INVESTIGATING THE GENERATION OF BIOPHOTONS INDUCED BY LOW-DOSE BETA-IRRADIATION AND THEIR ROLE IN THE RADIATION-INDUCED BYSTANDER EFFECT

INVESTIGATING THE GENERATION OF BIOPHOTONS INDUCED BY LOW-DOSE BETA-IRRADIATION AND THEIR ROLE IN THE RADIATION-INDUCED BYSTANDER EFFECT

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TITLE: Investigating the Generation of Biophotons Induced by Low-Dose Beta-Irradiation and their Role in the Radiation-Induced Bystander Effect

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Abstract

The communication of information between irradiated and non-irradiated bystander cell populations and the subsequent expression of radiation-like responses in the non-irradiated population, formally referred to as the radiation-induced bystander effect, is a very well established phenomenon in the study of radiobiology. Intercellular communication of bystander signals is known to occur via the exchange of soluble factors through biological fluids and via the transfer of molecules between adjacent cells via gap-junctions. Both of these communication methods require some degree of physical contact between biological entities. However, observations made in the literature demonstrating the induction of radiation effects in optically-coupled, yet chemically-separated organisms raises the hypothesis that alternative radiation bystander communication mechanisms may exist that have not yet been explored. Following the detection of significant photon emission from human keratinocyte cells exposed to ionizing beta (β)-radiation by Ahmad in 2013, the involvement of an electromagnetic bystander signal was proposed. While not yet established in the field of radiobiology, intercellular communication via electromagnetic signalling is widely studied in the field of biophotonics. The emission of electromagnetic radiation from biological material, called biophoton emission, and the subsequent communication of effects using those signals has been characterized both spontaneously and as a result of perturbation by various stressors. This thesis therefore aimed to investigate intercellular communication via electromagnetic signalling stimulated by low-dose ionizing radiation to identify a possible convergence between the fields of biophoton communication and radiation-induced bystander effects.

The characterization of biophoton emission from human cell cultures was accomplished using a single photon counting photomultiplier tube. The results revealed that biophoton emission is exacerbated by external stimulation (β-radiation), it possesses a dependence upon the activity of radiation delivered, the density of the irradiated cell culture, and cell viability. These results suggest that biophoton emission is governed by physical transitions between excited and ground states and may further be modulated by metabolic processes.

An effect of β-radiation-induced biophoton emission upon non-irradiated bystander cells was identified and manifested as a reduction in cell survival. The modulatory

effects observed following the application of photomodulating agents to the bystander cultures support ultraviolet electromagnetic radiation as a responsible factor in the communication of bystander signals. Observation of photon emission across the entire ultraviolet, visible and infrared spectra lead to the suggestion that ultraviolet wavelengths are only a portion of the signal responsible for eliciting bystander responses and that coherent interaction of multiple wavelengths is probable in the intercellular exchange of information.

The possibility of a link between biophoton bystander signalling and soluble-factor mediated bystander effects was investigated next by isolating exosomes from biophoton-exposed bystander cultures. Positive bystander responses were exhibited by secondary reporter cells incubated with the exosomes isolated from the biophoton-exposed bystander cultures, thereby suggesting that biophoton signalling is a possible form of biological redundancy where it acts as an intermediary to trigger soluble factor release and further reinforce intercellular communication.

Finally, the effect of β-radiation-induced biophoton signals upon mitochondrial activity was assessed and revealed the capacity for biophotons to downregulate Complex I and ATP synthase activity. The demonstrated effect of biophotons upon mitochondria elucidates a candidate mechanism worthy of further exploration to determine how biophotons may trigger responses in bystander cells.

Overall, this thesis elucidates an additional mechanism for intercellular communication between biological systems perturbed by low doses of ionizing radiation, in the form of an electromagnetic signal. This work contributes to the current perspective on biophoton bystander signalling as a potential source of biological redundancy, facilitating a means of intercellular communication when optical coupling but not chemical contact is available in a given system.

Dedication

Mom and Dad, I dedicate this thesis to you. The hardships you endured during your journey to Canada, the example of hard work you have always demonstrated, and the immense support you've given me are the reasons for any success I have been fortunate to experience. I couldn't have made it here without you.

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Contents

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List of Abbreviations & Symbols

α alpha

β beta

γ gamma

^{3}**H** Tritium

^{90}**Y** Yttrium-90

^{90}**Zr** Zirconium-90

^{222}**Rn** Radon-222

8-oxo-dG 8-oxo-7,8-dihydro-2'deoxyguanosine

ATP adenosine triphosphate

CHO chinese hamster ovary

CNSC Canadian Nuclear Safety Commission

COX-2 cyclooxygenase-2

D_2O deuterium oxide

DNA deoxyribonucleic acid

DPI diphenyleneiodonium

DSB double-strand break

EM Electromagnetic

EM electromagnetic

ETC electron transport chain

GJIC gap junction intercellular communication

H_2O_2 hydrogen peroxide

ICCM irradiated cell-conditioned medium

IL-8 interleukin-8

IR infrared

IRR induced radioresistance

LET linear energy transfer

LNT Linear No-Threshold

NCRP National Council on Radiation Protection and Measurements

NTE non-targeted effects

$O_2^{\cdot -}$ superoxide ion radicals

$^1O_2$ singlet oxygen

PET positron emission tomography

PMA phorbol myristate acetate

PMT photomultiplier tube

PTP mitochondrial permeability transition pore

RIBE Radiation-induced bystander effects

RNase ribonuclease

ROS reactive oxygen species

SOD superoxide dismutase

TBARS thiobarbituric acid reactive substances

TEM transmission electron microscopy

TNF tumour necrosis factor

UPE ultra-weak photon emission

US United States

UV ultraviolet

UV-A ultraviolet-A

UV-B ultraviolet-B

UV-C ultraviolet-C

Chapter 1

Introduction

1.1 Background

The study of biological effects as a result of low-dose ionizing radiation (doses up to 0.5 Gy [1, 2]) is considered highly valuable in today's scientific community due to the relevance of such doses for occupational, medical diagnostic, and environmental radiation exposures. While there is a considerable amount of research on the effects of low-dose radiation, there remain significant gaps in our understanding of the implications of low-doses upon biological risks and how we can estimate those risks on a whole-organism level. Many different phenomena have been observed in biological systems exposed to low-dose radiation which challenge the currently accepted Linear No-Threshold (LNT) Model used for assessing cancer risk at low doses. The LNT hypothesis is characterized by the extrapolation of risk observed at high doses, from epidemiological studies conducted predominantly on the Japanese Atomic Bomb survivors, down to low doses (below 200 mGy) such that the dose-response relationship exhibits a linear behaviour with an absence of a minimum dose below which there is an assumption of no risk [3]. While the LNT model is currently employed for radiation protection practices [3], many scientists do not consider it to be an appropriate model to describe biological risk due to the observation of low-dose effects which show a non-linear dose response. Hormetic responses to low doses of radiation manifest as beneficial effects whereby reductions in cancer incidence have been observed in nuclear industry workers [4–6]. Immune responses also demonstrate enhancement in the form of augmented immune cell proliferation [7, 8] & triggering of cytokine release in response to irradiation [9]. The concept of induced radioresistance (IRR)

or adaptive responses whereby an initial low-dose radiation exposure event triggers cellular communication which results in resistance to adverse effects upon successive radiation exposure events [10, 11] can be considered similar to hormetic responses such that the dose-response relationship in these situations is overestimated by the LNT hypothesis (infralinear classification). On the contrary, effects have also been observed whereby supralinear responses to low doses are exhibited. In such instances, adverse modulation of biological entities is expressed to a greater magnitude than that which is expected by a linear dose-risk estimate. For all of the phenomena described here which express deviations from the LNT model, the responses can be attributable to a variety of intracellular processes such as mis-repair, genomic instability, hyper-radiosensitivity [12], and the action of signalling from cells, tissues, or organisms directly traversed by radiation to those which are not [13]. The latter effects pertaining to cellular signalling are collectively referred to as non-targeted effects (NTE). The observation of NTE support the idea that deoxyribonucleic acid (DNA) is not the only critical target within the cell for inducing biological modifications subsequent to radiation exposure [14, 15].

RIBE are encompassed by the NTE classification and are exhibited as an expression of biological responses resembling direct effects by radiation in cells which have not been directly targeted (bystander cells) by the primary radiation, itself [16]. Two well-documented mechanisms by which bystander effects are propagated are communication via the transfer of soluble factors through biological fluids [17] and gap junction intercellular communication (GJIC) [18]. Both of these mechanisms of bystander mediation require a degree of physical interaction between biological matter whether that interaction occurs via medium exchange between an irradiated and non-irradiated bystander culture or organism [17, 19] or cellular proximity such that channels (gap junctions) may be formed between adjacent cells to facilitate the exchange of molecules through those channels [18, 20]. While these two mechanisms of bystander communication are very well-established, they cannot be used to explain some of the radiation-induced bystander effects observed in the existing literature which do not facilitate direct contact or biological fluid exchange between two biological systems [19, 21, 22]. While the organisms investigated in these studies were physically-separated from each other, bystander responses were still observed. Such an observation leads to the suggestion that there may be additional mechanisms of bystander communication which have not yet been elucidated and thus warrant further exploration.

An alternative mechanism of bystander effect mediation taking the form of an electromagnetic (EM) signal was proposed by Ahmad *et al.* following the detection of significant ultraviolet-A (UV-A) biophoton signals from human keratinocyte cells exposed to ionizing radiation [23]. This proposal was based upon previous observations that UV-A radiation, itself, is capable of generating damage in UV-irradiated cells as a result of reactive oxygen species generation [24]. It thus follows that a secondarily-generated UV-A source should be capable of eliciting similar effects in a bystander culture which is subjected to photons of this same energy by a cellular source. The rationale for investigating electromagnetic signalling as a form of radiation-induced bystander communication is supported further by extensive documented evidence for intercellular and inter-organism communication by light in situations not involving ionizing radiation [25, 26]. Documentation of electromagnetic signalling as a form of communication under normal circumstances [26] and as a result of perturbation by other stressors [25] provides confidence in the possibility of its participation in intercellular and inter-organism signalling subsequent to ionizing radiation exposure.

1.2 Outline and Objectives

This thesis aims to explore the role of electromagnetic signalling in the mediation of the radiation-induced bystander effect. The ultimate goal of this work is to elucidate further the mechanisms by which the RIBE can be communicated and the implications of the effects they elicit in non-targeted populations. This knowledge will enable us to gain further insight into the risks associated with low-dose ionizing radiation exposure. This work employs ^{3}H as the primary source of ionizing radiation since it is an environmentally- and occupationally-relevant radionuclide that is often present in quantities which would subject those exposed to doses which would be considered low. The use of ^{3}H was also advantageous since the short range of tritium's β-particles [27] allowed for irradiation of the primary cell culture but could not itself reach the bystander cells.

The experimental component of this thesis begins in Chapter 3 where the work presented by Dr. Bilal Ahmad [28] was extended with the objective of characterizing the UV photon emission from human keratinocyte cells (HaCaT cell line) in response to ionizing beta (β) radiation exposure. The results presented in this chapter illustrate the photon emission signal strength from cells, referred to throughout the text as

biophotons, subsequent to the modification of various independent variables including radioactivity (expressed in Curies, Ci), cell density, and cell viability. The motivation for studying the effects of these variables upon biophoton signal strength was to identify the conditions under which we could detect an optimal signal such that any subsequent efforts to investigate communication between cells using biophotons may also be optimized.

The primary objective of the investigations carried out in Chapter 4 was to address the question of whether biophoton signals from directly-irradiated cells may be able to elicit bystander effects in non-irradiated cell populations. The results confirmed a role for biophotons in bystander signalling by demonstrating clonogenic survival responses characteristic of direct irradiation in biophoton-exposed bystander cells. The experiments were also focused upon confirming the wavelength range of the electromagnetic photons responsible for inducing the effects observed in the bystander cells via the application of UV absorption filters and photomodulating substrates to the system under investigation.

Following the finding that bystander effects can be communicated via electromagnetic signalling in HaCaT human keratinocyte cells, we aimed to determine whether this mechanism of bystander communication could also be expressed by other cell types. To pursue this question, five different human cell lines possessing different p53 functional status were employed in an investigation encompassing biophoton emission characterization from each cell line and bystander response characterization by each cell line in response to signals emitted from each of the other cell lines. Consequently, the investigation became focused upon the dependence of both the bystander *signal* and *response* properties on p53 status. The focus upon p53 status was driven by the knowledge that wild type p53 is required to observe a bystander response in the bystander system mediated by soluble factors [29]. Therefore, the next logical step was to determine whether biophoton-mediated bystander effects were also dependent upon p53. The main biological endpoint investigated in this chapter was clonogenic survival while western blots were employed to confirm the expression of p53 and p21 proteins in each of the cell lines investigated in the experiments (HaCaT, SW48, HT29, HCT116 p53 +/+, HCT116 p53 -/-). This work is presented in Chapter 5.

Given the similarity in the dependence of the biophoton-mediated and soluble factor-mediated bystander responses upon p53 function of a given cell type, it was hypothesized that a link may exist between the two modes of bystander effect mediation.

The objective for the experiments presented in Chapter 6 was therefore to investigate a possible relationship between the radiation-induced biophoton signal and soluble factors released from cells. The focus was placed specifically upon exosomes since protocols for isolating this soluble factor are well documented and more importantly, the Lyng and Kadhim research groups have each identified exosomes as the critical bystander factor acting to mediated bystander communication [30, 31]. Therefore, the purpose of our work was to determine if there was a possibility of reconciliation between two seemingly mutually-exclusive mechanisms. Exosomes extracted from biophoton-irradiated bystander cells were used to investigate clonogenic survival and mitochondrial membrane potential in secondary reporter cells. Validation of exosome isolation was confirmed using western blot and transmission electron microscopy (TEM).

Chapter 7 investigates the effect of the biophoton signal upon mitochondrial function as a follow-up to the results identified in Chapter 6 regarding mitochondrial membrane potential. This was accomplished through the investigation of the activity of ATP synthase and Complexes I-IV of the electron transport chain (ETC) subsequent to biophoton exposure of bystander cells. Investigating the effect of the biophoton signal upon mitochondrial function may provide insight into the mechanisms by which biophotons interact with bystander cells to produce observable responses. This chapter also extends its investigation to the further characterization of the biophoton emissions across the UV and visible wavelength spectra subsequent to β-irradiation.

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Chapter 2

Literature Review

This chapter will first review the difficulties faced when investigating the biological effects and risks associated with exposure to low-doses of ionizing radiation. A brief discussion on the various dose-response models will be presented and will extend to exploring the literature which challenges the conventional framework for modelling the biological effects of low-dose radiation exposure. To this end, non-targeted effects are identified as a large contributor to non-linear responses to radiation in the low-dose region.

Non-targeted effects, radiation-induced bystander effects, and the mechanisms by which bystander effects are mediated will be reviewed. The review will subsequently extend to the exploration of literature evidencing intercellular and interorganism communication via electromagnetic signalling.

Because β-radiation was used throughout the experiments conducted in this thesis, the β-emitting radioisotopes and their presence in the environment, as well as their use in medicine will be discussed here. Beta particle interaction in matter will also be outlined and the relationship between β interactions and production of photons will be described.

The literature review will then revisit the purpose of this thesis by stating the relevance of elucidating radiation-induced bystander mediation mechanisms. Emphasis will be placed upon the goal of understanding phenomena observed in organisms as a result of low-dose exposures that cannot be explained solely by what we know of direct irradiation effects.

2.1 Estimating the Risk of Low-Dose Ionizing Radiation

Low doses of ionizing radiation are often defined as those which fall below 0.5 Gy [1, 2], and are regarded as highly relevant to scientific investigation due to their possible effect upon the general population. Recent exposure data collected by the National Council on Radiation Protection and Measurements (NCRP) indicates that a given member of the United States (US) population is exposed to an average of 6.2 mSv of radiation each year where background radiation (radon) (37%; 2.3 mSv) and medical exposures (48%; 3.0 mSv) are the largest contributors [3]. While occupational exposures contributed less than 0.1% of the overall US population's radiation dose, the occupational exposure among the 1.22 million radiation workers in 2006 was found to be 1400 person-Sv, resulting in an estimated average dose of 1.15 mSv to each worker from occupation-related activities [3]. The high probability for public, medical and occupational exposure to low doses of ionizing radiation emphasizes the importance for ongoing investigation of the biological effects that low doses elicit.

In the past, radiation effects had been classically explained using the framework referred to as the Target Theory [1]. The Target Theory ascribes to nuclear DNA as the critical target responsible for perpetuating damage in response to irradiation [4]. While the role of DNA in eliciting radiobiological effects has been validated by numerous studies [4–9], literature has emerged over the past 20 years to suggest the role of critical targets alternative to DNA in eliciting radiation-induced biological effects, particularly at low doses [2]. Consideration of the involvement of extranuclear targets in radiation responses was proposed in the 1990s when researchers observed that the percentage of cells showing sister chromatid exchanges was much greater than the percentage of cells that were theoretically-expected to express damage based upon direct traversal by alpha (α)-particles [10]. Subsequent research employing microbeams further supported this effect by delivering charged particles to the cytoplasmic volume of a cell resulting in the observation of persistent bystander effects [11–13]. These radiation responses which challenge the traditional DNA-centric paradigm are referred to as non-targeted effects (NTE) whereby the magnitude of the biological effects observed are not necessarily linearly proportional to the energy deposited in the DNA [2]. Evidence for the involvement of extranuclear targets in mediating radiation responses has led to a paradigm shift in the approach that is taken for estimating

radiation risk to include models which suggest both infra- and extra-linear dose-response relationships. Because the manifestation of many non-targeted effects is prevalent following low dose irradiation [2], continued investigation of NTEs are very important in improving our understanding of the biological implications at low doses.

2.2 Non-Targeted Effects

Non-targeted effects of ionizing radiation are radiation effects observed in cells, tissues and organisms in which energy was not directly deposited [14]. Non-targeted effects also encompass biological effects which do not directly result from energy deposition in the DNA of a given cell [2]. NTEs thus characterize models that support intra- and intercellular signalling to produce radiation responses which deviate from the model of linear dose-response. Abscopal effects [15, 16], clastogenic effects [17–19], genomic instability [20, 21], adaptive/hormetic responses [22, 23], and radiation-induced bystander effects (RIBE) [24, 25] are among the effects that are classified within the paradigm of NTEs.

The first evidence of NTEs was documented in 1915 when radiation effects were observed in non-irradiated organs of mice distant from the site of direct x-ray exposure [15]. Until 1953 such effects were described as "indirect effects". This effect is representative of that which is currently referred to as an *abscopal effect* whereby a modification is induced by radiation in a non-targeted tissue or organ within the same organism as the directly-irradiated tissue or organ [26]. The communication of effects between two biological systems via blood-borne factors (*clastogenic effects*) was first described in the 1960s by Goh and Summer whereby they observed breaks in the chromosomes of unirradiated lymphocytes cultured *in vitro* which had been incubated with plasma from individuals who had received whole-body irradiation [17]. Beginning in the 1980s, evidence of NTEs occurring on the cellular level were presented. In these studies, lethal mutations were found in the descendants of irradiated cells, an effect which characterizes the phenomenon of *genomic instability* [20, 21]. Further study of cellular radiation effects in the 1990s described the expression of sister chromatid exchanges in unirradiated cells following direct α-irradiation of cells within the same population [24]. This phenomenon describing the communication between cells to elicit responses characteristic of radiation exposure, was referred to as the *radiation-induced bystander effect* beginning in the 1990s. Abscopal effects and clastogenic effects are

often considered to be subclassifications of the RIBE [1]. The NTEs described thus far encompass a supra-linear dose-response model where the biological effects elicited exceed the degree of damage that is expected. An opposing model which has also been validated in the literature is that which is described by an infra-linear dose-response relationship. Hormetic and adaptive responses to radiation cause effects perceived to be beneficial in response to low doses of radiation. The first observation of an *adaptive radiation response* was reported in 1984 as the development of fewer chromosomal aberrations in lymphocytes cultured in low concentrations of radioactive thymidine before receiving a high challenge dose, compared with lymphocytes only receiving an acute high dose exposure [27]. This phenomenon is often explained by the initiation of proliferative and repair responses by a non-lethal and stimulatory dose of radiation [22, 28]. Yet another beneficial effect observed following low-dose radiation exposure is hormesis. The driving force behind *radiation hormesis* is hypothesized to be a low-dose exposure acting to initiate the activity of protective cellular functions [29]. Hormetic responses follow the inverted U-shaped dose-response curve suggesting that low doses of a given stressor can stimulate protective responses while high doses act to inhibit cellular functions [30].

Among the NTEs described, this thesis focuses upon RIBEs and the mechanisms by which intercellular communication is elicited in order to develop a greater understanding of the biological significance of NTEs.

2.2.1 Radiation-Induced Bystander Effects

Radiation-induced bystander effects (RIBE) are described by the expression of responses in non-irradiated cells or biological systems that resemble direct irradiation effects after the receipt of signals from directly-irradiated cells [14, 24]. These bystander signals are communicated via GJIC during cell-to-cell contact [31, 32] and via the exchange of soluble factors through biological fluids [25, 33]. These well-established mechanisms of bystander communication will be discussed in greater detail in section 2.2.2.

As previously alluded to, the first modern study citing RIBE was that published by Nagasawa and Little in 1992. They demonstrated that the direct traversal of less than 1% of a given cell population with α-particles was able to elicit sister chromatid exchanges in 30% of the cell population [24]. Following Nagasawa and Little's report, many studies followed supporting the influence of bystander effects in modifying cellular responses in various biological endpoints. In 1997, Mothersill and Seymour explored

the effect of bystander signals upon the clonogenic survival of non-irradiated cells subjected to cell culture medium harvested from directly-irradiated human epithelial cells exposed to Cobalt-60 γ-rays [25]. The results showed that the irradiated cell-conditioned medium (ICCM) was effective in reducing the bystander cells' clonogenic survival by approximately 40% compared to controls which received medium that was not cell-conditioned. The endpoint employed by Mothersill and Seymour, the clonogenic survival assay, was developed by Puck and Marcus in 1956 [34] and is an assessment of reproductive death in a cell population. Reproductive cell death, also referred to as mitotic catastrophe, is expressed as the loss of replicative potential following the attempt to undergo one or a few cell divisions following insult by radiation or another stressor [35]. The *in vitro* incidence of mitotic catastrophe can also be identified by the assessment of chromosomal aberrations and micronuclei formation using microscopy [36]. Chromosomal aberrations can be formed as a result of radiation-induced double-strand breaks (DSBs) and when left unrepaired, lead to cell death following a few cell divisions [37]. The assessment of chromosome rearrangements by Marder and Morgan revealed a marked correlation between the incidence of chromosomal instability induced by direct x-ray irradiation and delayed reproductive cell death [38]. The chromosomal aberration endpoint has also been studied in bystander cultures where direct irradiation of chinese hamster ovary (CHO) cells with α-particles yielded a 4-fold increase in the number of aberrations over that which was expected based upon the number of cells directly-irradiated [39]. Micronucleus formation is another commonly assessed endpoint in bystander investigations to indicate the potential for reproductive cell death in bystander populations. It manifests when a chromosome fragment does not get incorporated into a daughter nucleus during cell division [40]. Kashino and colleagues scored the formation of micronuclei in wild-type CHO and repair-deficient xrs5 bystander cells and found that micronuclei formation in the repair-deficient cells was significantly greater than that scored in the wild-type cells [41]. The results further demonstrated independence of micronuclei formation from the repair capabilities of the directly-irradiated cells from which the bystander signal originated, thus suggesting that the repair phenotype of the cells receiving the bystander signal is more influential in determining the overall bystander response than is that of the directly-irradiated cells [41].

Role of p53 in the bystander effect

Extensive research has been focused upon investigating the factors and intracellular pathways that are influential in mediating both the generation of and response to the bystander signal. The role of p53 in the bystander effect is investigated in the current thesis, therefore previous bystander studies involving p53 will be one focus of this review. The p53 protein, also referred to as tumour protein 53 (TP53), is an intracellular protein that functions to initiate DNA repair processes [42], promote cell death pathways such as apoptosis [43, 44], and regulate the cell cycle [45–47] in response to stress. p53 is considered a tumour suppressor gene because it functions to prevent continued proliferation of cells possessing aberrant DNA and functionality. Its role in suppressing the propagation of damage is evident when considering the functional capability of wild-type p53 mice in suppressing tumour formation compared to the prevalence of tumour growth in p53-null mice [48]. Apoptosis is triggered by p53 in cells which have incurred substantial DNA damage in order to maintain the integrity of the cell population [49]. In contrast, cell cycle arrest and repair mechanisms are initiated by p53 in cases where the damage is repairable so as to preserve cell viability [50]. Its role in initiating protective cellular responses leads to its title as the *guardian of the genome*.

