can also provide a plausible explanation for the low treatment efficacy of cancer in LFS and LFL patients by means of radiation therapy. A weak or absent response to radiation-induced bystander signals would manifest as decreased cell killing and therefore lower overall treatment efficacy.

The p53 sequence codons most frequently mutated in LFS are codons 248, 273, 245, 175 and 282 (*72*). Two of the five most prevalent p53 mutations in LFS are found in HT29 (Arg273His) and HaCaT (Arg282Trp) cells. The Arg282Trp mutation has also been observed in LFL cases (*73*). In the current study, the HT29 cell line exhibited no response to the emitted UV signals, whereas the HaCaT cell line was able to demonstrate a significant cell death response. However, the magnitude of cell death in HaCaT cells was weaker than that exhibited by p53 wild-type cells. The discrepancy between the magnitudes of response exhibited by HT29 and HaCaT cells to a UV-induced bystander signal may be due to the presence of multiple p53 mutations in HaCaT cells. The observed involvement of codon 282 mutations in a large proportion of LFS/LFL cases suggests that this mutation elicits a loss of normal p53 function. However, HaCaT cells also possess a mutation on codon 281, which, as discussed previously, is associated with a gain of function manifesting as the initiation of an alternative apoptosis pathway. In this regard, the weak response exhibited by the HaCaT cell line may be explained by the balance between a loss of function mutation on codon 282 and a gain of function mutation on codon 281.

Alternatively, it is also possible that the magnitude of response to the UV-induced bystander signal is differentially modulated depending on the specific p53 mutation present. That is, different point mutations can result in different magnitudes of effect. Based on this hypothesis and the observations conferred in the current study, we may expect a greater number of radiation-induced cancer in LFS/LFL patients possessing the Arg273His mutation compared to those with an Arg282Trp mutation. This is supported by the published study of Bougeard *et al.* in which earlier onset of cancer was observed in patients with Arg273His mutation compared to patients with Arg282Trp mutation

(*74*). The mean and median age of cancer onset for patients affected by an Arg273His mutation was 17.9 years and 17.0 years, respectively, compared to 19.6 years and 22 years, respectively, in patients affected by an Arg282Trp mutation.

Although the factor(s) responsible for the discrepancy in response magnitude between HaCaT and HT29 cells have not yet been clarified, an observed reduction in cell killing and an observed abolishment of cell death in response to a given UV-induced bystander signal in both of the p53-mutated cell lines is suggestive of a role for p53 mutations in contributing to radiation-induced cancer susceptibility and reduced efficacy of radiotherapy.

CONCLUSION

We investigated the UV-mediated bystander effect in the context of bystander signal generation and response to those bystander signals. It is important to elucidate the factors responsible for mediating the UV signal generation and response, since these factors may be used in predicting the bystander effect. Knowledge of the characteristics of a cell that mediate its ability to produce or respond to a UV bystander signal will facilitate preliminary assessment for predicting and evaluating the extent of the RIBE elicited in a specified cell type prior to the actual administration of radiation. In the context of therapeutic or even diagnostic radiation, the predicted outcome may either confirm radiation as the modality of choice or prompt consideration of an alternative method for treatment. The focus of the current investigation was on the potential role of p53 in mediating the UV-mediated bystander signal and response. Since p53 is a proven influential factor in mediating the media-borne bystander effect, it was chosen as a starting point for this study (*28–31*).

In the study of UV signal generation, as it pertains to p53 status in various cell lines, the pattern and strength of UVA (340 ± 5 nm) emission was comparable among the p53 wild-type and null cell lines exposed to beta radiation (^{3}H). The UV-photon emission signals detected from the ^{3}H-irradiated p53-mutated cells were significantly lower than the signals detected from the p53 wild-type and null cell lines. The slight signal strength observed may be attributed to the mutation of p53. However, further investigation is required, since the unexpectedly strong signal emitted from the p53-null cell line raises questions regarding the actual involvement of p53 in the process of bystander signal generation.

Bystander responses to the UV signals emitted from ^{3}H-irradiated cells appear to be strongly linked to the p53 status of the cell line. Cells possessing wild-type p53 reliably expressed diminished cell survival after exposure to the bystander UV. Accordingly, p53-null cells did not demonstrate the ability to generate responses regardless of the UV-photon emission rate or sensitization. The two p53-mutated cell lines that were tested conferred different responses to the same given signals. It is suggested that the discrepancy between their responses is attributed to the specific p53 mutation(s) that the given cell lines possess. Because each mutation is unique in terms of modulation of function, it is possible for two different mutations to elicit unique responses. For this reason, the importance of investigating p53 mutations in the context of the bystander response to cell-emitted UV signals is emphasized.

SUPPLEMENTARY INFORMATION

Fig. S1. Bystander cell survival of HaCaT, SW48, HT29, HCT116$^{+/+}$ and HCT116$^{-/-}$ cells exposed to photon signals emitted from ^{3}H-irradiated cell culture media and petri dish.

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