In directly-irradiated cells when DNA is traversed by radiation, p53 becomes activated in response to the DNA damage via site-specific phosphorylation [51, 52]. In bystander environments where DNA has not been directly targeted by radiation, reactive oxygen species (ROS) have been shown to play a role in upregulating the activity of proteins involved in the p53 pathway such as p53 and p21 [53]. The influence of p53 in modulating the generation of and the response to the bystander signal is well documented in the literature. In a study by He and colleagues, bystander *signal generation* was shown to be dependent upon p53 functional status. In their study, HepG2 cells (wild type (wt) p53), PLC/PRF/5 cells (mutated p53), and Hep3B cells (null p53) were irradiated with γ-rays and co-cultured with Chang liver bystander cells (wt p53) where it was found that only the p53 wt cells could induce bystander effects in the form of micronuclei formation in the Chang liver bystander cells [54]. This bystander effect was linked to p53-triggered release of cytochrome c since the treatment of HepG2 cells with cyclsporin A, an inhibitor of cytochrome c release from the mitochondria, resulted in a diminished induction of micronuclei formation in the bystander cells [54]. Further evidence supporting the influence of p53 upon bystander

signal production was demonstrated by Komarova and colleagues in their investigation of the generation of growth-inhibitory factors following γ-irradiation of multiple cell lines possessing various p53 statuses. The results demonstrated marked upregulation of growth-inhibitory factor, transforming growth factor-β2 (TGF-β2) in wt p53 cell lines, as well as significantly greater growth suppression rates in p53 wt cells cultured in ICCM compared to p53 null cells [55]. He and colleagues also assessed bystander signal generation by cells of wt (TK6 lymphocyte cells) and mutated (HMy2.CIR cells) p53 status but extended their investigation to bystander induction by both high- and low-linear energy transfer (LET) radiation. Their experiments demonstrated an enhancement of micronuclei formation in reporter cells co-cultured with γ-irradiated TK6 cells but not in reporter cells co-cultured with γ-irradiated HMy2.CIR cells. However, this p53 dependence was not observed when reporter cells were co-cultured with carbon-irradiated (high-LET) cells [56]. Such a result suggests that bystander signalling may only be p53 dependent in the case of direct-irradiation with low-LET radiations. Bystander *responses* have also been shown to exhibit a dependence upon p53 status. Mothersill and colleagues showed that while the production of bystander signals did not appear to be influenced by the p53 status of HCT116 cells (wt and null), clonogenic survival assessed using the medium transfer technique demonstrated that bystander effects could only be exhibited by HCT116 cells possessing wt p53 [57]. In a study by Widel *et al.*, HCT116 wt and null cells were also employed to investigate bystander responses following direct x-ray irradiation. The results demonstrated that while both p53 wt and p53 null cell lines expressed elevated frequencies of cell death, apoptosis was preferably expressed by the null cell line and senescence predominated in the wild type cell line [58]. These studies provide substantial evidence for the involvement of p53 in mediating the RIBE. While it is clear that bystander effects can be induced in cells without normally functioning p53 (due to the involvement of alternative pathways in eliciting the bystander effect), it is apparent that p53 status does play a role in modulating the bystander effect given the selection of certain factors such as cell type and the lineal energy of the radiation source.

Role of mitochondria in the bystander effect

In considering potential non-DNA targets for the induction of NTEs, evidence has emerged supporting the role of reactive radical species in driving the RIBE [31, 53, 59]. Because mitochondria are known to generate higher concentrations of free radicals

in response to stress [60–62], they are considered to be a prime source of bystander signalling factors as a result of ionizing radiation traversal through the cell [63]. Intracellular sources of reactive radical species are also derived from water radiolysis and biological sources such as NADH(P)H-oxidase [53]. However, mitochondria are considered to be one of the major biological sources of radiation-induced radical species [64, 65].

As the metabolic centre of the cell, mitochondria produce free radicals derived from oxygen, referred to as reactive oxygen species (ROS), during routine respiration. The generation of superoxide ion radicals ($O_2^{\cdot -}$) by mitochondria occurs as a result of electron transfer from the semiquinone anion (Q^-, an unstable intermediate of ubiquinone) to molecular oxygen (O_2) at complexes I [62] and III [66] of the electron transport chain (ETC). The steady state concentration of ROS produced by mitochondria under normal conditions are scavenged by mitochondrial antioxidant defences such that the oxidative status of the cell is homeostatic [60]. Present within the mitochondrial matrix is an endogenous form of superoxide dismutase (SOD) containing manganese in its active site (MnSOD) which catalyses the dissociation of $O_2^{\cdot -}$ into hydrogen peroxide (H_2O_2) or O_2 [60]. The antioxidant substrates acting within the mitochondrial intermembrane space include copper-zinc-containing SOD (CuZnSOD) [67], glutathione peroxidase (to dismutate H_2O_2) [68], and electron carriers such as cytochrome c [60]. Upon irradiation with ionizing radiation, the balance between the production of ROS and antioxidants within the mitochondria can be disrupted resulting in a state of excess ROS, referred to as oxidative stress. Direct irradiation has been shown to elevate the production of ROS by mitochondria [64]. The effect of ionizing radiation on mitochondrial function has been shown to stem from the greater susceptibility of mitochondrial DNA to incur damage compared to nuclear DNA [69, 70]. Richter and colleagues extracted mitochondria from γ-irradiated rat livers and found that mitochondrial DNA expressed 6 times as many 8-hydroxydeoxyguanosine lesions as nuclear DNA [69]. Similarly, May and Bohr observed 2 times as many strand breaks in mitochondrial DNA as compared to nuclear DNA from γ-irradiated colon cancer cells, also reporting that repair in nuclear DNA was 55% more efficient than that in mitochondrial DNA [70]. Because mitochondrial DNA encode for 13 enzymes involved in the ETC [71, 72], modifications to mitochondrial DNA can lead to dysfunction in the process of cellular respiration. In a study by Ishikawa *et al.*, missense and frameshift mutations in mitochondrial DNA resulted in NADH dehydrogenase (Complex I)

dysfunction leading to overproduction of ROS [73]. Furthermore, Voets *et al.* identified an increase in ROS levels in fibroblasts expressing a defect in the mitochondrial DNA encoding Complex I but not in fibroblasts with a defect in the POLG1 gene (encodes a subunit of mitochondrial DNA polymerase) [74]. The mitochondrial ROS produced in response to ionizing radiation can affect other enzymes and molecules within the directly-irradiated cell itself, but has also proven to be an influential mediator of biological changes in bystander cells [65, 75].

The study of bystander cells in response to RIBE signalling has identified a role for mitochondria in the expression of biological effects. Mitochondrial DNA appear to be crucial in a cell's ability to produce bystander signals since Zhou *et al.* demonstrated a lack of signal production by fibroblast cells depleted of mitochondrial DNA and marked signal production by fibroblasts with fully functioning mitochondria [76]. The expression of bystander effects has also been linked to the interaction of bystander signals with the mitochondria of bystander cells, thereby suggesting a central role for mitochondria in propagating bystander responses. The impact of bystander signals upon mitochondrial DNA is apparent following a report by Murphy *et al.* that 0.5 Gy γ-ICCM induced deletions and point mutations in the mitochondrial genome of HPV-G human keratinocyte reporter cells [77]. The role of mitochondria in RIBE was further clarified when Rajendran and colleagues reported that cell lines possessing functional mitochondria had the capacity to exhibit significant bystander responses (micronuclei formation) following ICCM transfer while cells with mutated mitochondrial function did not [78].

In an effort to identify the induction of mitochondrial dysfunction in bystander populations, mitochondrial membrane potential is commonly investigated as an endpoint. Among studies assessing mitochondrial membrane potential, it was found that mitochondrial membrane depolarization was induced in bystander cells by medium transferred from γ-irradiated human keratinocyte cells [79–81], UV-A-irradiated melanoma cells [82], and γ-irradiated colorectal tissue explants [83]. Mitochondrial membrane depolarization can be indicative of the opening of the mitochondrial permeability transition pore (PTP), found on the inner mitochondrial membrane, which can be triggered by the presence of free radicals [84] or an influx of intracellular calcium levels [85] subsequent to the receipt of bystander signals. An open PTP facilitates the influx of ions less than 1.5 kDa and water into the mitochondrial matrix, subsequently leading to the intracellular release of mitochondrial apoptogenic factors

such as cytochrome c [86]. Cytochrome c then binds to Apaf-1 to activate caspase-9 and caspase-3, leading to the downstream initiation of apoptotic cell death [86]. The role for apoptosis in the bystander effect was confirmed by Maguire and colleagues when they showed the ability of a caspase-9 inhibitor to prevent clonogenic death in bystander cells treated with ICCM [87]. Yang *et al.* have shown that cytochrome c originating from mitochondria, is also critical in detecting and responding to bystander signals since murine fibroblast cells deficient in cytochrome c exhibited attenuated expression of micronuclei in response to co-culture with α-irradiated cells compared to fibroblast cells expressing wild type cytochrome c [88]. Alternative to the indication of apoptosis, mitochondrial membrane depolarization can also be used as an indicator of mitochondrial dysfunction in regard to its capacity for synthesizing adenosine triphosphate (ATP). A loss of mitochondrial membrane potential directly affects the function of ATP synthase since the electrochemical gradient used to shuttle protons through the ATP F_0 and F_1-ATPase subunits has been compromised. Reduction in homeostatic intracellular ATP levels by approximately 30% has proven to initiate cells to undergo apoptosis [89]. In cases where cellular ATP levels are reduced even further, the cell initiates an alternative mode of death called necrotic cell death, which ascribes to bioenergetic deficiency [89, 90]. Necrotic death is considered to be uncontrolled and lacks the order and characteristic features that apoptosis does since the cell is deprived of the ATP required to carry out the orderly energy-dependent steps characteristic of apoptosis [89]. Vast amounts of literature have thus confirmed the importance of mitochondrial participation in the RIBE.

2.2.2 Bystander Effect Communication Mechanisms

There is consensus in the bystander community that the initiation of bystander effects occurs as a result of communication from directly-irradiated cells to unirradiated bystander cells (also referred to as reporter cells) [1, 91]. There are two well-established mechanisms of bystander communication that both operate on the basis of intercellular soluble factor exchange. Gap-junction intercellular communication (GJIC) facilitates the exchange of bystander factors through channels formed between adjacent cells by two hexameric connexons across the intercellular space [92] (figure 2.1, left). The second mode of bystander communication is accomplished via the sharing or exchange of biological fluids, such as cell culture medium, between irradiated and bystander cell populations. In the latter form of bystander communication, direct cell-to-cell

contact is not required between an irradiated cell and a bystander cell for an effect to be observed [33]. This mode of communication can be evaluated *in vitro* by the harvest of cell culture medium from irradiated cells and subsequent transfer of that medium onto bystander cells in a method called the medium transfer technique (figure 2.1, right). Alternatively, a directly-irradiated cell population can be co-cultured with a bystander population such that the two cultures share an analogous source of medium (figure 2.1, middle).

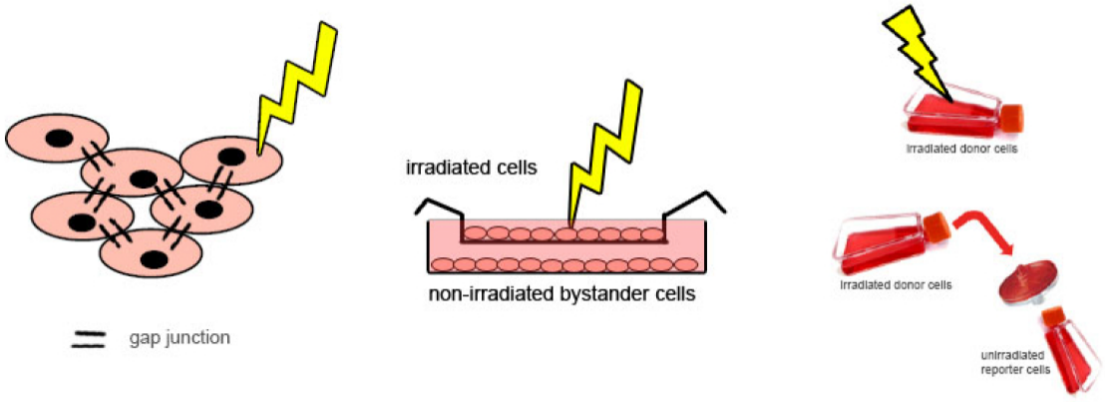

Figure 2.1: Methods of assessing RIBE chemical factor communication. Left: gap-junction intercellular communication, middle: co-culture technique, right: medium-transfer technique.

The involvement of gap junctions in the bystander effect was first proposed by Azzam and colleagues in 1998. They showed that direct-traversal of a small fraction of fibroblast cell nuclei by α-particles produced expression of the CDKN1A gene (G1 cell cycle arrest regulator) to a magnitude that was 4 to 20 times greater than expected, based on the fraction of cells whose nuclei were directly traversed by α-particles [31]. The participation of gap junctions in bystander signalling was later confirmed by their research group when lindane, an inhibitor of connexin-43 function, was proven effective in inhibiting the transduction of bystander effects to non-irradiated cells [32]. Azzam *et al.* had already shown in their 1998 study that the same connexin-43 inhibitor was unable to alter the biological responses exhibited by directly-irradiated cells [31], thus confirming that the modification of gap junction intercellular communication only influenced bystander radiation responses and not direct radiation responses. Following these pioneer studies on GJIC, the dependence of the bystander effect on gap junctions was further solidified by several research groups showing that the

expression of bystander endpoints could be suppressed by the application of non-toxic concentrations of gap-junction inhibitors [93, 94] or by the use of cells deficient in gap junctions [32]. These studies collectively demonstrated a crucial role for GJIC in the communication of radiation-induced bystander effects.

Communication of the bystander effect has also been demonstrated via a method that does not require direct cell-to-cell contact. The initiation of bystander effects via the exchange of filtered cell culture medium was first demonstrated by Mothersill and Seymour in 1997 when they reported the significant reduction of clonogenic survival in non-irradiated epithelial cells treated with γ-ICCM [25]. The possibility that the culture medium, itself became toxic as a consequence of irradiation was eliminated since a response was not observed in bystander cells treated with medium irradiated in the absence of cells [25]. Further details regarding the nature of this bystander system were elucidated in the following years. It was found that a response could be induced in bystander cells following incubation of non-irradiated cells with ICCM for as little as 30 minutes [33]. Furthermore, an effect was sustained in bystander cells even after the ICCM was subjected to multiple freeze-thaw cycles [33]. However, after heating the ICCM to temperatures exceeding 70°C, the effect in non-irradiated cells was effectively abolished [33]. The degree of clonogenic cell killing in the bystander population could also be abrogated by maintaining the directly-irradiated cells at 0°C during exposure to γ-rays [33]. These observations led Mothersill and Seymour to suggest that firstly, the bystander factor released from irradiated cells could be a protein due to its susceptibility to abrogation at high temperatures, and secondly, the mechanisms involved in bystander communication are likely dependent upon metabolism as opposed to a passive release of molecules.

This work was followed by research conducted in several laboratories seeking to identify the extracellular mediators involved in communicating a bystander response. As it turns out, many candidates have been identified, which is not surprising considering the marked presence of redundancy in biological systems [95]. Following the hypothesis that a protein-like molecule could be the responsible bystander factor [25], the extracellular release of interleukin-8 (IL-8) was shown to result from α-irradiation of human fibroblasts cells [96]. Since then, other cytokines such as IL-6, TGF-β1 and tumour necrosis factor (TNF)-α, have also demonstrated roles in the bystander response [76, 97, 98]. The involvement of cytokines in bystander signalling was further clarified when the inhibition of TNF-α by inhibitory monoclonal antibodies proved

to be effective in reducing the activation of the cyclooxygenase-2 (COX-2) signalling pathway in bystander cells [76, 99]. In addition to cytokines, reactive radical species such as ROS have also been implicated as one of the responsible bystander factors. The production of $O_2^{\cdot -}$ and H_2O_2 was found to follow direct α-particle irradiation [100]. Accordingly, studies followed to elucidate the involvement of ROS in bystander signalling to find that the treatment of ICCM with radical scavengers, SOD and catalase, was effective in reducing micronucleus formation in bystander cells [53]. These studies also showed that diphenyleneiodonium (DPI), a NAD(P)H oxidase inhibitor, is effective in inducing a pronounced decrease in the responses exhibited by bystander cells thereby implicating NAD(P)H oxidase as a primary source of bystander ROS [53, 100]. Most recently, the involvement of exosomes in communicating bystander effects has been proposed in the literature [101]. Exosomes are 50-150 nm membrane-bound extracellular vesicles that encapsulate cargo unique to the cells from which they originated [102]. The action of exosomes from irradiated cells is capable of modifying intracellular calcium levels, intracellular ROS levels, and cell viability in recipient cells whereas exosomes from non-irradiated cells could not [103]. Exosome concentration in ICCM has also been shown to share a positive relationship with increasing γ-radiation dose [103]. Perhaps the most intriguing relationship found between exosomes and ionizing radiation to date is the observation that the contents characterized within the exosomes derived from irradiated cultures differ from those derived from non-irradiated cultures of the same cell type [104]. It is therefore believed that the action of the contents carried within exosomes are responsible for eliciting biological modifications in bystander cells [101, 105, 106]. Xu *et al.* reported that microRNAs delivered between irradiated and bystander cells by exosomes were able to functionally modulate gene expression in the recipient cells [105]. Furthermore, Al-Mayah and colleagues were able to demonstrate partial inhibition of bystander responses in reporter cells by treating exosome fractions with either ribonuclease (RNase) or by boiling at 98°C for 10 minutes prior to placement onto bystander cells [106]. However, when both RNase and boiling were employed, nearly complete inhibition of the bystander effect was observed [106]. Such an observation implicates both RNA and proteins as factors critical to the exosome-mediated bystander effect. The demonstrated involvement of exosomes in communicating the RIBE, while still relatively novel, is evidently an important finding that will undoubtedly spark ongoing future investigation.

While GJIC and soluble factor mediation are both very well-established and in-

disputable bystander communication mechanisms, there is animal work reporting radiation bystander effects that may not be explainable by the two existing bystander communication mechanisms. The first observation of inter-organism bystander signalling was reported by the Surinov research group where significant reductions in leukocyte count were observed in non-irradiated rats and mice that were placed in the same cage as irradiated mice [107]. In following publications from Surinov's group, immunosuppression was exhibited by non-irradiated cagemates of irradiated mice [108, 109]. Since the mice did not come into physical contact with each other, the researchers deduced that the effect may have been attributed to the exchange of volatile molecules through an airborne route between the cagemates [108]. Further suggestion of the existence of a physical bystander signal came when both Mosse *et al.* and Marozik *et al.* observed protection of reporter cells from the bystander effect by treatment with the photoprotector, melanin [110, 111]. Mosse was one of the first to suggest that the bystander effect may be influenced by a physical component that was electromagnetic in nature due to the ability of melanin to modulate the response. This suggestion was further corroborated by experiments conducted by Mothersill *et al.* whereby physical separation of two fish by a partition still resulted in the observation of increased calcium flux in the non-irradiated bystander fish [112]. The possibility that the effect was attributed to volatiles and exchange of soluble factors through the water were eliminated since the two fish were held in isolated tanks [112]. Most recently, Fernandez-Palomo explored inter-animal communication between synchotron microbeam-irradiated Wistar rats and non-irradiated cagemates after co-habitation for 48 hours [113]. Bladder and brain tissues were harvested from both irradiated and non-irradiated animals, and the growth medium used to culture the tissue explants were incubated with HPV-G human keratinocyte reporter cells. Interestingly, the clonogenic survival exhibited in the reporter cells which received the non-irradiated cagemates' explant-conditioned medium was reduced even moreso than the cells that received irradiated explant-conditioned medium [113]. This result very clearly confirmed the transmission of a signal from irradiated rats to non-irradiated rats [113]. The collective existence of these observations suggest that there may be alternative forms of RIBE communication that have yet to be explored.

From an *in vitro* perspective, Ahmad *et al.* suggested that one possible mechanism of RIBE communication could be the action of UV photons emitted from directly-irradiated cells upon non-irradiated bystander cells [114]. This inference was drawn

following the observation of significant UV photon emission by human keratinocyte cells upon exposure of those cells to ionizing β-radiation [114]. Photon emission was also observed upon exposure of oyster tissue and citrus leaves to proton radiation [115]. To explore the hypothesis posed by Ahmad *et al.*, the current thesis investigates the possibility that signalling to non-irradiated cells occurs through electromagnetic waves emitted from cells irradiated with ionizing radiation. These electromagnetic waves produced by cells are referred to in the literature as *biophotons* [116]. While communication by electromagnetic waves has not been previously demonstrated in the field of radiation research, there is a wealth of literature dating back to the 1920s demonstrating that both cells and organisms communicate with each other using the light referred to as biophotons [117]. The involvement of biophotons in intercellular and inter-organism communication under circumstances not involving radiation provides some support that it could also be involved in cellular signalling when radiation is the perturbing agent.

2.3 Light/Electromagnetic Signalling in Biological Systems

Electromagnetic (EM) energy is energy which exhibits properties of both particles and waves. The emission and absorption of EM energy occurs in quanta called *photons*. Photons are referred to as *light* but this term is frequently interpreted to encompass only the visible wavelength range of the EM spectrum, falling between 400 and 700 nm. Despite this, the term can extend to include the entire spectrum of EM radiation (figure 2.2). For the purposes of this thesis, subsequent use of the term *light* will be intended to refer to the UV (100 to 400 nm), visible, and infrared (IR) (700 to 1000 nm) wavelength regions since higher energy electromagnetic radiations such as x-rays and γ-rays are also a topic of importance in the current thesis.

The energy and the frequency of a given photon shares an inverse relationship with its wavelength. For example, the energy of a 100 nm UV photon is 12.4 eV and its corresponding frequency is 3 PetaHz, whereas a 1000 nm IR photon only possesses an energy of 1.24 eV and a frequency of 100 TeraHz. Following this characteristic, we can deduce that the shorter wavelength photons such as those within the UV range are more energetic than visible and IR photons which possess longer wavelengths.

While it is intuitive to assume that higher energy photons are capable of inducing greater biological effects in a given target, the biological role of lower energy photons in the visible and infrared range cannot be discounted following the report of extensive biomodulation by light in the visible and infrared wavelength regions. The following section will discuss evidence for the modulation of biological processes and cellular functions by UV, visible, and IR light.

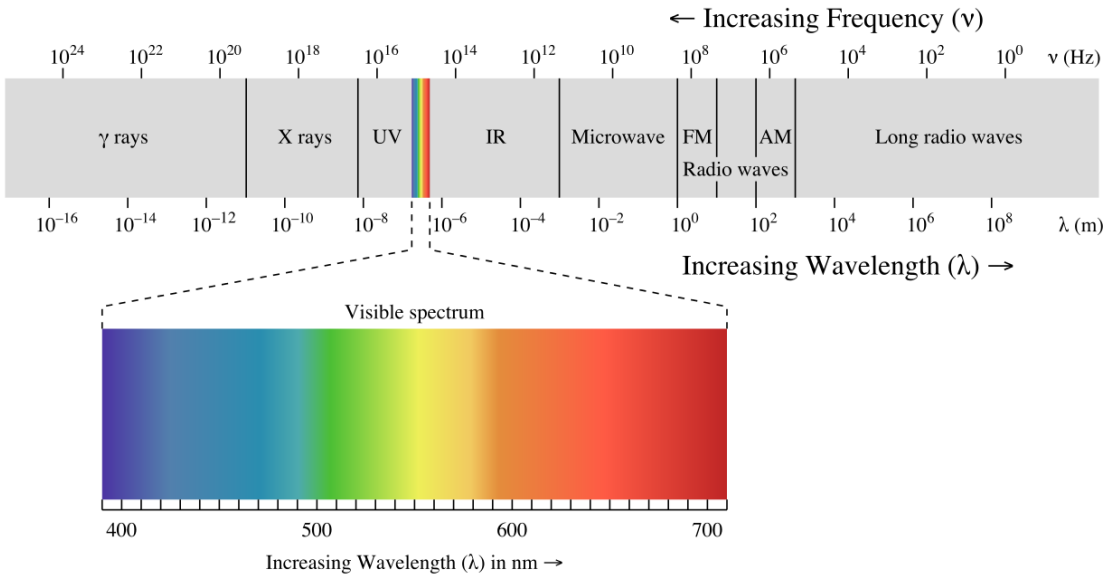

Figure 2.2: The electromagnetic spectrum. Figure retrieved from [118].

2.3.1 The Effect of Exogenous Light on Biological Functions

Modulation of biological systems by exogenous sources of light is a well-documented phenomenon. The action of UV light upon the cell can be detrimental [119–121] whereas certain wavelengths of visible light are used in low-level light therapy applications to stimulate biological processes leading to beneficial effects [122]. The effects of UV light are widely studied since UV constitutes approximately 8% of all solar emissions [123] where approximately 90% of the UV that reaches the Earth's surface is UV-A (320-400 nm) and 10% is ultraviolet-B (UV-B) (280-320 nm) [123]. UV-A possesses lower energies than the rest of the UV spectrum and acts to induce cellular damage in an indirect manner. Interaction of UV-A photons with photosensitizers facilitate the generation of ROS which subsequently act upon DNA to produce the characteris-

tic UV-A-induced lesion, 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxo-dG) [124, 125]. Guanine's susceptibility to oxidation is caused by its low redox potential [126]. An increased expression of 8-oxo-dG has been associated with an elevated susceptibility for the cell to undergo either uncontrolled proliferation or cell death since 8-oxo-dG preferably misinserts opposite adenine bases as opposed to cytosine bases, to produce mutagenic substitutions in the genome [119]. UV-B and ultraviolet-C (UV-C) radiation induce DNA damage directly via absorption of UV photon energy by DNA bases [127]. The wavelengths of UV-B and UV-C photons coincide closely with the absorption spectra for cytosine and thymine bases, therefore, the production of DNA lesions expressing base modifications is not surprising [128]. Cyclobutane pyrimidine dimers and 6-4 photoproducts are lesions characteristic of direct UV action that have been linked to mutagenesis when DNA encoding p53 has been targeted [120]. Pyrimidine dimer formation has also been shown to initiate immunosuppression in UV-irradiated mice [121].

Shifting over to the visible spectrum leads to the consideration of lower energy photons and their action upon biological systems. Blue light is characterized by wavelengths between 450 and 490 nm and has proven to induce modulatory effects in various biological systems. In mammals, the photoreceptor cells responsible for regulating the circadian rhythm (intrinsically photosensitive retinal ganglion cells, ipRGC) function to suppress melatonin levels in response to blue light [129]. These cells are most responsive to blue light, which is most prominent during daytime sunlight, than it is to red light which is prevalent as the sun approaches the horizon [130]. It has therefore been demonstrated that the exposure of ipRGC cells to extraneous sources of blue light during irregular hours have the capability of disrupting human sleep-wake cycles [130]. Blue light has also been used in phototherapy as it has been shown to induce anti-proliferative effects due to its capacity for generating ROS [131]. In a 2017 study by Oh *et al.*, blue LED light was used to inhibit cancer cell proliferation whereby the authors reported that the light-induced ROS acted by upregulating the phosphorylation of p38 mitogen-activated protein kinases (MAPK), a pathway involved in initiating cell differentiation and cell death [132].

In contrast to the inhibitory effects observed as a result of blue light exposure, laser and LED sources of red light (620-700 nm) and IR light (700-1000 nm) are widely used in therapeutic applications as they have been shown to stimulate cellular repair and proliferation [133]. While the mechanisms driving the biostimulatory effects by

red and IR light have not yet been clearly elucidated, it is believed that the absorption of the red and IR photons by mitochondrial cytochrome c oxidase (Complex III) is a key factor [134]. The absorption of these photon wavelengths by cytochrome c oxidase leads to accelerated electron transport [135], enhanced enzyme activity [136] and subsequently increased mitochondrial ATP production [122]. The increase in ATP production is thought to drive transcription [137] and subsequent upregulation of proteins involved in cell repair, proliferation and survival [138].

Overall, the demonstration that exogenous sources of light have the capability of modulating biological functions provides support for the possibility that biomodulatory effects can also be induced via the exchange of endogenously-sourced photons.

2.3.2 Biophoton Production in Living Systems

The phenomenon of spontaneous ultra-weak photon emission by biological systems was first documented in the 1930s where the photons from biological materials were measured using Geiger-Mueller counters fit with quartz windows [139, 140][1]. These pioneer experiments revealed that the fluences emitted from biological materials were relatively low, ranging from 10^1 to 10^3 photons cm^{-2} s^{-1} [139, 140][1], the emitted photons were either UV or contained a UV component [141], and the photon emissions could be detected from a variety of biological materials including blood [142][1], bacteria [143][1], and tumour tissue [117]. These photon emissions were distinguished from luciferin-luciferase-attributed bioluminescence since the photon yields in this system were 10^3 to 10^6 times weaker than the levels characteristic of luciferin-luciferase luminescence [144]. During the 1930s, the reports of positive photon emission were also challenged by negative results observed by other researchers [145, 146]. In the 1930s, the detection systems were all developed and constructed manually, thus it is likely that the detectors in each research group possessed different sensitivity and possibly explains the variability among the results observed [147]. Nevertheless, presence of evidence disproving the phenomenon led to a diminishing interest in the effect and scientific investigation into photon emission from biological materials was not revived until the 1950s when photomultiplier tubes (PMTs) became widely available for measuring photon sources with relatively low fluence [148, 149]. Only following the application of PMTs did detection of biological emissions become more reliable and reproducible [147]. Further

[1]Articles published in German.

characterization of these photon emissions demonstrated a qualitative dependence upon biophoton signal strength that did not adhere to the laws of linear physics. VanWijk *et al.* demonstrated significantly greater photon emission from tumour cells than normal cells even when each type of cell was maintained at the same quantity [150]. Such results suggested that photon emission linked to biological processes could not be explained purely based upon physical expectations. The phenomenon of biological photon emission was given a name in 1984 when German biophysicist, Dr. Fritz-Albert Popp, coined the term *biophotons* to refer to the weak fluence of UV and visible range photons emitted from biological systems [151]. Up until then, this phenomenon was often referred to as *ultra-weak photon emission (UPE)* [147], although the name did not indicate specificity to the photons' biological origin. Presently, the two terms are used interchangeably in the field of biophotonic research.

Following the confirmed existence of biophotons, the question of how the photons were being produced and whether they had any downstream biological effects arose as the two predominant lines of inquiry. Since the latter question will be addressed in section 2.3.3, the current discussion will focus upon presenting literature relevant to elucidating potential biophoton sources. Based upon the available literature, the leading candidate for intracellular biophoton production is the generation of excited species and their subsequent relaxation to a stable state. Lipid peroxidation and oxidative metabolism in the mitochondria can both lead to the production of electronically-excited states in biological systems [152]. Both singlet oxygen ($^1O_2$) and carbonyl compounds in the triplet state have been identified as sources of biophoton emission by the measurement of spectral emission following the use of various excited species scavengers (i.e. β-carotene) and sensitizers (i.e. rotenone) [153]. In the case of lipid peroxidation, both $^1O_2$ and triplet state carbonyls are confirmed products of the process [154]. In lipid peroxidation, oxidants react with lipids containing carbon-carbon double bonds resulting in the abstraction of hydrogen from a carbon (L·) and subsequent insertion of O_2 in its place to form a lipid peroxyl radical (LOO·). The LOO· then abstracts hydrogen from another lipid molecule to produce lipid hydroperoxides (LOOH) and another L·, which will subsequently function to carry on the reaction [155]. The main products of the lipid peroxidation process are the lipid hydroperoxides (LOOH). These LOOH molecules react with peroxynitrite (generated in the cell when nitric oxide interacts with $O_2^{\cdot -}$) or hypochlorous acid (reactive species present at sites of inflammation) to produce $^1O_2$ [156]. Alternatively, LOOH can

interact with peroxynitrite or heme proteins to produce excited triplet carbonyls [157]. Mitochondria are considered another important source of biophoton emission because of the abundance of molecular oxygen (ground state triplet oxygen, $^3O_2$) present in the mitochondrial respiratory chain. The oxygen involved in the respiratory chain therefore predisposes the mitochondria to an increased probability for $^1O_2$ generation. Excitation and subsequent change in electron spin of one of the electrons in $^3O_2$ results in the production of $^1O_2$ [60]. Because the probability for excitation of $^3O_2$ is associated with oxidant activity in the mitochondria, a relationship therefore exists between photon emission and mitochondrial metabolic activity [60].

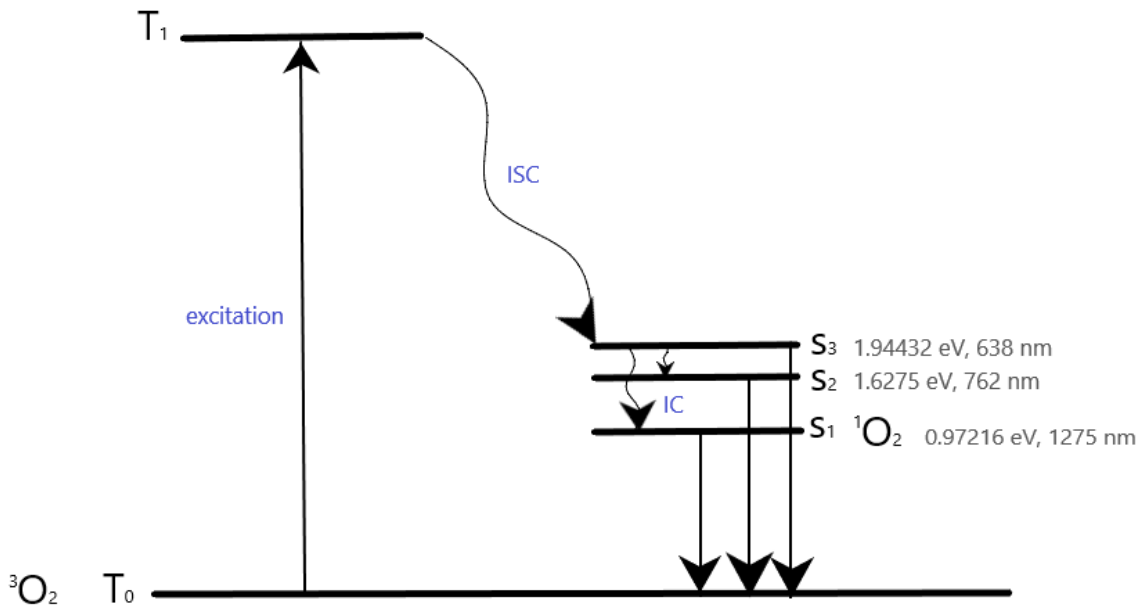

Figure 2.3: Jablonski diagram depicting electronic transitions following the absorption of energy by molecular oxygen which possess a triplet ground state. All excited triplet states are not depicted. S: singlet state, T: triplet state, IC: internal conversion, ISC: intersystem crossing. Adapted from diagram distributed by Columbia University [158].

Biophoton emission manifesting as a result of lipid peroxidation and mitochondrial oxidative metabolism occur by the radiative decay of excited $^1O_2$ and triplet carbonyls. Singlet oxygen emits photons via phosphoresence following the transition from the singlet excited state to the triplet ground state in a process called intersystem crossing [159] (figure 2.3). Phosphoresence (10^{-3} to 10^2 seconds) is characterized by a longer time scale than is fluorescence (10^{-9} to 10^{-6} seconds) due to the required transition between the singlet and triplet states compared to an intrasystem transition between

an excited and ground state within one of the singlet or triplet domains [159]. The radiative decay of $^1O_2$ and dimerized $^1O_2$ occur via reactions 2.1 and 2.2 to produce infrared (1275 nm) and red (638 nm, 762 nm) photons, respectively [159, 160]. Excited triplet state carbonyls can exhibit phosphorescence in the visible region between 380 and 450 nm via reaction 2.3 [161]. Alternatively, the excess energy possessed by the excited carbonyl can be transferred to another molecule such as a fluorophore and the energy can be subsequently emitted by the de-excitation of the recipient molecule [161] (figure 2.4). This energy transfer process can therefore result in photon emission at wavelengths alternative to those specified here.

$$^1\Delta O_2 \rightarrow^3 \Sigma O_2 + h\upsilon_{1275nm} \tag{2.1}$$

$$2\,^1\Delta O_2 \rightarrow 2\,^3\Sigma O_2 + h\upsilon_{638nm,762nm} \tag{2.2}$$

$$^3[> C = O]^* \rightarrow\ ^1[> C = O] + h\upsilon_{380-450nm} \tag{2.3}$$

Nakano and colleagues also provided evidence to suggest that excited tryptophan and excited tyrosine, produced as a result of oxidation by peroxidase, are candidate sources of biophoton emission. Their research supports these amino acids as potential biophoton sources since the emissions they observed from sea urchin eggs undergoing fertilization (a process associated with lipid peroxidation) coincided very closely with the reference emission spectra of excited tyrosine (280-350 nm) and excited tryptophan (300-450 nm) [162]. Konev and colleagues also attributed the luminescence detected from yeast cells to the relaxation of excited tryptohan following the observation of striking resemblance between the optical spectrum of excited tryptophan fluorescence and the spectrum characterized from the yeast cells [163]. Given the evidence presented here, it is likely that biophoton emission can originate from the excitation and subsequent de-excitation of many intracellular molecules that may be currently unexplored. Nevertheless, the contribution of tryptophan, tyrosine, singlet oxygen, and excited triplet carbonyls collectively demonstrate the ability of cells to emit biophotons

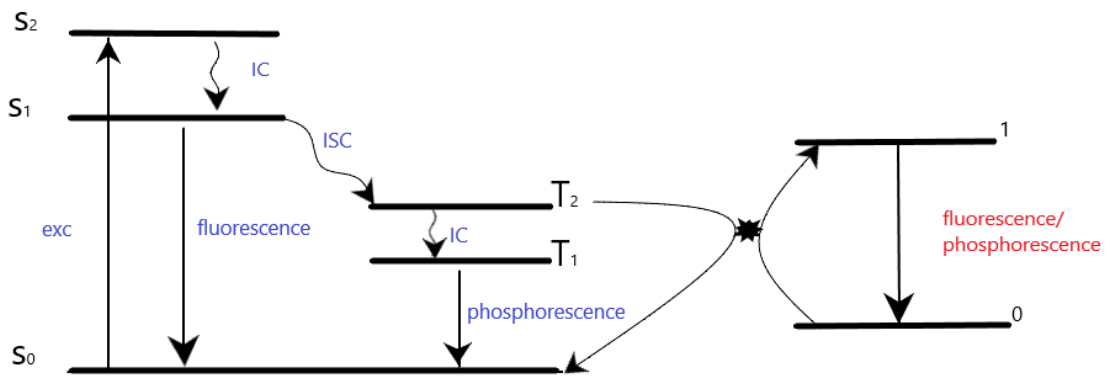

Figure 2.4: Jablonski diagram depicting electronic transitions following the absorption of energy by a given molecule possessing a singlet ground state. Energy can also be transferred from the excited triplet state to induce excitation in another molecule as exhibited here. This diagram would be representative of the electronic transitions possessed by a carbonyl, for example. S: singlet state, T: triplet state, IC: internal conversion, ISC: intersystem crossing.

ranging from UV all the way to IR wavelengths [164]. While it is apparent that the spectral distribution of biophoton emission spans the UV, visible and IR regions, specific details pertaining to the signal strength and fluence rate at each wavelength still remain difficult to determine.

In the 1980s, Fritz-Albert Popp suggested that DNA should be an important factor in the consideration of a potential source of biophotons [151]. His research using soybean cell cultures treated with ethidium bromide (a DNA intercalation reagent expressing minimal reactivity with other biomolecules) illustrated maximum photon emission at the concentration known to be most effective at decondensing tertiary DNA structures (30 μg/mL) [151]. This work demonstrated evidence for the involvement of DNA conformation in mediating biophoton emission. Subsequent research corroborated Popp's DNA-centric hypothesis when Niggli and colleagues observed effective biophoton emission by normal fibroblasts following illumination with incident visible light and a lack of efficient light emission by DNA repair-deficient Xeroderma Pigmentosum fibroblasts [165]. A hypothesized rationale to explain DNAs role in biophoton emission is the susceptibility for DNA to form polynucleotide excited dimers (excimers) in long-lived triplet states [164]. These excimers, in turn, have an affinity for photon trapping since their free energy is lower than that of the molecular fragments [164]. Nuclear DNA appears to be essential in the storage of photons since Rattemeyer *et al.*

reported an absence of biophoton detection from human erythrocytes which lacked nuclei [166]. Despite the evidence supporting a role for DNA in biophoton emission, the mechanisms by which nuclear DNA receive and store light have yet to be clearly elucidated.

In addition to investigating the potential sources of photon emission, research was extended to exploring the stimuli that were capable of triggering biophoton emission. It was found that biophoton emission could be observed both spontaneously [167], that is without external perturbation, and following the application of an exogenous stressor such as ultraviolet light [165, 168], visible light [169], chemicals [151, 170], and mechanical destruction [171–173]. The collective conclusion drawn following the exploration of these various triggers of biophoton emission was the suggestion that its measurement it could be used as a means of non-invasively determining the oxidative status within an organism or biological system having undergone stress or under normal conditions [174, 175]. The baseline oxidative activity during normal respiration, excess ROS production, and diminished antioxidant activity could therefore be monitored in real-time and used as a pathophysiological biomarker [176–178].

2.3.3 Biophoton-Mediated Intercellular Communication

There is a strong repertoire of literature supporting the capacity for biological systems to communicate with each other via the exchange of biophoton signals [117, 179–183]. The first documented evidence of intercellular communication via optical signalling was reported by Dr. Alexander Gurwitsch in 1923. Gurwitsch reported the ability of an onion root meristem to induce a 20-25% increase in the mitosis rate of a neigbouring onion root meristem placed 2 mm away for a 2 hour duration [117]. He noted that the surface of the recipient onion root that was exposed to the sender onion root exhibited a greater number of dividing cells than did the opposite surface. Further investigation led to the suggestion that the responsible factor was light in the UV range since the insertion of a glass plate 0.3 mm thick resulted in the elimination of the effect while insertion of a thinner (tens of μm) glass plate did not [184]. Gurwitsch called this observed phenomenon the *mitogenetic radiation theory* after the observation that light could stimulate mitosis [117]. It was later confirmed that UV light is indeed emitted from living cells upon detection with sensitive photon counters (PMTs) [185, 186].

The involvement of UV biophotons in eliciting optical intercellular communication has been further supported by subsequent studies. For instance, Wainwright and

colleagues demonstrated the induction of visible light emission by bacteria separated from fungus by a layer of quartz glass (permits transmission of wavelengths greater than 150 nm) [187]. However, upon separation of the bacteria from the fungus by UV-impermeable glass (which permitted the transmission of wavelengths greater than 340 nm), the effect was no longer observable [187]. Beloussov and Baskakov also reported a marked induction of accelerated frog egg division (cleavage) upon exposure of bystander eggs to the biophotons emitted from a dense cluster of cleaving eggs; the two egg populations were separated by quartz glass [188]. In addition to quartz, polystyrene is also effective in transmitting UV wavelengths, although only those greater than approximately 260 nm [189]. The use of polystyrene petri dishes by Kirkin proved to be effective in stimulating the proliferation of a sparse culture of rat cancer cells following optical exposure to a dense culture of the same cell type [190]. In contrast with the induction of proliferative effects by biophotons, Shen *et al.* have also shown that biophotons are capable of triggering stress responses in optically-coupled cell populations. The measurement of ferricytochrome c reduction, employed as an indirect indicator of $O_2^{\cdot -}$ production, revealed a significant elevation in $O_2^{\cdot -}$ levels within the bystander population of neutrophils optically-coupled to a neutrophil population that was treated with phorbol myristate acetate (PMA), a respiratory burst stimulator [179]. Rossi and colleagues also conducted experiments on human endothelial cells and mouse fibroblast cells chemically separated from each other by stacking polystyrene petri dishes on each other to find that cells of the same type were able to induce significant reductions in reporter cell viability [191]. They also reported that upon assessing the morphology of the reporter cells, adherence to the dish was poor and morphology was modified. These effects, however, were not observed when a black sheet of cellulose acetate was placed in between the two petri dishes [191]. Since it has become apparent that not all biophoton-mediated intercellular interactions result in proliferative endpoints, it is suggested that it may be more suitable to refer to these effects collectively as *biophoton-mediated communication effects* as opposed to *mitogenetic effects*.

Further research has demonstrated a role for biophotonic exchange of information by wavelengths extraneous to UV in inducing effects in biological detectors. Albrecht-Buehler conducted experiments upon baby hamster kidney (BHK) cell cultures separated by a glass coverslip. He observed the ability of a confluent layer of BHK cells to induce perpedicular alignment in the optically-coupled, sparsely-plated layer

of bystander cells [180]. Plating of the glass with Nickel-Chromium metal revealed an inhibition of the effect manifesting as randomly oriented bystander cells, thus suggesting that the signal was not chemical in nature but electromagnetic. In another experimental permutation, treatment of the glass with a silicone filter, optically capable of strong absorption of blue light while allowing transmission of red and infrared light, resulted in perpendicular orientation of the bystander BHK cells and led to the conclusion that red and infrared wavelengths were responsible [180]. In another study, Fels assessed the response of paramecia placed in cuvettes of quartz or glass to determine biological response to biophotons under conditions permitting and impeding the transmission of UV wavelengths, respectively [181]. When paramecium populations were separated by glass, the reporter population demonstrated accelerated cell division whereas separation by quartz resulted in a reduction in the cell division rate compared to controls that were not coupled to another cell population. The author hypothesized that cell populations probably employ two or more electromagnetic wavelengths in the process of information exchange since the interference of discrete portions of the spectrum appeared to modulate the biological responses exhibited, but not abolish the response completely [181].

The idea that multiple biophotonic frequencies may be employed in the endeavour to communicate cellular information is one that has been proposed widely in an effort to explain the ability of such low fluence signals to induce significant and observable biological modifications [151, 192–194]. Popp was the first to propose the coherence of light as a means of communication and information transfer within biological systems [151]. The concept of coherence describes the cooperative action of multiple subunits in a given system to carry out a function [194]. Following this framework, Bajpai proposed the role of quantum coherence in the ability of biological systems to communicate with each other via biophotons and subsequently regulate biological processes [194]. While extensive discussion on the topic of quantum biology is beyond the scope of this review, a brief explanation of the observed effects that are difficult to rationalize using classical mechanisms prescribes to the quantum coherence of light following absorption by a recipient cell, and the use of that coherence to maximize the efficiency of a given biological process by enacting simultaneous pathways in an effort to achieve the desired outcome or effect [195]. A hypothesis such as that proposed by Bajpai is now conceivable in a biological setting based upon the elucidation of a role for quantum coherence in explaining the efficiency of photosynthesis [196]. Another

proposed mechanism of cellular communication via biophotons is the encoding of information in photon bursts resembling binary-encoded data. Mayburov characterized photon emission from fish and frog eggs using a photomultiplier tube sensitive to both UV and visible light to find that the photon emission signal integrated over all of the wavelengths exhibited fluctuations in strength over time [197]. The time-dependent signal output over 400 seconds revealed distinct bursts of light at randomly interspaced intervals and subsequent analysis of reporter egg populations revealed an ability of the biophotons to synchronize growth among those bystander eggs [197]. While typical biophoton fluence rates are relatively low, the proposed mechanisms for communication provide a means of ascertaining how they may be capable of eliciting effective signalling between biosystems.

The research on biophoton communication presented thus far has focused primarily upon effects that result following exposure of bystander systems to spontaneous, or unperturbed, sources of biophotons. However, biophoton emission leading to intercellular communication of effects can also be induced by various stressors. Kaznacheev reported distant intercellular electromagnetic interaction between optically-coupled healthy human fibroblast cells and human fibroblasts which were infected with the Coxsackie A-13 virus or injured with mercuric chloride [182]. Interestingly, the unperturbed bystander fibroblasts exhibited mitotic inhibition, nuclear condensation, and subsequent cellular fragmentation; effects that are all characteristic of a cellular response to the Coxsackie A-13 virus. Further investigation was unable to isolate any viruses from the bystander cultures [182], eliminating the possibility that the virus could have travelled from the infected population to the bystander population via chemical means such as transfer of volatile molecules. It was noted that the effects mimicked by the bystander cells manifested following a 12-14 hour temporal delay. Reproduction of the same experiment using mercuric chloride revealed the induction of granular and vacuolar degeneration in the bystander cells following receipt of biophoton signals through quartz glass [182]. These cellular symptoms again reflected the manifestations characteristic of direct cytotoxicity by mercuric chloride. Similar observations were made by Galantsev *et al.* when mammary explants from lactating mice were treated with various hormones to elevate the level of thiobarbituric acid reactive substances (TBARS) in the directly-treated culture [183]. Subsequent optical interaction between the directly-treated culture and a non-treated bystander culture of the same tissue type revealed TBARS levels which reflected those observed in the treated culture [183]. TBARS formation

occurs as a by-product of lipid peroxidation and thus would not be expected in a chemically-separated culture of healthy tissue. In a study by Farhadi *et al.*, inducer cells treated with H_2O_2 were placed in an isolated container physically separated from untreated detector cells by a glass barrier to facilitate transmission of electromagnetic signals while preventing the exchange of chemical factors [198]. The results revealed significant reductions in cellular protein levels, indicative of compromised cell function, as well as activation of the transcription factor NFκB in the detector cells exposed to the signals emitted from the H_2O_2-treated cells [198]. In contrast, detector cells that were exposed to healthy untreated inducer cells did not exhibit significant differences in protein content nor NFκB activation [198]. A collective analysis of these results leads to the inference that the effects directly induced in the primary population by a nominal stressor can lead to the mirroring of such effects in a non-exposed bystander population that is optically-coupled to the former biosystem. As a follow-up, it is thus logical to suggest that the observation of synchronization effects would be expected in bystander cultures exposed to biophotons produced secondarily to insult of a primary culture by any biological stressor, particularly ionizing radiation.

2.4 Beta Radiation

Beta (β) radiation arises from the decay of an unstable isotope of a given element (a radionuclide) that possesses an excess neutron or proton within its nucleus [199]. The transformation of a neutron into a proton, or vice versa, is initiated to stabilize the isotope and thus is accompanied by the emission of a negatively charged electron and an antineutrino in the case of β^- disintegration or by the emission of a positively charged antielectron (positron) and a neutrino in the case of β^+ decay [200]. β^- and β^+ decay are represented by the general expressions presented below (equations 2.4 and 2.5, respectively). The antineutrino ($\bar{\nu}$) and neutrino (ν) function to carry the disintegration energy that remains when the β-particle does not possess its maximum possible energy [199], X represents the parent nuclide, Y represents the daughter nuclide, A is the sum of the protons and neutrons for a given species, and Z is the number of protons for a given species.

$$^{A}_{Z}X \rightarrow {}^{A}_{Z+1}Y + {}^{0}_{-1}\beta + {}^{0}_{0}\bar{\nu} \tag{2.4}$$

$$^{A}_{Z}X \rightarrow {}^{A}_{Z-1}Y + {}^{0}_{1}\beta + {}^{0}_{0}\nu \tag{2.5}$$

β-decay is exemplified by the parent nuclides ^{90}Y (equation 2.6) and Tritium (Hydrogen-3, ^{3}H) (equation 2.7). Decay of ^{90}Y to stable Zirconium-90 (^{90}Zr) is associated with the emission of a β^- particle with maximum energy of 2.28 MeV and average energy of 0.934 MeV [201], with a 64 hour half life. ^{3}H also decays by β^- emission (figure 2.5), producing only an 18.6 keV ($\bar{\beta}$=5.683 keV) β-particle per disintegration [201]. The half life of ^{3}H is 12.28 years. It is noted that while discrete β-particle energies have been specified here, the emission of β-particles is not monoenergetic, rather β-emission covers an entire spectrum of energies for a given radionuclide where the maximum energy specified is the greatest amount of energy that a β-particle can possess. Since the two radionuclides focused upon in this thesis, ^{90}Y and ^{3}H, decay via β^- emission, this discussion will emphasize the effects and interactions of β^- radiation, or more simply, electrons.

$$^{90}_{39}Y \rightarrow {}^{90}_{40}Zr + {}^{0}_{-1}\beta + {}^{0}_{0}\bar{\nu} \tag{2.6}$$

$$^{3}_{1}H \rightarrow {}^{3}_{2}He + {}^{0}_{-1}\beta + {}^{0}_{0}\bar{\nu} \tag{2.7}$$

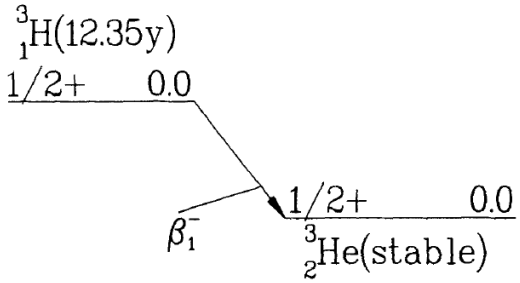

Figure 2.5: Tritium ^{3}H decay scheme illustrating only β^- decay. Tritium is often referred to as a pure β-emitter. Figure retrieved from ICRP 38, page 9 [201].

2.4.1 Photon Production associated with β-Decay and Interactions in Matter

Subsequent to irradiation of biological materials with β-radiation, photons may arise both as a result of classical physical transitions and biological interactions. Photon emission can often be associated with the process of β-decay, itself, if the disintegration of a radionuclide initially transitions to an excited state of the daughter nuclide. ^{90}Y decay, for example, is associated with the very low-yield (0.01%) release of 1.76 MeV γ-photons as a result of a transition from the ^{90}Zr nuclear excited state to the ground state [201] (figure 2.6). While the energy range of γ-rays far exceeds the energies characteristic of biophotons, the presence of γ-rays should still be considered since these photons have the potential to interact with biological matter or interfere with photon detection systems employed in experimentation.

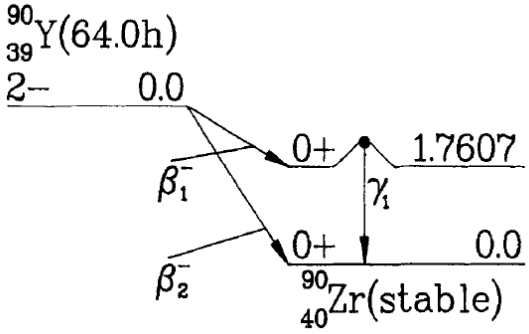

Figure 2.6: Yttrium-90 ^{90}Y decay scheme illustrating β^- decay associated with the emission of a 1.76 MeV γ-ray. Figure retrieved from ICRP 38, page 206 [201].

After the emission of a β-particle by the disintegration of a radionuclide, the focus shifts to the interaction of that β-particle with matter. When light charged particles such as electrons interact with matter, two types of interactions associated with energy loss must be considered: radiative and collisional losses [200]. Radiative energy loss occurs when a charge undergoes a significant change in velocity in the form of acceleration or deceleration upon interaction with the Coulomb forces of the target material's nucleus. Electromagnetic energy, referred to as Bremsstrahlung radiation, is consequently emitted with energy equal to the kinetic energy lost by the incident electron while the resultant reduced-energy electron exits the electric field of the target nucleus at an angle from its incident direction. The efficiency of bremsstrahlung

production is related to the atomic number of the target material by approximately Z^2 and also possesses a positive linear dependence upon β-particle energy. Energy loss by radiative stopping only occurs when β-energies exceed 200 keV [200], therefore, they are not produced following interaction of ^{3}H β-particles with matter. However, we *would* expect a low yield of bremmstrahlung radiation following ^{90}Y β-particle interaction. When compounded with a low Z target material such as water-equivalent tissue, the radiative stopping power becomes negligible compared to collisional losses, thus bremsstrahlung radiation is not considered a large contributor to the overall photon yield upon ^{90}Y beta-particle interaction with biological material.

Collision of β-particles with the orbital electrons of a target atom are the predominant mode of energy loss upon interaction with tissue. Collisional interactions can result in both elastic and inelastic scattering whereby the former results in the re-direction of an incident electron but no loss of energy, and the latter leads to electron redirection *and* energy loss within the target material. While elastic scattering in water-equivalent materials predominates at energies above 200 eV, a form of inelastic scattering referred to as ionization also has a high probability of occurring at energies greater than 200 eV. Ionization is characterized by the ejection of an orbital electron from the target nucleus by incoming energy. In our case, the ionizing agent is a β-particle. The vacancy created by ejection of an orbital electron will result in the transition of an outer shell electron down to the vacancy, which is associated with the emission of a photon called a *characteristic x-ray*. The energy of the x-ray produced will possess an energy equivalent to the difference between the binding energies of the outer and inner shell electrons. As such, the x-ray energies will be *characteristic* of the given target material. Following the ionization event, the β-particle will scatter off in a different direction with an energy equal to the incident β-particle, less the binding energy of the ejected orbital electron. Both the ejected electron and the scattered β-particle can then continue to interact within the medium until they experience significant reductions in energy. When electrons have lost enough energy to enter the realm below 30 eV, excitation interactions (another form of collisional interactions) will become more prevalent in tissue [200]. In the energy region between 7.4 eV and 30 eV, electrons have an increased probability of reacting chemically with water molecules, the precursor to water radiolysis and free radical formation.

β-radiation is a low-LET type of ionizing radiation, meaning that the rate of energy deposition and thus ionization along its traversal path is sparse relative to some other

types of radiation [202]. The frequency of ionization tracks is not sufficient to induce DSBs in nuclear DNA, thus the predominant mode of action by β-radiation is via interaction with water molecules and subsequent damage of cellular structures such as DNA and mitochondria via the products of water radiolysis [202]. The process of water radiolysis begins by the formation of ionized water molecules (H_2O^+), excited water (H_2O^*), and free subexcitation electrons (e^-) [200]. These three species will induce changes reflected in expressions 2.8, 2.9, and 2.10. The chemically-reactive products of the former interactions can diffuse from their initial locations of formation to undergo the reactions outlined in expressions 2.11 to 2.17 in an effort to become chemically stable[2]. These reactions can either occur between the free radical reactants themselves or with other molecules in the target tissue to perpetuate biological damage. The action of the free radicals upon intracellular constituents such as mitochondria could lead to functional impairments and subsequent oxidative stress. As discussed earlier in section 2.3.2, states of oxidative stress have been linked to emission of biophotons in the UV, visible, and IR wavelength ranges following the transition of excited species to their stable states.

$$H_2O^+ + H_2O \rightarrow H_3O^+ + OH \tag{2.8}$$

$$\begin{aligned} H_2O* &\rightarrow H_2O^+ + e^- \\ H_2O* &\rightarrow H + OH \end{aligned} \tag{2.9}$$

$$e^- \rightarrow e^-_{aq} \tag{2.10}$$

[2]Chemical reactions from *Atoms, Radiation, and Radiation Protection* textbook [200]

$$OH + OH \rightarrow H_2O_2 \tag{2.11}$$
$$OH + e^-_{aq} \rightarrow OH^- \tag{2.12}$$
$$OH + H \rightarrow H_2O \tag{2.13}$$
$$H_3O^+ + e^-_{aq} \rightarrow H + H_2O \tag{2.14}$$
$$e^-_{aq} + e^-_{aq} + 2H_2O \rightarrow H_2 + 2OH^- \tag{2.15}$$
$$e^-_{aq} + H + H_2O \rightarrow H_2 + OH^- \tag{2.16}$$
$$H + H \rightarrow H_2 \tag{2.17}$$

2.4.2 Environmental, Occupational and Medical Presence

The investigations in this thesis have focused on β-radiation as a primary source of exposure since it has a pronounced presence in the environment, and in both medical & occupational settings. Medical exposures to β-radiation can come in the form of diagnostic exposures such as the intake of positron emission tomography (PET) radiotracers or as a result of therapeutic delivery of β-emitters for treatment of tumours. Radiotracers employed for PET scanning are delivered intravenously and take advantage of preferential molecular localization to identify pathological processes *in vivo* [203]. Detection of the radiotracer accumulation sites is employed by measuring the photon emissions that are associated with β^+ decay. The internal radiation doses delivered by the β-particles and photons resultant to decay of the radionuclide are maintained in the low dose region, reaching up to 30 mSv for a single scan [203]. β-emitters are also employed in radiotherapeutic practices whereby insertion of encapsulated radioisotopes are employed to deliver localized treatments of high-dose radiation. In a technique employing ^{90}Y, microscopic beads containing the radioisotope are delivered interstitially to embolize the vasculature feeding the target tumour while also delivering ionizing radiation in an effort to destroy the cancerous cells [204]. The tumour dose from this procedure can range from 50-150 Gy and thus far exceed the low-dose characterization [205]. However, concern of low-dose effects should be considered in neighbouring healthy tissue since research has reported the receipt of doses between 0.1 and 0.5 Gy by bone marrow, kidneys and lungs when tumours of hepatic origin are targeted [206].

Occupational exposure to the medical radioisotopes employed in PET and radio-

therapy can occur during handling of the materials by medical professionals where typical whole-body doses received by PET technologists average at approximately 2-3 mSv per annum [207]. In addition to medical occupational exposures, radiation dose can also be received occupationally by workers in the nuclear power industry. The most prominent β-source in a heavy-water nuclear power reactor is ^{3}H [208]. ^{3}H is produced as a result of neutron activation of the heavy water moderator, or deuterium oxide (D_2O), by thermal neutrons originating in the reactor core [208]. Because ^{3}H β-particles possess such low energies, they do not penetrate tissue sufficiently to induce adverse biological effects externally. However, it becomes an internal radiation hazard when there are weaknesses in the integrity of the moderator system and ^{3}H is exposed to air, subsequently becoming airborne tritiated water vapour. Two thirds of the tritiated water vapour can enter the body via inhalation while the other one third is internalized via absorption through the skin [208]. Tritium contributes to approximately 20% of the 3 mSv annual dose received by Canadian nuclear operations workers [209], thereby validating the need to further investigate the effects of low-dose β-exposures.

Tritium is also contained in the effluents released from nuclear power plants, therefore it is evident that ^{3}H is both occupationally and environmentally relevant. In 2006, airborne and waterborne releases of D_2O into the environment by Canadian nuclear power generating facilities resulted in contamination of the environment surrounding nuclear facilities to concentrations between 0.38 Bq/m^3 and 35.88 Bq/m^3 [210]. Upon assessment of ^{3}H transfer through the environment, the Canadian Nuclear Safety Commission (CNSC) determined that the general public living in the vicinity of nuclear generating stations were exposed to very low ^{3}H doses ranging between 0.00045 to 0.00236 mSv per annum [210]. A natural source of β-radiation in the environment comes from Radon-222 (^{222}Rn) daughters found in the earth, thus spending extended periods of time in basements raises concern for exposure to ^{222}Rn and its daughters. ^{222}Rn is a noble gas that occurs naturally in the environment as a part of the Uranium-238 decay series. Its decay leads to the production of short-lived daughters, three of which are β-emitters (Lead-214, Bismuth-214, Lead-210) releasing β-particles ranging from 0.017 to 2.37 MeV in energy [201]. While the alpha-emitting daughters of ^{222}Rn often receive most of the focus when dose estimates are concerned, Markovic *et al.* were able to show that the β-emitting daughters also contribute dose to the lungs when inhaled (1.73 mGy delivered over a duration of 170 hours when airborne radon

concentration is 3700 Bq/m^3) [211]. The presence of low-dose β-radiation is apparent in each of the public, medical and occupational settings. The susceptibility for public and occupational exposure to these sources of radiation provide, at the very least, some justification for the continued study of their biological implications.

2.5 Relevance of the Thesis Work

While the RIBE is widely studied and is now accepted as a mechanism for inducing radiation effects in a manner that is alternative to direct DNA damage [2], investigations of the RIBE have thus far been strictly limited to the exploration of chemical mediators as a means of facilitating intercellular communication. Biophotonic research has demonstrated a role for electromagnetic radiation, or light, as a mechanism used by biological systems to communicate information with each other [117, 179–181]. However, to our knowledge, this form of intercellular signalling has not yet been explored as a means of bystander communication following exposure to ionizing radiation, although it has been shown to occur following cellular exposure to other biological stressors [182, 183]. The objective of this research is therefore to explore the potential for a role of electromagnetic signalling in propagating RIBEs. Elucidating a capacity for electromagnetic signalling to communicate radiation effects may provide a plausible mechanism for explaining observed effects resembling radiation insult reflected in non-irradiated animals co-habitating with irradiated animals [112, 113].

The choice to employ β-radiation as the primary source of direct irradiation throughout the experiments presented in the current research was motivated firstly by the fundamental characteristics of the chosen radionuclides, themselves. To elaborate, the low yield of γ-radiation emitted upon ^{90}Y decay and the absence of γ-ray emission upon the decay of ^{3}H conferred these radionuclides attractive candidates for measuring biophoton emission owing to the relative lack of interference expected by photons generated as a result of the disintegration of the radionuclide, itself. The selection of these specific radionuclides was therefore a conscious decision based upon efforts to minimize the probability for false positive biophoton detection. Secondly, it is clear that there is a relatively high susceptibility for members of the public and individuals working in the medical & nuclear power sectors to encounter low-dose β-radiation on a regular basis. There is also an awareness that non-targeted effects manifesting at these low doses lead to the potential for biological effects to deviate from the

linear dose-response paradigm [30, 212]. The high probability for public, medical and occupational exposure to low doses of β-radiation is therefore a primary driving force for further elucidating the mechanisms by which low-dose radiation communicates non-targeted effects.

In addition to medical exposure to β-radiation, this work can also be considered important to the implications for exposure to x- and γ-radiation. Electromagnetic forms of ionizing radiation are ionizing because they produce secondary electrons upon interaction with matter. These secondary electrons are analogous to β-particles and furthermore, the secondary electrons produced in tissue by the low x-ray energies applied in mammography would possess similar energy to the β-particles associated with tritium decay. The work conducted in the current thesis can thus also be considered relevant to the potential effects occurring as a result of exposure to low-energy x-rays.

While this project is still in the exploratory phase and was therefore limited to *in vitro* work, we hope that it will contribute to gaining a more developed understanding of the communication mechanisms of the RIBE, such that it will aid in bringing us a step closer to clarifying the biological consequences of the low-dose ionizing radiation that is all around us.

2.6 References

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Chapter 3

Factors Affecting Ultraviolet-A Photon Emission from β-Irradiated Human Keratinocyte Cells

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The initial concept of the research presented in this chapter and the experimental methodology was conceived by Dr. Syed Bilal Ahmad, Dr. Carmel Mothersill, Dr. Fiona McNeill, and Dr. Colin Seymour. Radioisotope production of ^{90}Y was conducted by Dr. Andrea Armstrong. Cell culture, photon quantification experiments, statistical analyses, and manuscript writing were carried out by the first author. All authors contributed to the revision of the manuscript prior to submission and following receipt of reviewer comments from the journal.

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Factors affecting ultraviolet-A photon emission from β-irradiated human keratinocyte cells

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Abstract

The luminescence intensity of 340 ± 5 nm photons emitted from HaCaT (human keratinocyte) cells was investigated using a single-photon-counting system during cellular exposure to ^{90}Y β-particles. Multiple factors were assessed to determine their influence upon the quantity and pattern of photon emission from β-irradiated cells. Exposure of 1×10^4 cells/5 mL to 703 μCi resulted in maximum UVA photoemission at $44.8 \times 10^3 \pm 2.5 \times 10^3$ counts per second (cps) from live HaCaT cells (background: 1–5 cps); a 16-fold increase above cell-free controls. Significant biophoton emission was achieved only upon stimulation and was also dependent upon presence of cells. UVA luminescence was measured for ^{90}Y activities 14 to 703 μCi where a positive relationship between photoemission and ^{90}Y activity was observed. Irradiation of live HaCaT cells plated at various densities produced a distinct pattern of emission whereby luminescence increased up to a maximum at 1×10^4 cells/5 mL and thereafter decreased. However, this result was not observed in the dead cell population. Both live and dead HaCaT cells were irradiated and were found to demonstrate different rates of photon emission at low β activities ($\leqslant$400 μCi). Dead cells exhibited greater photon emission rates than live cells which may be attributable to metabolic processes taking place to modulate the photoemissive effect. The results indicate that photon emission from HaCaT cells is perturbed by external stimulation, is dependent upon the activity of radiation delivered, the density of irradiated cells, and cell viability.

It is postulated that biophoton emission may be modulated by a biological or metabolic process.

Keywords: radiation-induced luminescence, ultraviolet radiation, beta radiation, yttrium-90, intercellular signaling, bystander effect, phosphorescence

(Some figures may appear in colour only in the online journal)

1. Introduction

Biological entities are known to emit photons and the photon emission can be linked to signaling processes between entities. For example, since the early 1920s, multiple reports of low-intensity photon emission from plant (Popp *et al* 1984, Gurwitsch 1988, Bajpai *et al* 1991), animal (Devaraj *et al* 1991, Evelson *et al* 1997, Van Wijk *et al* 2014) and human-derived material (Niggli 1993, 1996, Niggli *et al* 2008, Van Wijk *et al* 2013) have been made and referred to as ultraweak luminescence or biophoton emission.

Biophoton emission at very low fluxes has been observed in the absence of a stimulus, a phenomenon termed spontaneous photon emission (Bajpai *et al* 2013). Weak electromagnetic fields such as these can act as informational signals. Further, these signals are intended for inter- and intra-cellular communication within a population of cells, a tissue system or within an organism (Borodin 1930, Rahn 1936, Gurwitsch 1988, Slawinski *et al* 1992). Of interest to us, was the fact that photon emission can also be elicited in response to a stimulus. The stimulated emission is generally in quantities that are orders of magnitude greater than those of a spontaneous nature.

Previous work conducted in our laboratory (Ahmad *et al* 2013, Le *et al* 2015) has confirmed that stress in the form of β-radiation elicits significant photon emission from human keratinocyte cells, HPV-G and HaCaT, in the ultraviolet (UV) range. Photon emission from biological materials has been shown to be induced not only following exposure (in our hands) to ionizing radiation but also (in other hands) by chemical agents (Popp *et al* 1984, Devaraj *et al* 1991), ultraviolet radiation (Niggli 1993, Niggli *et al* 2008) and intense white light (Niggli 1996).

Biophoton emission is seen as a response to stress or stimuli (Slawinski *et al* 1992) and therefore has, in fact, been utilized as a diagnostic indicator of disease and stress states in animal (Van Wijk *et al* 2013) and human (Van Wijk *et al* 2014, 2008) models. Similar to reported data regarding spontaneously emitted photons, the previous work from our lab has shown that the radiation-induced UV photons have cell communication capabilities (Le *et al* 2015). That particular experiment is detailed in full but we summarize the information here.

Our previous experiment had a layer of tritium-incubated cells, a barrier, and then another layer of cells. The upper were only exposed to the subsequent radiation-induced UV, not the initial β-radiation from the tritium. We state that UV induced cell communication occurred, because a bystander effect was observed in the upper layer of non-β exposed cells. When a UV filter was inserted between the layers, the bystander effect i.e. the induced cell communication, was not observed.

To date therefore, our data show that an external stressor of β-radiation is indeed able to initiate biophoton emission. We previously observed UV photon emission from a number of organic materials including plastics and dried tissues (Ahmad *et al* 2014), so one mechanism of UV emission is probably simply a consequence of electron rearrangement after ionization

events in atoms and/or molecular structures. In addition, our previously reported data show that the subsequent emitted photon signal is capable of communicating with bystander cells to produce observable responses.

We decided to investigate factors that can modulate the UV signal. This manuscript therefore reports on a series of experiments that were designed to investigate potential factors that can affect the magnitude of the β-induced UV emission. We explore the pattern of photon emission and the relationship to factors such as cell viability, activity, of radiation delivered, density of irradiated cells and concurrent versus post-irradiation photon quantification.

2. Materials and methods

2.1. Cell line

The HaCaT cell line was obtained from the lab of Dr Orla Howe (Dublin, Ireland). HaCaT cells are immortalized, non-transformed human skin keratinocyte cells which express p53 mutations on both of its alleles (Boukamp *et al* 1990, Lehman *et al* 1993, Datto *et al* 1995). Cells were confirmed to be free of mycoplasma using PlasmoTest mycoplasma detection kit (catalog no: rep-pt1, Invivogen, San Diego, CA, USA).

2.2. Cell culture

All reagents were obtained from Gibco unless otherwise stated. HaCaT cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Life Technologies Inc., Grand Island, NY, USA) containing a final concentration of 10% fetal bovine serum, 0.5 μg mL^{-1}hydrocortisone (Sigma-Aldrich, St-Louis, MO, USA), 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin sulphate. Cell culture work was performed in a class II laminar flow cabinet to maintain sterility of the cells. Subculture of cells was performed at 80–90% confluency using a 1:1 solution of dissociation reagent, 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA), for detachment of cell monolayer from the flask substrate. Cell stock was subsequently seeded into 250 mL stock flasks and grown in 15 mL medium. Cells were incubated at 37 °C, 95% relative humidity, 5% CO_2 and medium renewed every 2–3 d. Stock flasks received full volume medium renewals 24 h prior to set-up of experimental cultures.

2.2.1. Preparing living cell cultures for experiments. HaCaT cells were plated onto 60×15 mm polystyrene petri dishes (BD Falcon, Mississauga, ON) and supplemented with RPMI culture medium (without phenol red) to a total volume of 5 mL. A 5 mL volume placed into the dish created a 3 mm thick layer of liquid. Living cells were plated at 7 different densities ranging from 5×10^2 to 5×10^5 cells per 25 cm^2 petri plate containing a total volume of 5 mL. From now on, *cell density* throughout this article refers to the number of cells per 5 mL volume of cell culture medium in a petri-dish. As the volume is fixed for all experiments, we quote total number of cells/5 mL when we use the term *cell density*. Cells were incubated at 37 °C, 95% humidity, 5% CO_2 for 6 h before experimentation took place to ensure cell attachment to the dish substrate. Cells destined for irradiation in a living state were irradiated in the petri dish containing 5 mL of RPMI 1640.

2.2.2. Preparing dead cell cultures for experiments. For experiments conducted upon dead cells, cells were seeded in the same manner as previously described. Cells were plated at

densities ranging from 5×10^5 to 2×10^6 cells per 5 mL. Following 6 h incubation, cells were killed by treating the HaCaT monolayer with 5 mL of 100% ethanol for 30 min. Following ethanol treatment, the ethanol was aspirated carefully from the cell culture dish and left to stand for an additional 10 min to facilitate complete evaporation of residual ethanol from the petri dish. 5 mL of RPMI 1640 without phenol red was then added to the petri dish containing dead cells in order to ensure consistency with the live-cell conditions.

2.3. Source Preparation

The radioactive source, Yttrium-90 (^{90}Y) decays by β-emission (maximum beta energy = 2.28 MeV; average beta energy = 0.9337 MeV; half life = 64.1 h). It was chosen because it is an almost pure β-emitter with no γ-signal which could be used as an external source of radiation. We manufactured the ^{90}Y in house: Yttrium salt was irradiated in the core of the McMaster Nuclear Reactor and prepared into a liquid solution by dissolving the Yttrium-salt in 0.5 M HCl. The resultant ^{90}Y possessed a high specific activity to reduce self-shielding effects. The stock source was always prepared to a final volume of 400 μL, however, the physical dimensions and mass of the source were not always consistent for a given activity. For each preparation, the target activity was 1000 μCi per 400 μL, however this could not always be achieved and resulted in variability in the ^{90}Y activity between 839.3 μCi to 1100 μCi per 400 μL. Activity of the prepared source was measured using an AtomLab 400 Dose Calibrator possessing an expected uncertainty of $\pm$3% (Biodex Medical Systems, Shirley, NY).

In regard to the volumes actually used during irradiation, different volumes were required to achieve each of the desired radiation activities. Therefore, for activities ranging from 14 to 703 μCi, the required volume from the 400 mL stock, varied from 5.09 to 335 μL. The variability in the physical dimensions and mass of the source may have contributed to uncertainties in the activity delivered to the cells (table 1) and therefore the observed counts.

2.4. Irradiation and Photon Quantification

Photons emitted from keratinocyte cells were quantified using a single photon counting apparatus (Ahmad *et al* 2013) comprised of a Hamamatsu R7400P photomultiplier tube (PMT) (Hamamatsu Photonics, Bridgewater, NJ, USA) fitted with an optical filter (Edmund Optics Inc., Barrington, NJ, USA) specific to 340 nm $\pm$ 5 nm. It is noted that the transmission of incident light through this optical filter is reduced to 25% using a collimated beam as compared to a non-collimated light source. As determined previously (Le *et al* 2015), one reason we focused on the measured photon wavelength centered at 340 nm $\pm$ 5 nm for further experiments was because it demonstrated an optimal photon output compared to 300 nm and 280 nm wavelengths. An additional reason for choosing to focus on the 340 nm UVA wavelength is because it is biologically interesting since UVA, but not UVB, has been proven to induce bystander effects in human cells (Whiteside and McMillan 2009). In this manuscript, we use the term UV from now on to mean the emission we measured at 340 nm. This is not to suggest that there are no other frequencies of light emitted, merely that this is the wavelength we could measure with our apparatus, and also the wavelength we chose to measure because the stronger signal would result in greater statistical strength.

Photon counting was conducted in a light-tight aluminum container with the photomultiplier tube located within (figure 1). The radioactive source was placed into a 60×15 mm petri dish at the bottom of the light-tight box and a 3.2 mm thick aluminum collimator (2 mm diameter pinhole) was placed above the source. This collimator was used for the photon quantification

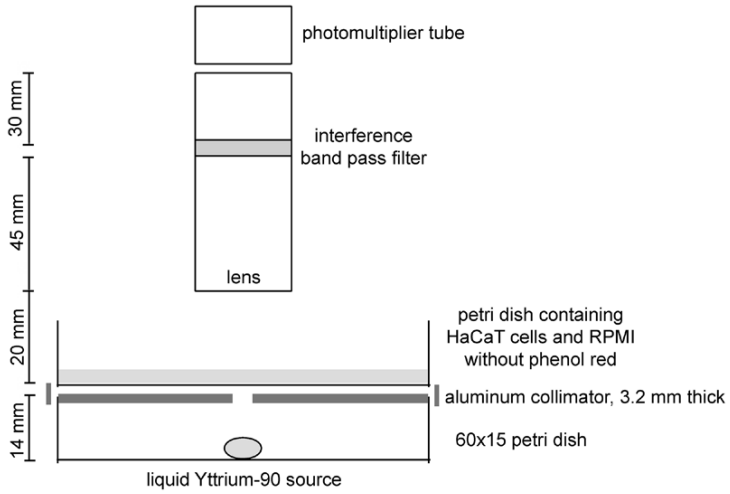

Figure 1. Experimental configuration for ^{90}Y irradiation of cells and quantification of photon emission from irradiated cells. Image not to scale.

experiments in order to limit scattering of photons within the lens system. Our previous work had shown that collimation was a necessary part of the experiment (Ahmad *et al* 2013). The 0.95 mm thick petri dish containing cells and a 3 mm layer of cell culture medium was positioned above the collimator. Although the cell culture medium was free of the pH indicator phenol red, it was still slightly colored (light orange) due to the natural orange pigmentation of the FBS supplemented into the medium. The pigmentation of the culture medium would have contributed to absorption and scattering of the UV photons. In addition, natural chromophores in cells, cell membranes and boundaries can all contribute to absorption and scattering of UV photons. Therefore the UV flux detected by the PMT does not accurately represent the number of photons actually emitted from the irradiated cells, and it will require validated Monte Carlo methods to accurately back calculate the UV emission at source.

The photon counting apparatus was developed in-house; apparatus specifications are described in detail in an existing publication (Ahmad *et al* 2013). The PMT was turned on 30 min prior to each experiment and the high voltage supplied to the PMT was set to −800 V. Following a 30 min warm-up period, three background photon counts lasting 3 min each were taken to determine the background noise level (no radiation, dishes or cells in the chamber).

2.4.1. Different activities. Cell irradiation was carried out at six different activities for live cells (14, 70, 140, 197, 351, 703 μCi) and eight activities for dead cells (same as live cells, also 165 and 540 μCi) and photon emission was quantified concurrently. Unirradiated controls with cells and without cells were also included. Photon quantification was conducted at each of the activities for a total of 9 min; each of three petri dishes were exposed for 3 min while photon quantification took place. In order to accomplish this, the high voltage was switched off between each 3 min exposure, the petri dish containing cells was removed, and another petri dish containing the same number of cells was placed into the chamber to be exposed by the same source. Once in place, the high voltage was switched back on and photon quantification resumed. After three dishes were exposed to a given activity, the activity was increased to the next specified level, taking into account decay over the last 9 min. Irradiation and photon quantification usually began within 15 min of the completion of source preparation. This time

Table 1. Estimated average beta particle flux seen by cells and estimated error in beta particle flux seen by cells due to pipetting uncertainty, source decay, activity measurement and differences in source geometry.

^{90}Y target activity (μCi)	Activity uncertainty (μCi)
14	1
70	3
140	5
165	6
197	7
351	13
540	20
703	27

Note: The estimate of error includes contributions from source decay, measurement of the source activity using a dose calibrator, pipetting uncertainties and an estimate of variation in source dimensions. This may, however, still be an underestimate of the overall error.

required to transport and set-up the source was taken into consideration when calculating the decay and thus the volume of ^{90}Y required for the next activity. It is noted that the source activity was only replenished every 9 min between each triplicate. It was not adjusted between irradiation of each petri dish (every 3 min), therefore variability in the activity stemmed from decay of the source, especially at the higher activities which were done later in the experiment (table 1). Experiments were always completed within 3 h of source formation, therefore, the source was always maintained above 96.81% of the initial activity.

2.4.2. Different cell densities. For dead cells, 3 different densities from 5×10^5 to 2×10^6 cells per 5 mL volume and for live cells, 7 different densities ranging from 5×10^2 to 5×10^5 cells per 5 mL volume were exposed to each of the activities described in the previous section. 0 cell controls were also included where petri dishes containing only 5 mL of cell culture medium and no cells were exposed to each of the activities ranging from 0 to 703 μCi.

2.4.3. Post-irradiation quantification. Photon quantification was also carried out for 92 min immediately *after* irradiation. In this experiment, photon quantification took place for 30 min during the exposure of 1×10^4 living cells/5 mL to 703 μCi. Irradiation set-up for the 30 min duration was identical to that in figure 1. Post-irradiation quantification for 92 min was then accomplished by turning off the HV supply, removing the petri dish containing the ^{90}Y source, and replacing the source petri dish with an empty petri dish thereby maintaining the geometry of detection from UV-emitted cells. It is noted that photon quantification could not be measured for the minute immediately following removal of the cells from the radiation source. This experiment was conducted with a freshly perpared source. Taking into consideration transport, preparation, and 30 min irradiation time, the activity delivered to cells in this experiment varied between 703 and 699.2 μCi (approximately 99.46% of the initial activity).

2.5. Statistical Analysis

Photon quantification experiments were conducted three times with a triplicate tested for each trial, $n = 9$. For each trial, a different source was prepared and used. The data are presented as the mean where error bars are representative of the mean $\pm$ the standard error. All data sets were found to be normally distributed. To determine the nature of the relationship between photon emission and the ^{90}Y activity applied, experimental data was assessed first using

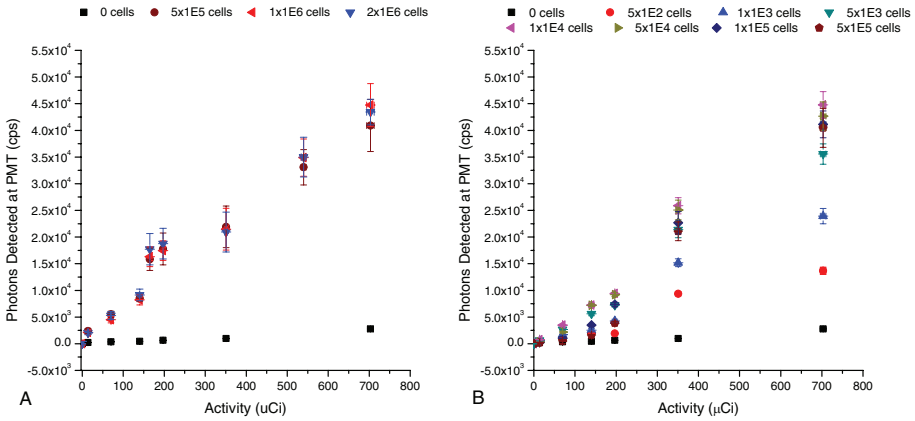

Figure 2. UVA (340 ± 5 nm) photon quantification from (A) ethanol-treated and (B) living HaCaT cells during exposure to different activities of beta emitter, Yttrium-90. Legend: reported numbers refer to total number of cells per 5 mL of medium. Error bars: mean $\pm$ standard error (SEM) for $n = 9$.

Pearson's correlation test and then linear regression fixed to a common y-intercept (y-intercept for unirradiated control). 2-way analysis of variance (ANOVA) tests were conducted to assess photon emission dependent upon activity and density. A p-value less than 0.05 was considered significant. Statistical analyses were conducted using SPSS Statistics 17.0 and Origin Pro 8.

3. Results

3.1. Background counts at 340 nm detection

Background photon counts, obtained in the absence of any materials in the path of the PMT, were quantified within the light-tight apparatus at the beginning of each experimental setup. The background counts were found to be between 1 and 5 counts per second (cps) (data not shown). As the background count values quantified were low, these quantities were considered negligible compared to count rates obtained following β-irradiation and were not subtracted from subsequent photon emission measurements.

3.2. 340 nm photon emission from irradiated ethanol-treated (dead) HaCaT cells

Ahmad and colleagues demonstrated significant photon emission resulting from the exposure of dead HPV-G human keratinocytes to ionizing radiation (Ahmad *et al* 2013). Dead cells were initially studied because the experiments could then be conducted in a non-biohazard approved laboratory. We previously did not have access to laboratories that were approved for work with both open liquid sources of radioactivity and biohazardous materials. To confirm this phenomenon in our chosen human keratinocyte cell line (HaCaT), photon counting was conducted during the irradiation of dead HaCaT cells with low LET-β-particles.

3.2.1. Relationship between activity and photon emission. The results of this experiment demonstrated an increase in UVA photon emission with increasing activity applied to the dead cells (figure 2(A)), this result is evidence of a positive relationship between ^{90}Y activity

and photon emission intensity (table A1). Given the relatively small number of activity points, we fitted linear relationships to the data. Other functions may be better representations of the underlying physical process, but there are too few degrees of freedom to determine this with certainty. The linear fits were conducted such that the regression fits for all of the cell densities intersected a common point along the *y*-axis (b) which corresponds to the intercept of the unirradiated control data ($b = 2.77$). Linear fits to the data were significant $p < 0.05$ (table A2) for all but one cell density. Among those that were significant, the lowest R^2 value of 0.800 provides the information that approximately 80 percent of the variation in UV photon emission can be explained by the activity in a linear model. The borderline significance (p=0.051) demonstrated by the linear regression fit to the 5×10^5 cell/5 mL data is probably because a linear fit is not the ideal model for this data and because the intercept has been forced.

Photon quantification of unirradiated controls, was limited to 1–2 cps and did not differ significantly from background counts ($p = 0.856$). Upon quantification of cells exposed to 14 μCi ($p = 0.023$) and all activities greater ($p < 0.0001$), emission was found to be significantly different from unirradiated and background controls.

As stated earlier, linear fits may not be the best model of the data pattern. The data may turn over and follow some other function. A greater number of data points would be required to evaluate the pattern fully. We can say that at these activity levels and densities, the numbers of detected UV photons increase with activity. There is, perhaps, a suggestion of a small systematic offset or an artifact that arises between 150 and 200 μCi. Our measurements were performed in the same activity order for each cell density series. An explanation is that there is perhaps some evidence of a time dependent transition. Another interpretation could be differences in source distribution. However, whether this is a true pattern arising from the interactions between UV and more and less dense layers of cells, or an artifact or systematic offset, is difficult to determine in a post hoc analysis. This small effect requires further investigation.

3.2.2. Relationship between cell density and photon emission. The control curve labelled 0 cells in figure 2(A) illustrates the UV photon emission from petri dishes containing no cells and only culture medium that received radiation. Upon exposure of culture medium in the absence of cells to the lowest activity level used (14 μCi), the detected emission was significantly different from background emission levels (up to 5 cps) at the 95% confidence level ($p = 0.037$). The observed emissions in the absence of cells can be attributed to interaction of the beta particles with the polystyrene petri dish and cell culture medium. However, the amount of emission observed upon irradiation of 0 cells (dish and medium only) was significantly less than that observed in the presence of cells ($p < 0.0001$) indicating that UV emission is significantly increased further when cells are placed in the path of the radiation. Therefore we conclude that the molecular structures of cells compared to the composition of medium make UV emission more likely.

It was also found that the emission count rates of all three dead cell densities (5×10^5 to 2×10^6 cells/5 mL) were not significantly different from each other. Upon analyzing micrographs of these three cell densities using ImageJ (figures 3((A)–(C)), it was determined that 5×10^5, 1×10^6, and 2×10^6 cells/5 mL covered 15.7%, 35.1% and 67.7% of the petri dish surface area, respectively. Although the plated densities are quite variable in confluency on the plate, emission magnitudes measured from each of these densities were comparable. This result is suggestive either of a threshold in density above which photon count rate does not change greatly, or that the large relative measurement uncertainties of approximately 19% mean that there is emission variation of less than 0.5% per 5×10^5 cells/5 mL.

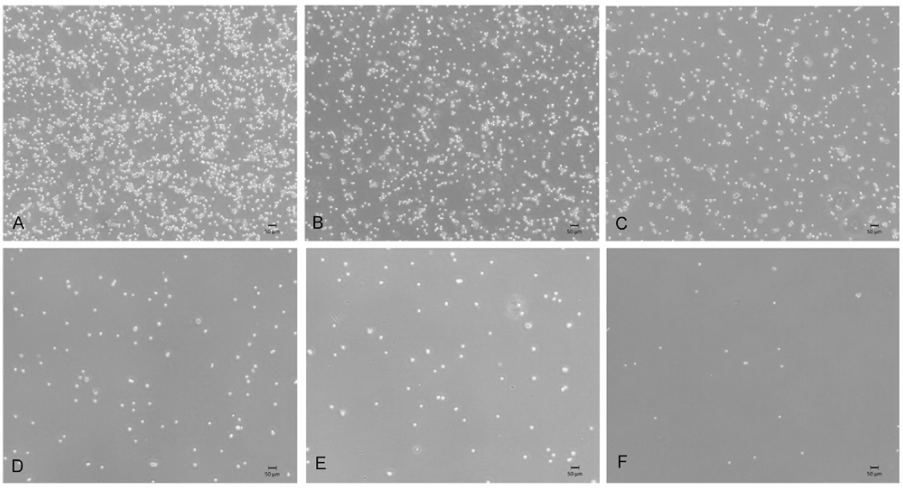

Figure 3. Micrographs of different cell densities plated on 60×15 mm petri plates. Cells per 5 mL volume: (A) 2×10^6, (B) 1×10^6, (C) 5×10^5, (D) 1×10^4, (E) 5×10^3, (F) 1×10^3.

3.3. 340 nm photon emission during irradiation of living HaCaT cells

Following gain of access to laboratory facilities approved for both open radioisotope work and biohazard work, we quantified the photon emission at 340 nm emanating from live HaCaT cells during irradiation at six different activities of ^{90}Y and seven cell densities to determine whether there was a difference between living and dead cells.

3.3.1. Relationship between activity and photon emission. Unirradiated cell controls demonstrated a minimum count rate of 1 cps and a maximum count rate of 4 cps for cell densities of 5×10^2 to 5×10^5 cells/5 mL, respectively. We conclude that because there is no significant increase ($p = 0.983$) in photon emission above background in the presence of unirradiated cells alone, living HaCaT cell cultures, like dead cells, do not luminesce strongly in the absence of stimulation. When live HaCaT cells were exposed to 14 μCi of the ^{90}Y low-LET β-radiation, photon emission was significantly greater than the 0 dose control ($p = 0.025$). For all activities greater than 14 μCi, $p < 0.025$ when a given activity was compared to the 0 dose control. As with dead cells, positive relationships between photon emission and activity were observed (table A3).

The curves do appear to be non-linear (figure 2(B)), with some evidence of a turn-over at the high activity end. However, with 6 data points, there are not enough degrees of freedom to determine the exact mathematical relationship. Linear fits were significant (p <0.05, table A4) for all but one data set. Among those that were significant, the lowest R^2 value of 0.803 suggests that just over 80% of the increase in light intensity can be explained by an increase in activity in a linear model. Similar to the dead cell analyses, the linear regression analysis for one of the data sets was nearly significant (5×10^4 cells/5 mL, $p = 0.050$), likely because the underlying pattern of the relationship between measured UV emission and activity in living cells is not completely linear. There may be an underlying pattern, but the possibility of artifacts in the data will need to be investigated. Future measurements will need to assess

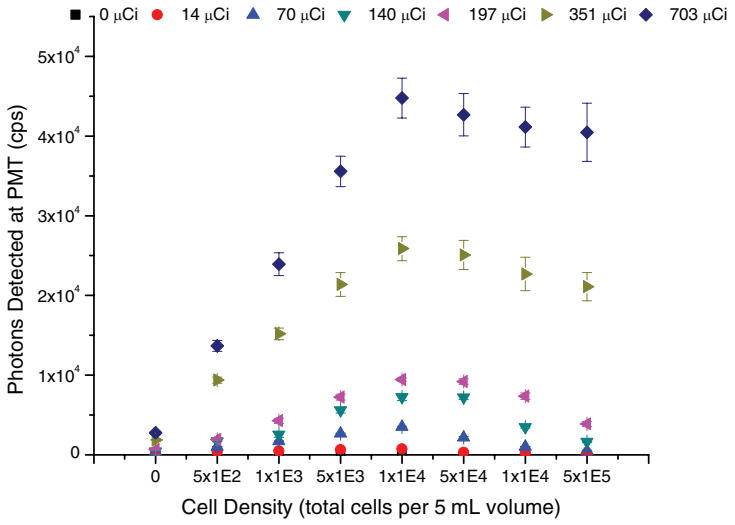

Figure 4. UVA (340 ± 5 nm) photon quantification from HaCaT cells plated at various densities during exposure of live HaCaT cells to Yttrium-90. Surface area of petri dishes upon which cells were plated: 25 cm^2, total volume: 5 mL. Error bars: mean $\pm$ standard error ($n = 9$).

source distributions, and issues such as phosphoresence in the lens arising from beta and bremmstrahlung interactions with the lens.

3.3.2. Relationship between cell density and photon emission. Figure 4 illustrates the effect of cell density upon the resultant photon emission quantity. Control irradiations consisting of irradiated petri dishes containing medium in the absence of cells were conducted at each activity level (6 activity points from 14 μCi to 703 μCi). The resultant light emission intensity was 219 cps to 2767 cps for activities of 14 and 703 μCi, respectively. In the absence of cells, irradiation of the polystyrene petri dish and the RPMI cell culture medium with 14 μCi produced a count rate of 219 cps. System background counts were quantified to be 1–5 cps in the absence of radiation, petri dishes, cells and medium. Photon emission from the 14 μCi cell-free control was significantly greater than background (1–5 cps) at the 95% confidence level, however this difference was just significant at $p = 0.046$. Despite significant emission from the cell-free controls, the cell-free counts were comparatively lower than those measured in the presence of cells. This data suggests that although there is some degree of interaction of the incident β-particles with the polystyrene and cell culture medium, the molecular structures of these materials result in lower UV emission than from cells.

As figure 4 shows, at low cell densities starting at 5×10^2 cells/5 mL (figure 3(F)), the photon emission demonstrates a positive relationship with cell density until reaching a density of approximately 1×10^4 cells/5 mL (figure 3(D)) where the UV emission starts to decrease slightly. Upon reaching 1×10^4 cells/5 mL, the photon emission quantity reaches a maximum; this observation was a consistent trend at all activity levels.

The relationship between measured photon emission and cell density is approximately linear up to densities of 1×10^4 cells/5 mL. While the relationship between measured UV emission and cell density may not be completely linear, and there may be better models of the behavior, this is an observation based on a set of 5 data points, so further analysis would

Table 2. Linear regression of measured UV emission versus cell density (for 0 to 10 000 cells) for each of the activities studied.

Activity (μCi)	Slope of light output(cps) versus cell density	p (for slope of light output versus cell density)	R^2 value for regression ($n = 5$)	Slope/ activity
70	0.28 ± 0.06	0.02	0.88	0.004 ± 0.001
140	0.63 ± 0.11	0.01	0.92	0.004 ± 0.001
197	0.81 ± 0.17	0.02	0.89	0.004 ± 0.001
351	2.02 ± 0.65	0.05	0.76	0.006 ± 0.001
703	3.59 ± 0.95	0.03	0.82	0.005 ± 0.001

possibly over interpret the results of this data. For each activity level, linear regressions were significant at the 95% confidence level, and the activity explained over 75% of the variation in measured light output in a linear model. Table 2 presents the results from linear regressions of measured UV emission versus cell density for each of the activities studied.

There is a positive relationship between measured UV emission and cell density up to 1×10^4 cells/5 mL. This would indicate that the cells are the source of the emission of a significant proportion of the measured UV. It can be seen in table 2 that the results of the slope of light output versus cell density increase linearly with increasing activity. If the slopes are normalized to activity, they are found to be the same to within uncertainties. Up to cell densities of 1×10^4 cells/5 mL, the measured level of emitted UV is a function of the source activity incident upon the cells and the number of cells in the dish.

Above cell densities of 1×10^4 cells/5 mL, small decreases in measured UV emission are observed. The decrease in measured UV photons is related to cell density: it is not a function of source activity. This is shown in figure 5 below. The decrease in measured photon counts per second is plotted against cell density in the figure for the region above cell densities of 1×10^4 cells/5 mL, where decrements are observed. The decrease is defined as the number of counts per second at a particular cell density minus the number of counts per second at the maximum signal recorded at a cell density of 1×10^4 cells/5 mL. It can be seen that the magnitude of the decrease is similar for all measured source activities, so this reduction is a function of cell density not activity. We suggest that the reduction is possibly a form of self-shielding. As cell densities increase, the amount of material which emits the UV signal increases. However, the amount of scattering and absorption also increases, and above cell densities of 5×10^4 cells/5 mL this reduces the signal that is measurable at an external detector.

3.3.3. Photon emission post-irradiation. An experiment was performed where UV photon emission was measured during irradiation of 1×10^4 living cells/5 mL to 703 μCi for 30 min and then was subsequently measured in the same cells as promptly as possible following removal of cells from β-exposure. Average photon emission during irradiation was 3.96×10^4 cps $\pm 1.52 \times 10^3$ cps (figure 6). The quantification of post-irradiation photon emission began one minute after the end of irradiation; measurement immediately following irradiation was not possible due to the need to turn off the PMT high voltage, remove the radioactive source from the light-tight unit and then replace the petri dish back into the counting apparatus. The average post-irradiation emission over a 92 min duration (figure 6 inset) was measured to be 1.74 ± 0.42 cps. In the absence of irradiation, it is evident that photon emission from live HaCaT cells is less than during irradiation. However, there is a slight suggestion that it is not quite to background levels. There is some evidence of a slow decay in light output post-irradiation in figure 6. This can be seen most clearly in the first 20 min. The light output in the

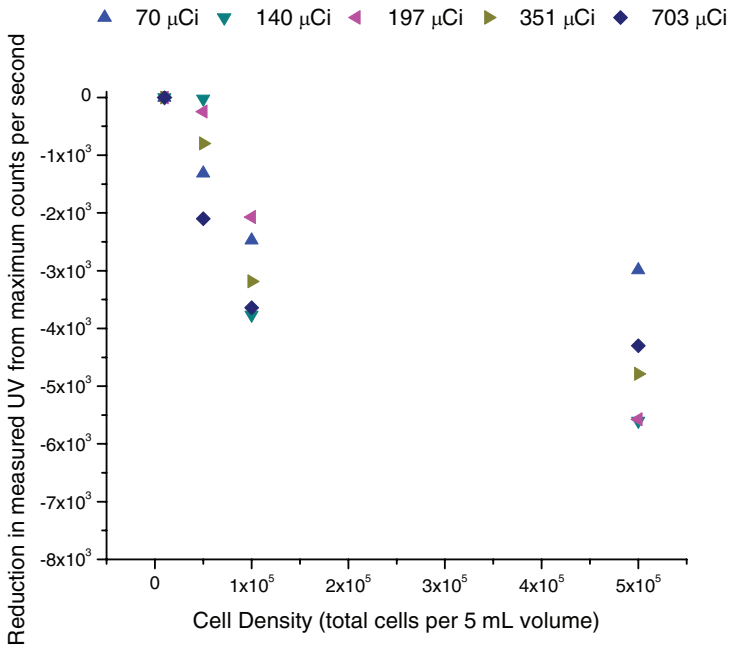

Figure 5. A plot of the reduction in measured UV counts per second from the maximum observed emission at cell density of 1×10^4 cells/5 mL plotted against cell density. All higher activities (which have high measured UV emission rates) show the order of magnitude of reduction indicating that the reduction is a function of cell density.

last time decile is significantly lower than in the first ($p < 0.004$), and fitting a linear decay results in a significant regression. If some of the UV photon emission is from scintillation in organic molecules, then it might be expected, through intersystem crossing, that in addition to scintillation, there could be phosphorescence. We do not suggest that scintillation and phosphorescence are the only mechanisms of photon emission under direct irradiation, but they would be expected to occur when certain types of organic molecules (which are present in cells) are irradiated.

It is also possible that the photon counts observed immediately following removal of the radioactive source is attributed to a systematic error. Gain variation (drift) over short operating times could explain the decay observed: Hamamatsu Photonics testing demonstrated an approximate 2% decay in relative output over 100 min of PMT operation (Hamamatsu Photonics 2007). To achieve more stable operation, Hamamatsu recommends warm-up for several ten minutes where voltage is set close to the operating voltage (Hamamatsu Photonics 2007). However, in the current experiment this was not possible due to the nature of the experiment. In order to account for this potential source of counts, a control experiment was performed where counts were detected for 90 min immediately following the application of high voltage at −800 V (figure A1). These measurements were taken without any cells or radiation in the light-tight chamber. The counts detected from the control's first time decile (mean = 1.219 ± 0.003 cps) were significantly different ($p = 0.001$) from the counts detected during the following 9 time deciles (mean = 1.205 ± 0.009 cps). The counts detected in the last 81 min of measurement were not significantly different from each other ($p > 0.994$). This result supports the idea that there is a noise contribution due to high voltage switch-on to the

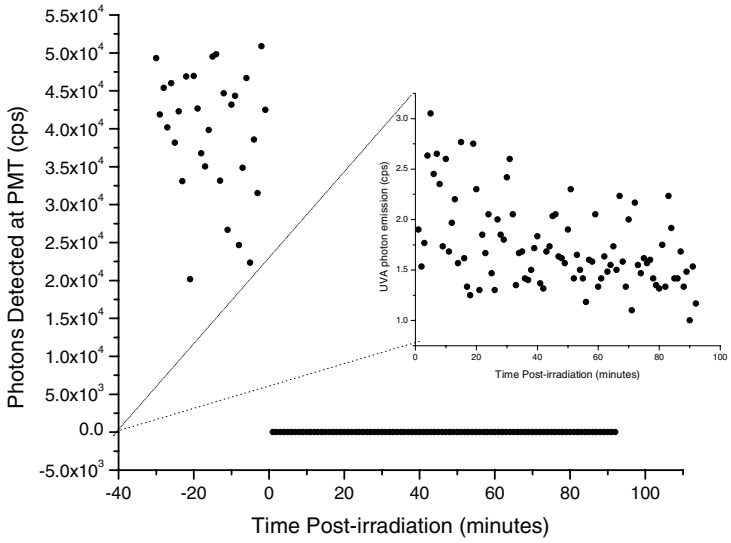

Figure 6. Photon emission during irradiation (1×10^4 cells/5 mL exposed to 703 μ Ci ^{90}Y for 30 min) and post-irradiation (92 min measurement). Inset: photon emission post-irradiation only.

observed light signal decay in the post-irradiation measurements. However, when the intensity of emission from HaCaT cells post-irradiation was compared to the intensity in the control experiment, it was found that the emission from the cells during the first time decile (mean = 2.23 ± 0.52 cps) remained significantly greater than the highest background control counts (1.219 ± 0.003 cps) ($p < 0.0001$). This would suggest that the photon emission observed in the post-irradiation experiment was not only attributed to noise in the system, but processes such as phosphorescence from cells may have also contributed a portion of the signal.

4. Discussion

4.1. Relationship of 340 nm photon emission quantity with ^{90}Y activity

Significant UVA photon emission at 340 ± 5 nm was detected emanating from both dead and living HaCaT cell cultures subjected to ionizing radiation. In both HaCaT cell populations, we noted a positive dependence of luminescence upon the activity of ^{90}Y applied. The underlying relationship between luminescence and activity may not be completely linear: more data will be required to establish the best model for the relationship with statistical certainty. The increase in emission related to an increase in activity seen in the results are in line with the ideas proposed by existing publications which suggest that greater photon emission from biological entities is prompted by a disruption of biological order (Bajpai *et al* 1991) or by any agents capable of insult or injury (Popp *et al* 1984). It also makes sense from a physical perspective; more ionizations resulting from a higher level of irradiation would be expected to result in a subsequently greater number of molecular and atomic level transitions which could produce photons in a range of wavelengths including the UVA range that we have measured here.

It is not surprising that irradiated materials can produce light, including that in the UV range, when irradiated. This is, of course, the physical basis by which both organic and crystal scintillation detectors can be used (Knoll 2011). These detectors emit light when irradiated, although by different mechanisms, within the material depending on whether they are organic or crystalline detectors. In addition, there is a whole field of materials analysis research, called ionoluminescence, which uses the phenomenon of emission of light as a consequence of charged particle irradiation, to interrogate and characterize materials. Our previous work showed that many materials used in radiation biology applications emit light when irradiated (Ahmad *et al* 2013) and that, in fact, dead cells also emit light. An important outcome of the data presented here, however, is that light output relates to cell density. This implies that the majority of the light detected in the presence of cells is coming from the cells.

These may be purely physical processes arising from transitions in the materials. However, an interesting question is whether these purely physical processes that lead to subsequent light emission as a consequence of irradiation, link to biological mechanisms and further, whether there is light emission in biological processes that may be changed, interrupted or enhanced as a consequence of irradiation. It has been hypothesized that reactive oxygen species (ROS) may be involved in the promotion of the photo-emissive process (Quickenden and Tilbury 1983, Devaraj *et al* 1991, Evelson *et al* 1997, Niggli *et al* 2001, Van Wijk *et al* 2008). Presence of reactive oxygen species (ROS) is one possible explanation for luminescence as it can explain the occurrence of the phenomenon in both dead and live cells via radical chain reaction processes or, in living cells, via oxidative stress mechanisms.

We have observed that at higher levels of radioactivity, dead and live cells (plated at 5×10^5 cells/5 mL) emit the same levels of UV per unit cell density and unit activity. However, it is particularly interesting to note that irradiation of 5×10^5 cells/5 mL produced significantly greater magnitudes of photoemission in dead cells than in living cells at beta activities up to and including 197 μCi ($p < 0.01$) (figure 7); the difference in photoemission was not significant at greater activities (351 μCi, $p = 0.146$; 703 μCi, $p = 0.987$). We speculate that the observation of weaker photon detection from living cells may be due to the ability of living cells to mediate intracellular reduction-oxidation (redox) reactions. This contrasts with dead cells where there is an absence of potential difference across cell membranes. Living cells possess active redox potential and therefore would be able to initiate antioxidant defenses to moderate the degree of cellular photoemission. At higher beta activities however, accumulation of ROS could cause antioxidant activity to decrease (Egea *et al* 2007) and therefore result in the inability to suppress ROS-driven photon emission. Despite uncertainty regarding the mechanism for reduction in photon emission, these results indicate that cellular viability may play some role in the measured level of emitted UV.

In previous findings by our research group (Le *et al* 2015), we had observed a lack of significant difference between HaCaT cell *survival* when exposed to signals from dead and living tritium-irradiated HaCaT cells. In the current study, we found that *signal* strength differed in dead versus living cells. These differences observed suggest that the response of cells to signals are likely mediated by mechanisms separate from those which mediate the actual signal production.

4.2. Relationship of 340 nm Photon emission quantity with cell density plated

No significant difference in photon emission rate was observed for the range of cell densities where irradiated ethanol-treated (i.e. dead) cells were studied. However, in the live cell

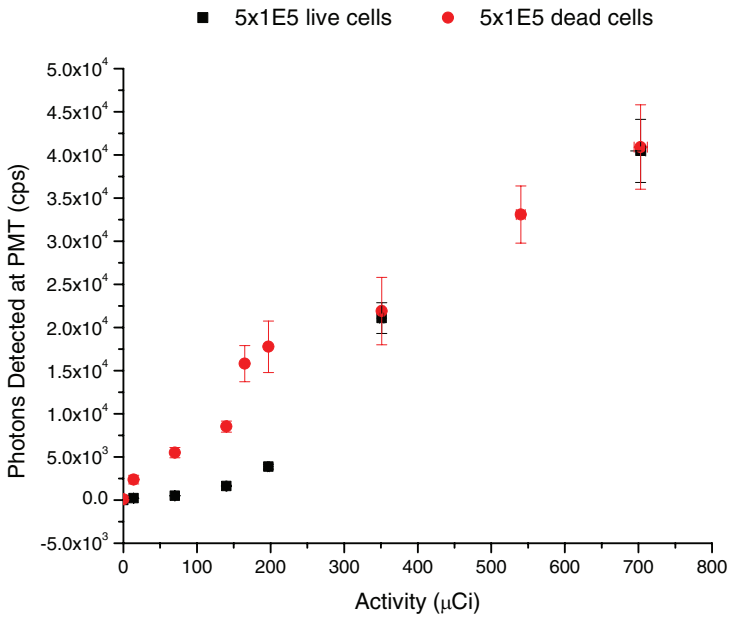

Figure 7. Comparison of the UVA photoemission from dead and live cells, each plated with 5×10^5 cells/5 mL and exposed to various ^{90}Y activities (n=9). Same data as that from figure 2.

population, where a different range of densities was studied, a trend was observed where the photon emission rate increased with increasing cell density up to a maximum at 1×10^4 cells/5 mL, after which a decrease in detected photons was demonstrated. This distinct pattern of photon detection in live cells was demonstrated at all six of the activity levels applied and the reproducible nature of these emissions strongly supports a relationship between the density of cells and the photon emission quantity. We suggest that the decrease in photon quantification at cell densities greater than 1×10^4 cells/5 mL could be attributed to a greater degree of interaction, such as scattering and absorption occurring between the photons emitted from the cells and structures within the cells. Scattering or absorption of the photons would effectively prevent the photons from reaching the detector to be read by the photocathode. The observed maximum at approximately 1×10^4 cells/5 mL may have biological implications if the emitted UV has a subsequent effect. It could be expected that observable damage or other biological effects would be evidently demonstrated when the density of cells is near 1×10^4 cells/5 mL. It is therefore expected that the effects of the cell-emitted UV would be relevant for cell signalling in all tissues which possess densities greater than 1×10^4 cells/5 mL (i.e. all tissues of the human body).

Importance of cell presence during irradiation. Exposure of the cell culture medium and petri dish in the absence of cellular material produced a relatively low count rate at the PMT (2767 cps at 703 μCi exposure activity). Upon irradiation of 5×10^2 cells/5 mL at 703 μCi, the photon emission quantity was 5 times greater than that emitted upon irradiation of 0 cells to the same activity. Upon irradiation of 1×10^4 cells/5 mL at 703 μCi, the luminescence rate increased to a maximum of just under 4.5×10^4 cps, a 16-fold increase over the photon emission levels

achieved in the absence of cells. It is clear that the presence of cells during irradiation induces a significantly greater photon emission signal than in the case of an absence of cells. This inference is further supported by the literature (Devaraj *et al* 1991, Ahmad *et al* 2013). Following removal of suspension medium from the cell culture, an increase in the photon emission intensity was identified (Devaraj *et al* 1991). Deveraj suggests that absorption of light emitted from the cellular material is attributed to the presence of suspension medium and therefore, upon removal of the absorbing medium, a greater photon signal could be detected.

4.3. Photon emission post-irradiation

In the assessment of photon emission from cells during and immediately after irradiation, we found that photon emission decreased sharply down to levels only marginally above background when measured only 1 min post-irradiation. Such results are supportive of direct cell irradiation being a causative factor for photon emission and indicates that the vast majority of photoemission ceases without coincident stimulation. The one caveat is that there may be a very small level of phosphorescence observed post-irradiation. In addition, some of the signal detected during the first tens of minutes of post-irradiation measurement is likely attributed to noise in the system caused by insufficient warm-up time following high voltage application. Even still, the contribution to the detected photon emission by cellular phosphorescence has not been dismissed since the post-irradiation emission intensity with cells was significantly greater than the high voltage controls quantified in the absence of cells.

5. Conclusions

The present study demonstrates significant secondary ultraviolet photon emission from both dead and living human keratinocyte HaCaT cells upon irradiation by low-LET β-particles. We have presented data showing various factors including cell density and intensity of irradiation that affect the level of secondary photon emission. In addition, the level of photon emission from dead cells was found to be significantly greater than that emanating from living cells over certain cell density ranges, leading us to suggest that photon emission may be reduced by antioxidant activity occurring within living cells. In living cells, a distinct relationship was observed between photon emission and the density of cells present within the irradiated field. It was also evident that the cells themselves were required to be present in order to achieve significant levels of photon emission. Overall, these results indicate that cells, when irradiated with β-particles emit secondary photons, some of which are emitted in the UV range. In addition, there may be a biological component which contributes to the level of measured photon emission. Further investigation is required to determine the responsible biologic or metabolic factor.

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Appendix A

Table A1. Pearson's correlation analysis output for assessment of activity-photon emission relationship in dead cells.

Cells/5 mL	R	p-value
0 (control)	0.948	<0.0001
5×10^5	0.985	<0.0001
1×10^6	0.988	<0.0001
5×10^6	0.980	<0.0001

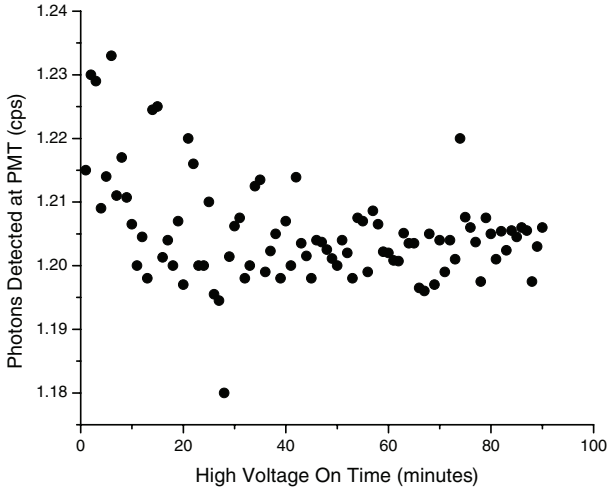

Figure A1. Background photon quantification conducted immediately following activation of the −800 V high voltage supply.

Table A2. Output for activity-dependent photon emission data fit to fixed-intercept linear regression (dead cells).

Cells/5 mL	R^2	p-value
0 (control)	0.914	0.003
5×10^5	0.895	0.051
1×10^6	0.893	0.044
5×10^6	0.800	0.031

Table A3. Pearson's correlation analysis output for assessment of activity-photon emission relationship in living cells.

Cells/5 mL	R	p-value
0 (control)	0.948	<0.0001
5×10^2	0.954	<0.0001
1×10^3	0.959	<0.0001
5×10^3	0.968	<0.0001
1×10^4	0.972	<0.0001
5×10^4	0.966	<0.0001
1×10^5	0.960	<0.0001
5×10^5	0.935	<0.0001

Table A4. Output for activity-dependent photon emission data fit to fixed-intercept linear regression (living cells).

Cells/5 mL	R^2	p-value
0 (control)	0.915	0.003
5×10^2	0.844	0.010
1×10^3	0.849	0.045
5×10^3	0.916	0.042
1×10^4	0.866	0.041
5×10^4	0.857	0.050
1×10^5	0.881	0.032
5×10^5	0.803	0.021

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Chapter 4

An Observed Effect of Ultraviolet Radiation Emitted from β-irradiated HaCaT cells upon Non-β-Irradiated Bystander Cells

Michelle Le, Fiona E. McNeill, Colin B. Seymour, Andrew J. Rainbow, Carmel E. Mothersill

The initial idea to investigate the effect of radiation-induced biophotons upon bystander cell populations was conceived by Dr. Carmel Mothersill, Dr. Fiona McNeill, and Dr. Colin Seymour. The experimental design was collaboratively established by the first author, Dr. Carmel Mothersill, Dr. Fiona McNeill, Dr. Colin Seymour and Dr. Andrew Rainbow. All experiments, statistical analyses, and manuscript writing were performed by the first author. All authors also contributed to the revision of the manuscript prior to submission and following receipt of reviewer comments from the journal.

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An Observed Effect of Ultraviolet Radiation Emitted from Beta-Irradiated HaCaT Cells upon Non-Beta-Irradiated Bystander Cells

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Previous research has shown that beta radiation can induce ultraviolet (UV) photon emission in human keratinocyte cells. Spectral analysis using a filter-based method in the ultraviolet range demonstrated that the strongest externally measureable photon emission was induced by beta radiation in the UVA range. In the current study, the potential biological implications of this UV photon emission from beta-irradiated cells were investigated. HaCaT human keratinocyte cells were irradiated with tritium (^{3}H) and the photon emission induced was concurrently measured at the strongest externally measurable wavelength, 340 ± 5 nm, using a combination filter-photomultiplier tube system. Unirradiated reporter HaCaT cell cultures were also placed directly above ^{3}H-irradiated cells so that they would receive the induced secondary photons emitted from beta-irradiated cells, and the clonogenic survival in reporter cells was then assessed. Maximum photon emission (1207.04 ± 107.65 counts per second) was observed during irradiation of 2,000 cells/cm^2 with ^{3}H and the maximum reporter cell death (23.2 ± 0.9% reduction in survival) was observed under the same conditions. The measured photon emission from beta-irradiated cells and reporter cell death were strongly correlated ($r = 0.977$, $P < 0.01$). Placement of a polyethylene terephthalate filter, designed to eliminate >90% of UV wavelengths below 390 nm, between the directly irradiated and reporter cell layers was effective in nearly abolishing both 340 nm photon detection and reporter cell death in treated groups. Concurrent treatment of reporter cells with lomefloxacin during exposure to the secondary photons resulted in significantly increased cell killing, indicating a potential synergistic effect, while melanin treatment resulted in decreased reporter cell killing regardless of irradiation. These results suggest that secondary photons in the UV spectral range induced by beta irradiation play a role in inducing a response in neighboring non-beta-irradiated reporter cells.

INTRODUCTION

Photon emission from biological materials has been reported by several investigators (*1–5*). The recent finding of significant ultraviolet (UV) photon emission induced by beta irradiation of human keratinocyte cells by Ahmad *et al.* (*2*) and our research group (unpublished data) prompted us to investigated whether this measurable UV emission had subsequent effects on non-beta-irradiated cells. This is of interest because UV photons alone are capable of inducing cell lethality either through direct methods such as substrate absorption (*6*) of UVB and C radiation resulting in covalent bonding of thymine or cytosine carbon–carbon bonds (*7*), or indirect methods such as UVA-induced generation of hydroxyl radicals ($^{\bullet}$OH) via Fenton-type reactions and subsequent action of cytotoxic $^{\bullet}$OH upon critical structures (*8*). Exposure of cells to UVA, UVB and UVC radiation has also produced bystander effects where various end points were assessed in bystander cells [micronuclei formation (*9*) and clonogenic survival (*10, 11*) for UVA; apoptosis, reactive oxygen species (ROS) generation (*12*) and oxidative stress (*13*) for UVB; and senescence (*12*) for UVC].

In addition, the risk that UV radiation poses to biological material can be further exacerbated by the type I and II photosensitizing reactions of photosensitizing agents. Both endogenous and exogenous photosensitizers become excited upon absorption of photon energy and interact directly with the cellular substrate or with molecular oxygen ($^3O_2$) (*14*). In type I photosensitization reactions, a sensitizer anion and a substrate cation are produced, which may then react with $^3O_2$ to produce oxidized species capable of inducing cellular damage. Conversely, in a type II photosensitization reaction, the interaction between the excited sensitizer and $^3O_2$ results in the generation of singlet oxygen $^1O_2$ which is then capable of reacting with DNA to form adducts and lesions.

Editor's note. The online version of this article (DOI: 10.1667/RR13827.1) contains supplementary information that is available to all authorized users.

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The above observation that UV radiation is capable of inducing such adverse effects in cellular material motivated our group to conduct the experiments in this current study. To be clear, the effect being investigated is not from a primary UV source but from a secondary UV source emitted as a consequence of beta irradiation of cellular substrates. This study investigates the relationship between this radiation-induced UV photon emission from cells and an observed response in neighboring bystander cells not exposed to beta radiation. A human keratinocyte cell line was assessed in terms of photon emission and clonogenic survival after irradiation, with the low-linear energy transfer (LET) radioisotope, tritium (^{3}H). Photosensitizer and photoprotector treatments were also employed to confirm the role of UV radiation in the observed bystander response.

MATERIALS AND METHODS

Cell Culture

The immortalized, nontransformed human keratinocyte cell line, HaCaT (*15*), was used in the current study. This cell line was a gift from Dr. Orla Howe (Dublin, Ireland) and was chosen for experimental study due to its past demonstration of the bystander effect (*16–18*). Cells were cultured in Roswell Park Memorial Institute (RPMI; Buffalo, NY) 1640 medium, free of phenol red (Gibco® Life Technologies Inc., Grand Island, NY). Supplemented RPMI 1640 contained 0.5 μg/mL hydrocortisone (Sigma-Aldrich® LLC, St. Louis, MO), 2 m*M* L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin sulphate. Cells were passaged in a Class II laminar flow biological safety cabinet when cultures reached 80–90% confluency.

To detach the adherent monolayer from the flask substrate we used 10 mL 1:1 solution of 0.25% trypsin and 1 m*M* ethylenediaminetetraacetic acid (EDTA) dissociation reagent. Trypsinized cells were neutralized with 10 mL culture medium. Cell suspension was pipetted into 75 cm^2 stock flasks containing 15 mL culture medium. Cultures were incubated at 37°C, 95% relative humidity and 5% CO_2 and received full volume medium renewals every 2–3 days. Experiments were performed on both live and dead cells to see if effects were different between the two. For the dead cell experiments, cell monolayers were treated with 5 mL 100% ethanol for 30 min. Ethanol was aspirated and cell cultures were left in the biological safety cabinet for 10 min to allow for complete evaporation of residual liquid. Culture medium (5 mL) was added to the dead cell culture to maintain consistent parameters with living cell experiments. All reagents were obtained from Gibco Life Technologies Inc. unless otherwise stated. Cell cultures were tested prior to use for mycoplasma contamination with the PlasmoTest™ mycoplasma detection kit (cat. no. rep-pt1, InvivoGen, San Diego, CA) and were confirmed to be mycoplasma free.

Photosensitizer and Photoprotector Preparation

Lomefloxacin hydrochloride (Sigma-Aldrich) is a fluoroquinolone antibiotic with photosensitizing properties. Solid lomefloxacin hydrochloride (38.8 μg) was solubilized in 0.776 μL of 1 *M* sodium hydroxide (NaOH) (Sigma-Aldrich) and 499.224 μL of distilled-deionized water (ddH_2O). This solution was then passed through a 0.2 μm pore filter (Pall Corporation, Ville St. Laurent, Canada) and added to 4.5 mL of cell culture medium to produce a total volume of 5 mL at a concentration of 20 μ*M* lomefloxacin hydrochloride. A 20 μ*M* sterile solution of lomefloxacin hydrochloride was used to treat cells since this concentration has been reported to produce photogenotoxic effects in human keratinocyte cells upon exposure to UVA (*19*).

A 10 mg/L solution of melanin of the eumelanin variety (Sigma-Aldrich) was prepared for uniform distribution upon addition into cell cultures. For each aliquot, 50 μg of solid melanin was dissolved in 2.5 μL 1 *N* ammonium hydroxide (NH_4OH) and 497.5 μL ddH_2O. This solution was pipetted into 4.5 mL cell culture medium and passed through a 0.2 μm pore filter (Pall Corporation) to ensure sterility. A melanin concentration of 10 mg/L was chosen due to the previously demonstrated modulation of the bystander effect upon addition of 10 mg/L melanin (*20*).

Chemical solutions were added to cell culture medium and 5 mL volumes were measured for significant deviations from normal pH using a VWR® sympHony™ B10P benchtop pH meter (electrode: 89231-580 saturated in potassium chloride; buffer calibration: pH 4.00 and 7.00) (VWR® International LLC, Mississauga, Canada). The pH for untreated, lomefloxacin-treated and melanin-treated samples of RPMI cell culture medium were measured as 7.40 ± 0.00, 7.416 ± 0.003 and 7.46 ± 0.00, respectively (n = 3, error represents standard error of the mean). Clonogenic survival was also tested in unirradiated cell cultures to determine the cytotoxicity of chemicals in the absence of photon exposure. Cultures received no treatment (clonogenic survival for 24 h and 7 days, respectively: 99.7 ± 4.1%, 100 ± 3.97%), lomefloxacin treatment (94.1 ± 4.2%, 8.1 ± 3.2%) and melanin treatment (97.5 ± 0.7%, 99.5 ± 2.3%) for 24 h or 7 days. Since these experiments confirmed the absence of the chemicals' cytotoxic action on HaCaT cell cultures when treated for 24 h. A treatment period of 24 h was chosen for successive experiments.

Irradiation

HaCaT cells that would be directly irradiated (DIR) by beta radiation were plated into 100 × 15 mm polyethylene petri dishes at seven different cell densities ranging from 20 to 2×10^4 cells/cm^2. A given volume of tritiated water (PerkinElmer® Inc., Woodbridge, Canada) corresponding to activities ranging from 1.71–857.5 μCi was pipetted into each petri dish where the final volume in each dish was 5 mL. Cell cultures containing tritiated water were sealed with Parafilm® (Bemis® Company Inc., Neenah, WI) to limit evaporation and were then incubated at 37°C for 24 h to achieve desired dose levels (Supplementary Table S1; http://dx.doi.org/10.1667/RR13827.1.S1). The dose was calculated using Eq. (1). The long half-life of ^{3}H (12.28 years) allows for the assumption that decay is negligible over the cell irradiation period, i.e. the dose rate is constant. Tritium is a pure β^- emitter with a maximum beta energy of 18.6 keV, average beta energy of 5.7 keV and a half-live of 12.28 years (*21*). The use of a low-energy beta emitter such as ^{3}H restricted the absorption of all beta particles to within the cells themselves. The maximum range of a beta particle produced from ^{3}H decay is 7 μm in tissue (*21*), thus eliminating the possibility of beta particles reaching the photomultiplier tube or adjacent bystander cell cultures. To emphasize this point, only cells incubated with ^{3}H are irradiated with beta particles. Cells referred to as bystander cells cannot be exposed to beta particles directly because the plastic layer between the irradiated and bystander cells is too thick to allow ^{3}H beta particles to pass.

$$D = \frac{N_o \lambda_R \overline{E}_\beta t}{m} \tag{1}$$

Single Photon Counting

Photon quantification was performed using a photomultiplier tube (PMT) (Hamamatsu Photonics, Bridgewater, NJ) fitted with an interference-type band filter (Edmund Optics Inc., Barrington, NJ) permitting only a narrow range of photon energies to pass. For the filter ultimately used on the measurement system in this series of experiments, the pass wavelength was centered at 340 ± 5 nm. This wavelength was chosen because it was the strongest externally measurable signal. This is not to suggest that this is the only emission wavelength, nor that this is the strongest emission wavelength within the cells and media. This

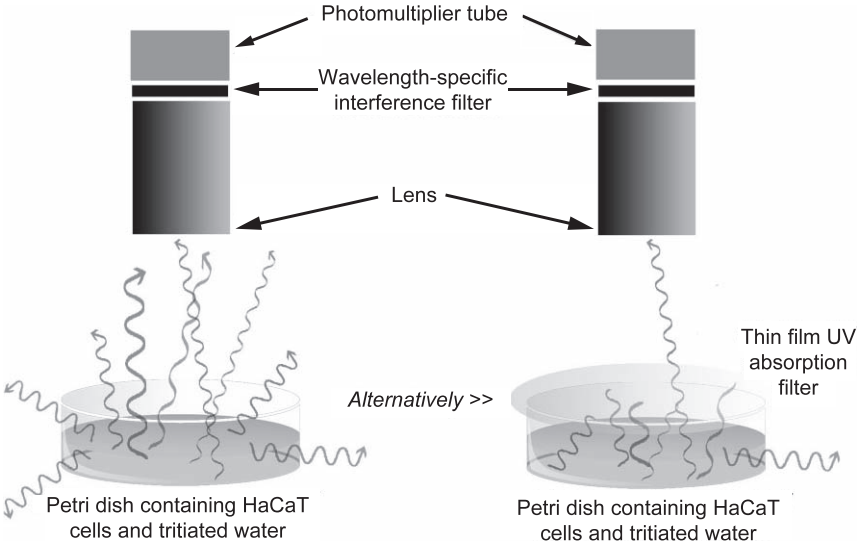

FIG. 1. Configuration for photomultiplier tube detection of photons emitted from a directly irradiated cell culture. Alternative configuration shown on the right demonstrating the addition of a thin film UV absorption filter superior to the irradiated cell culture.

equipment was contained within a light-tight aluminum container to eliminate ambient photons from the room from being detected. A closed petri dish containing HaCaT cells was placed inside the light-tight aluminum container at a distance of 3.5 cm from the lens of the PMT detector system. Figure 1 shows the experimental setup for photon counting from irradiated cell cultures.

Spectral Analysis of Photon Emission

Spectral analysis was initially performed in the ultraviolet range using three interference-type band pass filters (Edmund Optics Inc.) centered at 340 ± 5 nm (in the range of UVA), 300 ± 5 nm (in the range of UVB) and 280 ± 5 nm (in the range of UVC) placed in the path between the emitting cells and the photomultiplier tube photocathode. This permitted the study of photon emission across a spectral range that encompasses UV radiation. This methodology allows assessment of only very narrow windows onto the total spectrum and one window at a time, and assessment of the full spectrum emission cannot be done using this method. Cells (2×10^4/cm^2) were exposed to 857.5 μCi for 24 h while photon quantification took place concurrently.

Bystander Experiments and Treatments

To investigate the potential biological consequences of the cellular photoemission of UV photons, a 25 cm^2 reporter flask plated at clonogenic density (500 cells per flask) was placed directly superior to a petri dish containing DIR (^{3}H) cell cultures for 24 h. The distance from the radioactive cell culture surface to the reporter cell monolayer was approximately 1.5 cm and each flask–petri dish pair was placed into its own partitioned compartment within a light-tight box. Any effects observed in the reporter cells are presumed to result from cellular photon emission since, as previously described, the low-energy ^{3}H beta particles are insufficiently high in energy to escape the directly irradiated cell culture.

Various parameters of the experiment were changed to confirm the emission and transmission of UV light. First, a thin film UV filter (Edmund Optics Inc.) designed to absorb >90% of UV photons below 390 nm was placed between the petri dish and the reporter flask and clonogenic survival was subsequently assessed. The thin film UV filter is made from deep-dyed polyethylene terephthalate (PET) and is 0.04 mm thick. A spectrum of UV transmission data for the filter was not available from the manufacturer; we assume equal absorption of 90% for all photon wavelengths below 390 nm, i.e., 90% absorption of all UV wavelengths. Second, lomefloxacin, a fluoroquinoline antibiotic with photosensitizing properties was added to reporter cells, directly irradiated petri dish cells, or both for 24 h either during or after irradiation. Upon reaching the end of the 24 h incubation period with lomefloxacin, the reporter flask was given a full volume medium renewal to eliminate the drug from the reporter cell culture. Clonogenic survival was assessed 7 days after initial cell plating. Finally, this experimental design was repeated using the photoprotector and photon absorber, melanin. All experiments were performed with both living and dead cells in the directly irradiated petri dish compartment.

Clonogenic Assay

Subconfluent flasks (80–90% confluence) received full-volume medium renewals 24 h prior to seeding for experimentation. Cell monolayers were detached using a 1:1 solution of 0.25% w/v trypsin-1 m*M* EDTA and neutralized using an equivalent volume of cell culture medium. Cell concentration of stock solution was determined using a Beckman Coulter Z2 particle count and size analyzer (Beckman Coulter LP, Mississauga, Canada) previously calibrated using a hemocytometer. Clonogenic cell densities (500 cells per flask) were plated onto 25 cm^2 flasks, treated and incubated for 7–8 days until most colony-forming units contained at least 50 cells. Flasks were stained with carbol fuchsin (Sigma-Aldrich) and 50-cell colonies were counted according to the colony-forming clonogenic survival technique developed by Puck and Marcus (*22*).

Statistical Analysis

Analysis of variance (ANOVA) was used to determine the statistical significance of studies employing multiple independent variables and one dependent variable (cell survival). Post-hoc testing was performed using Fisher's least significant difference (LSD) test. Statistical analyses were performed using SPSS Statistics 17.0.

RESULTS

Spectral Analysis of Photon Emission

Upon ^{3}H irradiation of 2×10^4 cells/cm^2 to 857.5 μCi, average photon emission rates of 311.75 ± 3.16 counts per

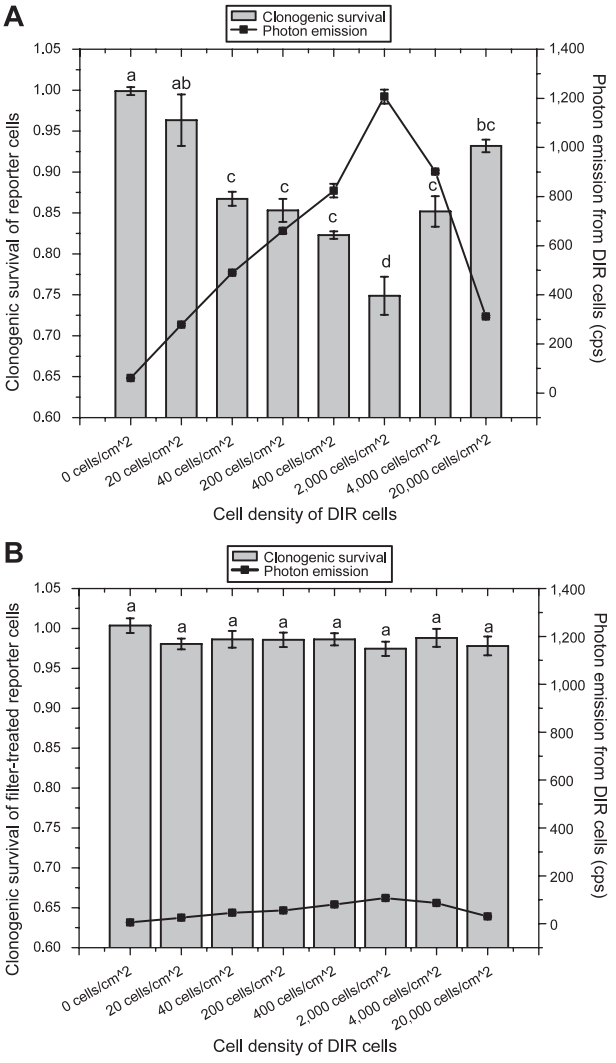

FIG. 2. Line plot: Quantity of photons detected upon ^{3}H 857.5 μCi (0.5 Gy) irradiation of live HaCaT cells plated at cell densities ranging from 20 to 2×10^4 cells/cm^2. Column graph: Clonogenic survival of bystander cells placed directly superior to directly ^{3}H-irradiated (857.5 μCi or 0.5 Gy) live HaCaT cells plated at different cell densities. Error bars represent SEM, n = 9. Letters (a, b, c, d) indicate similarities and statistical differences in surviving fraction. Analysis was performed using two-way Analysis of Variance (ANOVA) method. Post-hoc testing was performed using Fisher's least significant difference (LSD) test. Significance level: 0.05. Panel A: Directly irradiated (DIR) cell photon detection and bystander cell survival in the absence of a UV absorption filter. Panel B: Directly irradiated (DIR) cell photon detection and bystander cell survival in the presence of UV absorption filter between the directly irradiated and bystander cell cultures.

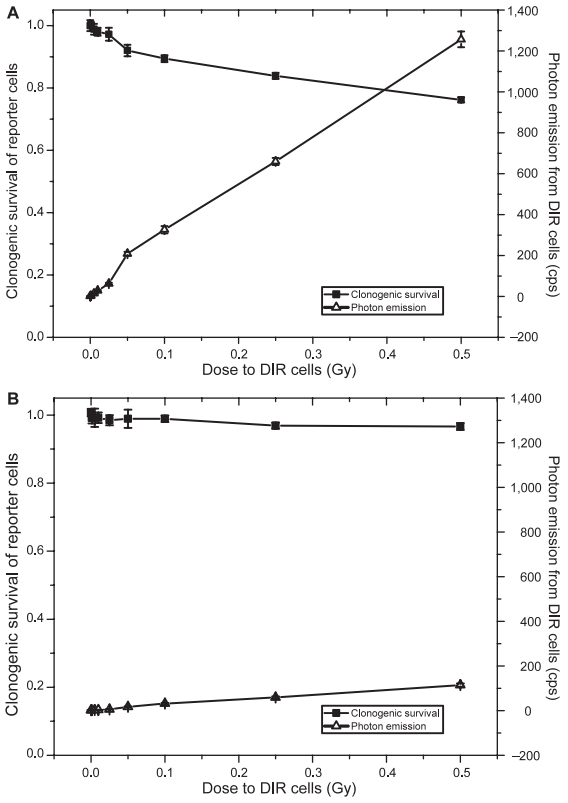

FIG. 3. Triangle data points: Quantity of photons detected from live HaCaT cell cultures plated at 2,000 cells/cm^2 upon irradiation with ^{3}H activities ranging from 1.7– 857.5 μCi (0.001–0.5 Gy). Square data points: Clonogenic survival of bystander cells placed superior to directly irradiated live HaCaT cells (2,000 cells/cm^2) exposed to different doses of ^{3}H. Error bars represent SEM, n = 9. Panel A: Directly irradiated (DIR) cell photon detection and bystander cell survival in the absence of a UV absorption filter. Panel B: Directly irradiated (DIR) cell photon detection and bystander cell survival in the presence of UV absorption filter between the directly irradiated and bystander cell cultures.

second (cps), 194 ± 5.43 cps and 86.48 ± 4.87 cps were quantified at the PMT for wavelengths of 340 ± 5 nm (within the UVA range), 300 ± 5 nm (within the UVB range) and 280 ± 5 nm (within the UVC range), respectively. We observed emission at all three filter wavelengths where the strongest externally measurable signal was at 340 nm. We therefore chose to use this as a ''marker'' of a wider, and as yet unknown, emission spectrum for further experiments, because it provided the best relative uncertainty in photon counting statistics. These measured signal magnitudes do not necessarily indicate the relative spectral shape either emitted by the DIR cells, or incident on the reporter cells, since our detector measures the transmitted spectrum that passes through the cell layers, folded through a detector response function. It is therefore not implied that 340 nm was the only wavelength emitted; rather it will be used as a measureable marker of overall UV emission in a broad spectral range.

Photon Quantification from Directly Irradiated Cultures

In the absence of thin film UV filter treatment, photon emission from ^{3}H-irradiated live HaCaT cells [857.5 μCi (0.5 Gy)] demonstrated an increasing number of photons detected with an increase in cell density up to 2,000 cells/cm^2. The maximum number of photons measured at 2,000 cells/cm^2 was 1,207.04 ± 28.23 cps. Irradiation of cell cultures plated at higher cell densities resulted in diminished quantities of detected photons (Fig. 2A). Cell cultures directly irradiated with various ^{3}H activities demonstrated a positive biphasic relationship with added radioactivity (Fig. 3A); the slope corresponding to the relationship between ^{3}H

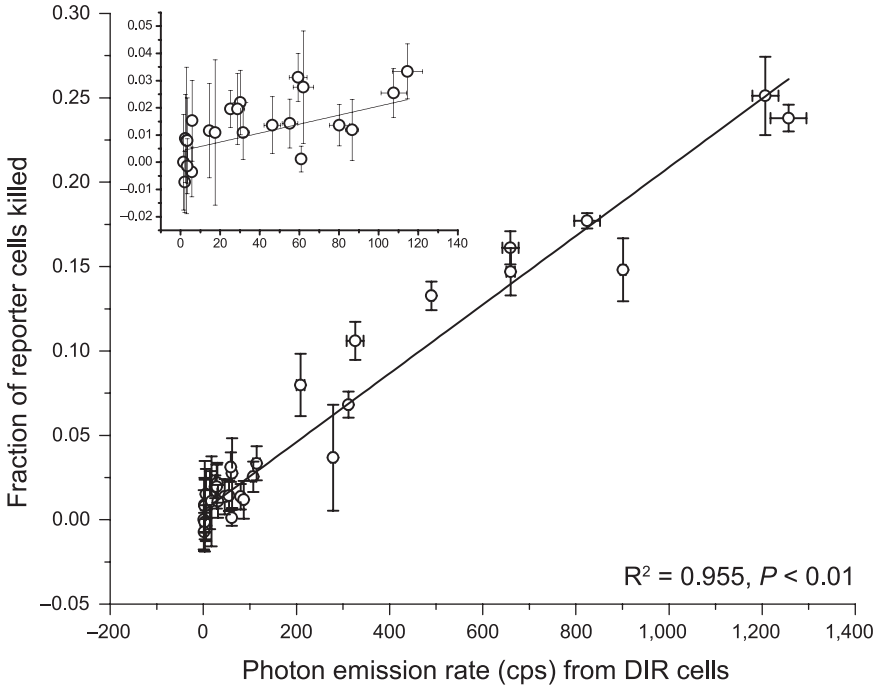

FIG. 4. Scatter plot of directly irradiated (DIR) photon emission rate and reporter cell killing. Scatter plot contains 34 data points, each of which represents the mean of n = 9. Error bars represent SEM for n = 9. Inset: Reporter cell killing at photon fluences below 200 counts per second (cps).

activity and photon emission was 2.34 from 0–85.75 μCi (0.05 Gy) and 1.36 from 85.75–857.5 μCi (0.05–0.5 Gy).

Clonogenic Survival of Reporter Cells

For bystander experiments, the reporter cells in the field of DIR cells that were plated with 2,000 cells/cm^2 demonstrated the greatest degree of cell death (Fig. 2A). That is, the greatest cell death was observed at the density that corresponded to the highest measured UV output at the detector. Reductions in survival were observed as cell density increased up to 2,000 cells/cm^2 while, upon further increase of cell density, an increase of clonogenic survival was seen. Such a pattern suggests an influence of cell density upon bystander cell survival. Clonogenic survival follows an inverse pattern to UV emission. For reporter cells in the field of directly irradiated cultures exposed to different ^{3}H activities, reporters demonstrated significant reductions in survival for doses $\geq$0.1 Gy compared to reporter cells that were placed superior to unirradiated cell cultures (Fig. 3A). Bystander survival demonstrates negative biphasic behavior as ^{3}H dose increases. From 0–85.75 μCi (0–0.05 Gy) the slope is −1.525 and between 85.75 and 857.5 μCi (0.05 and 0.5 Gy), the slope is −0.345. In the low-dose range below 85.75 μCi (0.05 Gy), the change in survival is more pronounced.

Thin-Film UV Absorption Filter Treatment

Placement of a thin-film UV absorption filter on a petri dish containing directly irradiated cells, and therefore in the path between the beta-irradiated cell media and the PMT detector, was effective in eliminating the detection of most 340 nm photons (Figs. 2B and 3B). The intensity of measured photoemission from UV-filter-treated cell cultures was on average 86% lower than the intensities measured for the non-UV-filter-treated cell cultures, and the degree of intensity reduction is closely representative of the 90% effectiveness in absorption stated by the manufacturer (Edmund Optics Inc.).

For experiments conducted with thin-film UV filters placed between directly irradiated and reporter cell cultures, cell survival was maintained near 100% (Figs. 2B and 3B). Elimination of the UV flux (for all wavelengths below 390 nm) by application of a UV filter was correlated with an increase in bystander cell survival. We emphasize that the use of the absorbing filter only allows us to conclude whether observed effects are eliminated when all UV wavelengths below 390 nm are eliminated.

Association between DIR Photon Emission and Reporter Cell Killing in Nonfilter-Treated Cells

Photon emission measured from directly irradiated HaCaT cells plotted against reporter cell killing is shown in Fig. 4. Assessment using Pearson's correlation test demonstrated a significant positive correlation between DIR cell photon emission rate (measured at 340 nm) and fraction of cell killing in reporter cells ($r = 0.977$, $P < 0.01$). Assessment using linear regression shown an $R^2 = 0.955$, $P < 0.01$.This indicates that at a fixed cell density, 95.5% of the variation in the fraction of cell killing can be explained

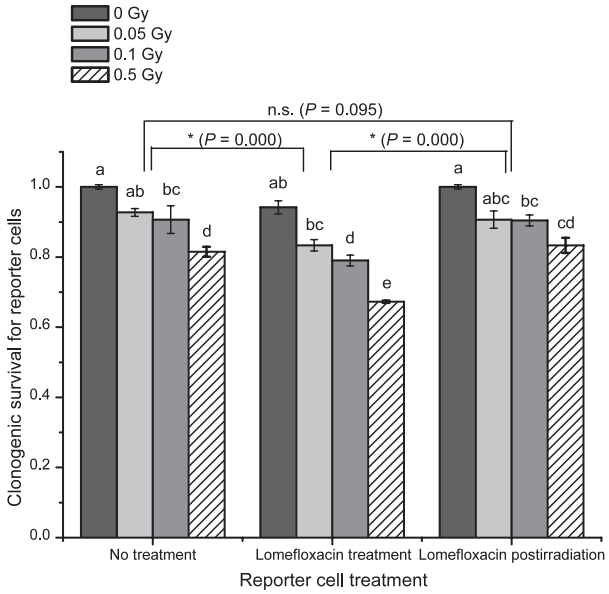

FIG. 5. Clonogenic survival for reporter cells receiving 20 μ*M* lomefloxacin that were in the field of ^{3}H-irradiated directly living (DIR) cells; DIR cells received no other treatment. Error bars represent SEM, n = 9. Letters (a, b, c, d, e) indicate similarities and statistical differences between reporter cell clonogenic survival. Significant differences between treatment groups is denoted by ''*'' (significance level = 0.05). Analysis was performed using two-way Analysis of Variance (ANOVA) method. Post-hoc testing was performed using Fisher's least significant difference (LSD) test.

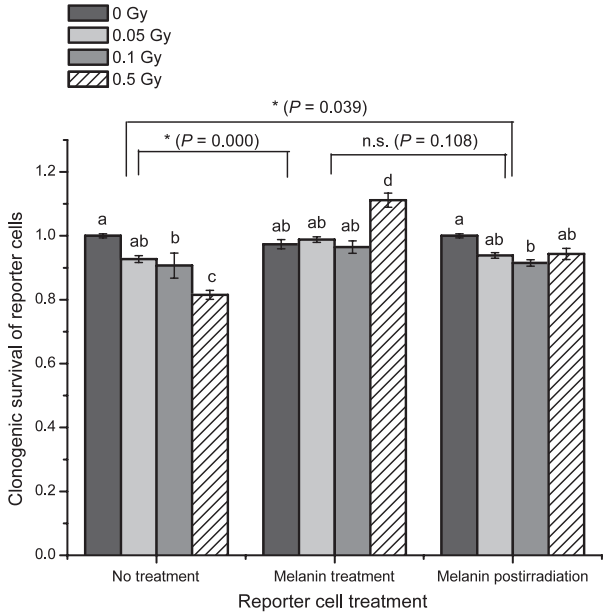

FIG. 6. Clonogenic survival for reporter cells receiving 10 mg/L melanin that were in the field of ^{3}H-irradiated directly living (DIR) cells; DIR cells received no other treatment. Error bars represent SEM, n = 9. Letters (a, b, c, d) indicate similarities and statistical differences between clonogenic survival of reporter cells. Significant differences between treatment groups is denoted by ''*'' (significance level = 0.05). Analysis was performed using two-way Analysis of Variance (ANOVA) method. Post-hoc testing was performed using Fisher's least significant difference (LSD) test.

in this correlation by the variable ''measured 340 nm UV emission''. This is not to say that we have established that UV photons only in the range 340 ± 5 nm cause the cell death. However, the fact that when all UV below 390 nm is filtered out, the cell death is reduced and cell death correlates with a measurable marker of UV emission, is highly suggestive, and should be further explored.

Lomefloxacin Treatment in Reporter Cells

Two thousand live cells/cm^{2} plated in petri dishes were directly irradiated with 0.05, 0.1 and 0.5 Gy ^{3}H. Reporter cells receiving 20 μ*M* lomefloxacin either before or after 24 h irradiation were placed directly above DIR cells (Fig. 5). Survival in the lomefloxacin-treated reporters decreased by 9.4, 11.7 and 14.2% when in the field of 0.05, 0.1 and 0.5 Gy ^{3}H-irradiated cells, respectively, compared to reporters that received no lomefloxacin treatment. The survival of the lomefloxacin treatment group was significantly different from both the no treatment and postirradiation treatment groups ($P < 0.001$). These results indicate that treatment of reporter cells with lomefloxacin in combination with their presence in the UV emission field of directly irradiated cells produces a synergistic effect where cell survival is further decreased below the survival levels demonstrated in untreated cells. We describe the effect as synergistic since lomefloxacin treatment alone, in the absence of UV exposure, did not produce significant reductions in bystander cell survival and so the effect is not merely additive. The surviving fractions of the no treatment and the lomefloxacin postirradiation treatment groups did not demonstrate statistically significant differences from each other ($P = 0.095$). Treatment with lomefloxacin for a 24 h duration after a 24 h irradiation did not confer any changes in survival.

Melanin Treatment in Reporter Cells

Reporter cells received 10 mg/L melanin during or after placement in the field of DIR cells exposed to 0.05, 0.1 or 0.5 Gy of ^{3}H (Fig. 6). In the untreated reporter group, cell survival decreased with increasing dose. In the melanin-treated group, survival at all doses was slightly greater than survival levels seen in the untreated group. Cell survival at 0.05, 0.1 and 0.5 Gy for the treated group were 6.1%, 5.8% and 29.7% greater, respectively, and differences in survival between untreated and melanin-treated cells were significant ($P < 0.001$). The postirradiation melanin-treated group also demonstrated slightly higher survival rates than the untreated group where survival was 1.2%, 0.8% and 12.8% greater at 0.05, 0.1 and 0.5 Gy, respectively. Thus, postirradiation melanin treatment resulted in survival levels that were significantly different from those of untreated reporter cells ($P = 0.039$).

Melanin increased the growth efficiency in cell cultures regardless of whether it was added during or after irradiation. It is possible that melanin absorbed photons that were emitted from the DIR cells since melanin is one of

TABLE 1
Clonogenic Survival of Reporter Cells in the Field of Living and Dead Directly Irradiated (DIR) Cells

Untreated reporters	Living DIR cells (I)	Dead DIR cells (J)	Mean difference (I–J)	Significance (*P* value)
0 Gy	1.00 ± 0.006	0.999 ± 0.018	0.001	0.975
0.05 Gy	0.927 ± 0.011	0.911 ± 0.020	0.016	0.483
0.1 Gy	0.907 ± 0.039	0.834 ± 0.012	0.073	0.095
0.5 Gy	0.815 ± 0.014	0.795 ± 0.0116	0.020	0.277

Notes. Reporter cells and DIR cells were not treated with any chemicals, drugs or filters. Errors represent SEM, n = 9.

the UV absorbing chromophores in human skin (*23*). Photon absorption by melanin could explain the increase in plating efficiency observed in the reporters treated with melanin during irradiation. This, of course, does not explain the effectiveness of melanin treatment postirradiation. We suggest this effect may be explained by melanin's radical scavenging properties, but it clearly warrants further investigation.

Lomefloxacin or Melanin Treatment in DIR Cells

DIR cells received either lomefloxacin treatment or melanin treatment to determine the effects of these drugs upon signal transduction to reporter cells. Treatment of DIR cells with 20 μ*M* lomefloxacin solution slightly modulated the cell death observed in untreated and lomefloxacin-treated reporter cells (Supplementary Fig. S1; http://dx.doi.org/10.1667/RR13827.1.S1). However the change induced by DIR cell treatment with lomefloxacin was not significant ($P = 0.067$) when compared with untreated DIR cells. It is possible that the photosensitizer's interaction with the cell-emitted UV photons resulted in weak photon absorption within the DIR cell culture, but this is a suggestion of an explanation for a mild effect that did not reach significance at the 95% level. Resultantly, reporter cells were subject to slightly weaker photon flux, however photon absorption by lomefloxacin was not quenched enough to significantly modulate the UV signal transduction.

Melanin treatment of DIR cells was effective in abolishing cell-killing effects previously found to correlate with UV emission, in reporter cells ($P < 0.001$) (Supplementary Fig. 2; http://dx.doi.org/10.1667/RR13827.1.S1). This observation is attributed by us to melanin's efficiency in absorbing a wide spectrum of photon wavelengths (*20, 24*). Efficient absorption likely occurred in the directly irradiated compartment thus preventing the overall transmission of UV photons, thereby resulting in fewer UV photons reaching the reporter cell compartment. In this respect, melanin was able to quench the photon signal and thereby modulate the photon signal emitted by the DIR cells. The survival comparisons were made in reference to untreated reporter and DIR cell controls.

Dead Cell Experiments

Experiments were performed where HaCaT cells plated in petri dishes were killed with ethanol prior to irradiation. This experiment was performed to determine whether UV emission and subsequent effect was different between live and dead cells. These dead cells were directly irradiated with tritiated water and were not treated with any other drugs. Reporter cells that were placed above these dead DIR cells were shielded with thin film UV filters, or treated with lomefloxacin or melanin. Filter, lomefloxacin and melanin treatment of reporter cells upon exposure to dead DIR cells produced similar trends to those exhibited by reporter cells that were exposed to live DIR cells: thin film filter and melanin treatments nearly abolished bystander cell killing and lomefloxacin treatment exacerbated cell death in bystander cells. The clonogenic survival in untreated reporter cells exposed to the field of dead DIR cells was reduced to a greater, but not significantly different, extent than those in the field of live DIR cells (Table 1). These results demonstrate a negligible difference in the measured photon signal (at 340 nm) between dead and living HaCaT cells. This suggests that the UV emission that results from irradiation of cell material primarily by beta rays is therefore not a biological phenomenon but a physical response. We presume from these experiments that UV arises because of electron rearrangement in atoms that are either ionized or excited by the interaction with beta particles. As electrons rearrange within atoms and molecular orbitals, photons of a range of energies, including UV, can be emitted. This will happen whether the atom is in a live cell, a dead cell or in the container material.

DISCUSSION

Confirmed Role of Ultraviolet Photons in the Observed Bystander Effects

The current study supports the hypothesis that UV photons emitted from directly irradiated cells may play a role in influencing the status of bystander cells. Our experimental design allowed for irradiation of a given cell population using tritiated water (a low-energy beta emitter) while preventing primary radiation exposure to bystander cells. This was possible because the beta particles emitted as a result of ^{3}H decay are sufficiently low in energy, and thus short in range, therefore that they will only irradiate the cell culture in which they are contained (*25–27*). Any effects observed in bystander cells placed above the directly irradiated culture are therefore presumed to be a result of factors extraneous to the primary radiation source.

The current study focused on measuring the 340 nm signal because it was the strongest signal and therefore

would produce the most robust measurements for statistical purposes. However, wavelengths at 300 nm and 280 nm were also detected. We do not know the photon spectrum incident on the reporter cells, and have measured only a few wavelengths external to the cells. We assume that many wavelengths of UV are emitted. The filter, which eliminated observed effects, reduced all wavelengths below 390 nm by 90%, i.e., all UV wavelengths. Each wavelength range within the UV spectrum may contribute a different proportion of action to the overall observed response. In contrast, it is also possible that there is a very narrow range of UV photon wavelengths that constitute the action spectrum. Further studies are planned, which will restrict the transmission to narrow UV bands through to the bystander cell compartment to resolve this issue. For the purpose of this study, the measured UVA radiation is meant to be a representative marker of UV emission below 390 nm.

Our experimental studies demonstrated a very strong correlation between the measured photon emission assessed at 340 nm from directly irradiated cells and the clonogenic survival of reporter cells in the field of directly irradiated cells. The strength of this correlation supports the suggestion that UV photons emitted from directly irradiated cells may influence a bystander response in cells subject to the emitted UV. To confirm that the modulating agent was indeed a photon in the UV range, a PET thin film UV filter was placed between the directly irradiated culture and the bystander culture. The effectiveness of the filter was specified by Edmund Optics to be 90% absorptive for wavelengths below 390 nm, thus encompassing all but 10 nm of the UV spectrum. The effectiveness of the filter was confirmed by our photon emission experiments (Figs. 2 and 3). Our experiments show that the UV filter, when placed between direct and bystander cell layers, was effective in nearly abolishing negative survival effects seen in the reporter cell population, also supporting the UV wavelength range used in this study.

The mediation of bystander cell survival by UV emitted from irradiated cells is a novel concept. We therefore conducted further investigations using photosensitizing and photoprotecting agents to garner more evidence as to whether UV truly plays a role in bystander signaling or whether our preliminary observations were attributed to a confounding factor. Unirradiated bystander cells were supplemented with a solution of the fluoroquinolone antibiotic, lomefloxacin. Fluoroquinolone antibiotics act as phototoxins that absorb and become excited in response to incoming UV photons, and with lomefloxin, maximum absorption occurs at 320 nm (*28*). Simultaneous lomefloxacin treatment with UV irradiation is capable of inducing DNA strand breaks (*19, 29*) and enhancing the severity of malignant squamous cell carcinomas (*30*). Our results are consistent with those seen in the current literature, where treated reporter cells subject to UV (although secondarily emitted from cells rather than directly emitted from a UV source) resulted in increased severity of effects. The observed reduction in survival in the current study confirms the presence of light incident upon reporter cells since photosensitizers require light to induce cytotoxic effects (*14*). We concluded that what we had observed was a synergistic effect: the action of UV photons and the presence of lomefloxacin in culture produced a bystander cell killing result that was more than additive. In the case where lomefloxacin was added into culture after UV exposure had already taken place, bystander cell survival was not significantly different from the untreated irradiated control. Upon UV irradiation of the cell culture only, the cell killing effect produced in the reporter population was not as strong as when both UV and lomefloxacin were applied. Thus UV and lomefloxacin acted together to produce an effect that was greater than the sum effect of the two individual treatments. In terms of signal modulation, the treatment of directly irradiated cells with lomefloxacin did not significantly change reporter cell survival. This result indicates a lack of effect in terms of signal modulation by lomefloxacin treatment.

Supplement of the photoprotector, melanin, into reporter cell populations resulted in increased cell survival compared to untreated populations. Interestingly, melanin-treated cells demonstrated increased cell survival regardless of whether melanin was added during or after exposure to cell-emitted UV. The modulation in survival of reporter cells treated with melanin after UV exposure may be explained by the presence of long-lived radical species. Koyama and colleagues demonstrated the existence of long-lived radicals that were capable of being scavenged by L-ascorbic acid but not dimethyl sulfoxide (DMSO) upon treatment 20 h after irradiation (*31*). Therefore, we suggest that it is possible that the generation of long-lived species could have resulted from cell-emitted UV exposure and that these species were scavenged by the melanin that was added post exposure. The presence of melanin in reporter cell cultures during irradiation resulted in reporter cell survival levels exceeding 100% efficiency. This finding indicates to us that there is an interaction between radiation and melanin, which is consistent with existing literature involving microorganisms (*32*) and fungi (*33*) whereby melanized organisms demonstrate enhanced growth with UV exposure. The proven modulation of cell survival in bystander cells treated with a photosensitizer and with a photoprotector supports the role of UV radiation as an influential factor mediating the bystander response observed in these experiments.

It has recently been proposed that the bystander effect may be mediated, in part, by a physical component (*20, 34*). Melanin is a pigment proficient in absorbing a broad spectrum of physical energies including UV (*20, 24*). A previous study demonstrated the ability of melanin to mitigate the effects of the bystander response (*20*). Treatment of irradiated cell cultures with melanin effectively reduced the degree of cell killing observed in both directly irradiated and bystander cell populations. Because

UV radiation is capable of inducing deleterious effects when it interacts with biological molecules (*6, 35, 36*), treatment of irradiated cell cultures with melanin could effectively reduce the degree of lethality potentiated by UV light. Furthermore, a marked demonstration of the bystander effect was present in unirradiated culture flasks that were merely placed beside flasks containing irradiated cells (*34*). This finding led Mothersill *et al*. to speculate on the contribution of a physical process independent of medium diffusible factors. The results garnered by the latter study (*34*) are similar to those in the current study in terms of experimental setup and configuration. Our results strongly support the existence of a physical component (which we attribute here to UV), to be a factor mediating the bystander effect. However, this physical component may be one of many factors working simultaneously to modulate the bystander response. Chemical signaling via diffusible factors (*37*) and gap junction communication (*38*) have also shown to be major mediators of the bystander effect. Thus, action by UV is not a sole explanatory factor, but one factor among several.

Mechanisms by Which UVC, UVB and UVA May Mediate the Observed Effects

It appears that the level of cellular photon emission measured in these experiments does not reach the levels of photons that result in erythemal doses and would not be considered strong enough to directly induce cellular damage via photon absorption. However, it is important to note that the measured photon fluence at the detector is only a narrow 10 nm wide window in an approximately 300 nm wide overall UV spectrum. If the UV emission across the spectral range is consistent, then the level of photon emission must be increased by a factor of 30. Also, this is the fluence measured at the detector, not the fluence incident on the reporter cells. UV photons generated in the DIR cells must be transported to the bystander compartment to have an effect. To be detected, they then must be transported through the bystander compartment, and air, before they are absorbed at the detector. Absorption and scattering can occur in the bystander compartment and this will significantly diminish the number of UV photons that exit the compartment. The penetration depth (i.e., the distance at which the fluence is reduced by 1/e) of this wavelength of UV in tissue is only a few hundred microns at best (*39*) and is highly dependent on cell type, color and scattering boundaries such as cell membranes. Our reporter cells are in a layer of cells and media that is 2 mm thick. From the middle of the layer to the air is 8 penetration depths, which would reduce the signal by a factor of 10,000. In addition, the fluence at the detector is subject to an approximate $1/r^2$ reduction compared to the fluence at the cells and the detector responds with different efficiency to different wavelengths of photons. Because it is difficult to accurately back-calculate the photon spectrum and fluence incident on the reporter cells from the measured detector data. We suggest that Monte Carlo methods would be needed to unfold the optical transport through cells and media and calculate the spectrum and fluence at the reporter cells with accuracy. However, we would estimate (very crudely) that the photon fluence at the cells will be many orders of magnitude higher than that measured at the detector, which means that it is nevertheless still in the milli-Joule per cm^2 range. Although the UV photon fluences delivered to bystander cells by beta-irradiated cells are estimated to be quite low, it is still very possible that subcellular effects will occur at these levels.

As stated earlier, we focused on measurements of UVA photon fluences of 340 nm wavelength outside of the cells because they provided a stronger signal, but this does not mean that other UV ranges were not incident on the cells. Spectral analysis conducted in the current study indicated that externally measurable photon emission in all of the UVA, UVB and UVC ranges were detected. UVB and UVC were measurable outside of the container, just to a lesser degree than UVA. This may not indicate less emission, but more absorption and scattering. It is therefore important to consider the action of lower wavelength UV photons as a possible explanation for the strong bystander response observed.

Short-wave UV is particularly detrimental to cells, since these wavelengths coincide with the DNA absorption spectrum (*12*) and peak absorption by thymine and cytosine is centered at approximately 260 nm (*40*). Because of the increased probability for direct interaction with DNA, UVB and UVC are more biologically active than UVA even at fluences that are orders of magnitude lower (*6*). Short-wave and middle-wave UV radiation act predominantly through direct interaction with nucleic acids to form cyclobutane pyrimidine dimers (CPD) and 6-4 photoproduct (6-4PP) (*41*). CPD and 6-4PP lesions are capable of inhibiting gene expression and halting cell division by blocking replication, respectively (*42*).

Alongside nucleic acids, aromatic amino acids such as tryptophan and tyrosine possess peak photon absorption in the low UV wavelength range at 280 nm (*40*). Absorption of UV results in the photo-oxidation of those amino acids thus producing photoproducts. UV-generated photoproducts have been shown to initiate the activation of transcription factors (*43*) involved in regulatory processes including differentiation (*44*), proliferation (*44*) and apoptosis (*45*).

Although the external flux from UVB and UVC were not measured extensively in the current study, it is possible that the action of UVB and UVC upon bystander cells has contributed to the overall observed bystander response due to the potent biological impact of short- and middle-wave UV radiation. To confirm this hypothesis in future investigations, it will be critical to focus upon analysis and assessment of the overall UV spectrum emitted from beta-irradiated cells and the fluence and spectrum incident on the reporter cells.

As to UVA-induced effects, current literature have cited that doses of 0.12 J cm^{-2} are required to induce detectable breakage of nuclear DNA (*46*) and 5 J cm^{-2} is effective in significantly decreasing cell viability by apoptosis (*47*). Although relatively high UVA doses are required to induce cell killing, sublethal doses, as low as 10% of the lethal dose, have induced transient cellular effects observed in the cell (*6, 48*). The ability of sublethal doses to induce changes in the cell such as delayed growth (*6*), inhibition of enzyme tryptophanase (*6*) and inhibition of tyrosine phosphatases (*48*) indicates that sublethal doses of UVA are also capable of modulating cellular function independent of direct DNA damage.

A possible mechanism of cell killing by cell-emitted UVA is the production of hydrogen peroxide (H_2O_2) in UVA-irradiated HaCaT cells or cell culture medium. It has been shown that H_2O_2 is a contributor to UVA-mediated DNA damage by conversion to the cytotoxic hydroxyl radical ($^{\bullet}OH$) (*8*). H_2O_2, formed by the dismutation of the superoxide anion ($^{-}O_2^{\bullet}$), is converted into $^{\bullet}OH$ by Fenton type reactions involving the reaction of H_2O_2 with iron (Fe^{2+}) (*49*). Fe^{2+} release from ferritin is initiated by UVA sensitization (*50*) and therefore the incidence of Fenton reactions is particularly favorable during UVA irradiation.

Another possible explanation for the significant cell-killing effects demonstrated in our results involves dose rate. Shorrocks *et al.* (*51*) showed that delivery of a given UVA dose using lower dose rates is more effective in achieving a greater degree of HaCaT cell killing than delivery of the same dose at comparatively higher dose rates. Although the detected UVA photon fluence of approximately 1,200 photons per second (and thus the many orders of magnitude higher fluence of photons reaching the reporter cells) would not be expected to induce any visible reductions in cell survival, the effects observed may be caused by the incidence of UV photons on reporter cells at a consistent low-dose rate over 24 h.

Taken together, photons spanning the entire UV spectrum are capable of inducing effects in exposed cells at low fluence levels. From the current results, we cannot yet conclude which portion(s) of the UV spectrum are responsible for the reported effects. However, the results presented here strongly support a role for secondarily emitted ultraviolet photons, in general, in the mediation of the bystander effect.

Limitations

We acknowledge that there are limitations to the current work that will need further investigation. Thus far, photon quantification covering the full ultraviolet spectrum has not been performed. While a preliminary spectral analysis with narrow band pass filters within each of the UVA, UVB and UVC wavelength ranges was performed upon beta irradiation of 2×10^4 cells/cm^2 at 857.5 μCi (0.5 Gy), photon emission at lower wavelengths (UVB, UVC) has not yet been investigated for the range of cell densities and radiation doses that were assessed for the UVA range. Further, although these are the photon levels measured outside of the cells and media, the necessary experiments and simulations have not yet been performed to determine the spectrum and fluence of UV photons incident on the reporter cells. We do not suggest that the specific bandwidth of UV radiation measured here (340 ± 5 nm) was solely responsible for the effects observed in bystander cells. However, the results presented here suggest a role for secondary UV photons in general, in mediating a bystander response. Further investigation of the full UV spectrum and a careful calculation of the photon spectrum and fluence incident on the reporter cells will be important. Experiments that permit only a narrow window of UV to be incident on the reporter cells will allow us to assess which wavelengths may be responsible.

Investigation of the origin of emitted light from cells would be another crucial experiment for future study. Although we have not yet been able to delineate the exact cell component from which UV photons originate, and there will probably be multiple sources found inside the cells, photon quantification experiments should be extended. Adding isolated cell extracts comprising different cellular components to media may help elucidate the exact cell constituents with which ionizing radiation interacts to produce a UV light signal. These experiments are considered important since delineation of both signal and response are equally important in understanding the bystander effect.

CONCLUSIONS

The results obtained in the current study are supportive of a role for UV radiation in the mediation of a response in neighboring cells that have not been directly irradiated with beta particles. This study showed a strong correlation between the quantity of measured UV photons emitted from ^{3}H-irradiated HaCaT cells and the degree of cell death seen in reporter cells that were subjected to the emitted UV. Further confirmation of the modulating effect of UV on cell survival in neighboring cells was exhibited by a lessening of cell death upon treatment with a PET UV filter in the path between UV-emitting cells and nonbeta-irradiated reporter cells. Furthermore, treatment with modulators such as the photosensitizer lomefloxacin and the photoprotector melanin produced supportive results. Taken together, these findings indicate the importance of further investigation into the consequences of cell-emitted UV. These results, at the very least, suggest one physical mechanism of bystander response mediation, which to our knowledge has not yet been discussed.

SUPPLEMENTARY INFORMATION

Table S1. Tritium (^{3}H) activities, exposure duration and corresponding dosimetry.

Fig. S1. Survival is demonstrated for bystander cells in the field of directly irradiated (DIR) living HaCaT cells treated with lomefloxacin.

Fig. S2. Survival is demonstrated for reporter cells in the field of directly irradiated (DIR) living HaCaT cells treated with melanin.

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Chapter 5

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

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The investigation of p53 in relation to the biophoton-mediated bystander effect was proposed by the first author and supported by the rest of the research team. The experimental methodology was developed by the first author, Dr. Carmel Mothersill, Dr. Fiona McNeill, Dr. Colin Seymour and Dr. Andrew Rainbow. All experimentation, statistical analyses, and manuscript writing were performed by the first author. All authors also contributed to the revision of the manuscript prior to submission and following receipt of reviewer comments from the journal.

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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,

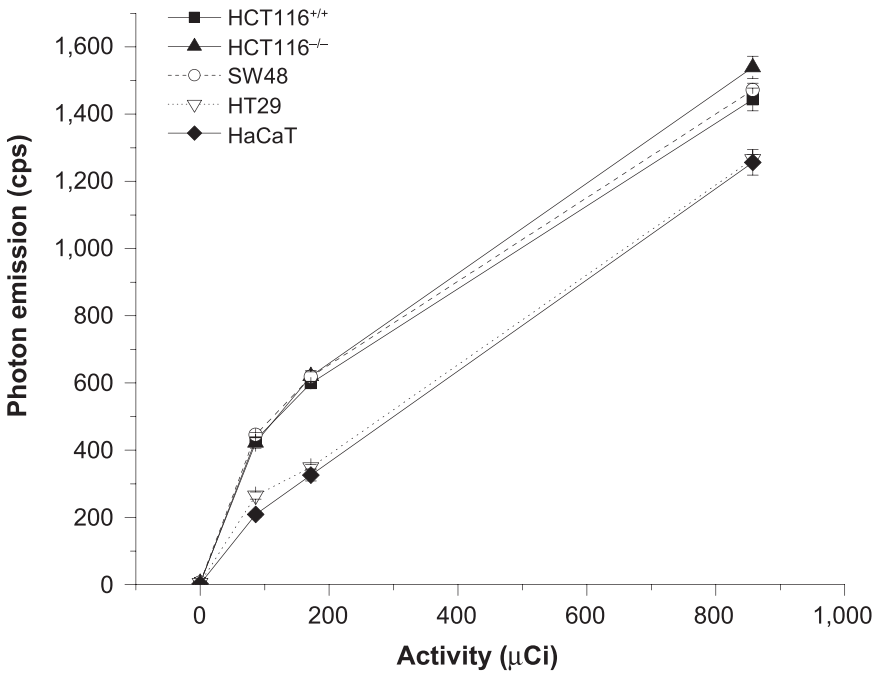

FIG. 1. Photon quantification at 340 ± 5 nm from HCT116$^{+/+}$, HCT116$^{-/-}$, SW48, HT29 and HaCaT cells that have been exposed to 85.7, 171.5 and 857.5 μCi ^{3}H beta particles. Errors represent the standard error of the mean (SEM), where n = 9 for three independent experiments.

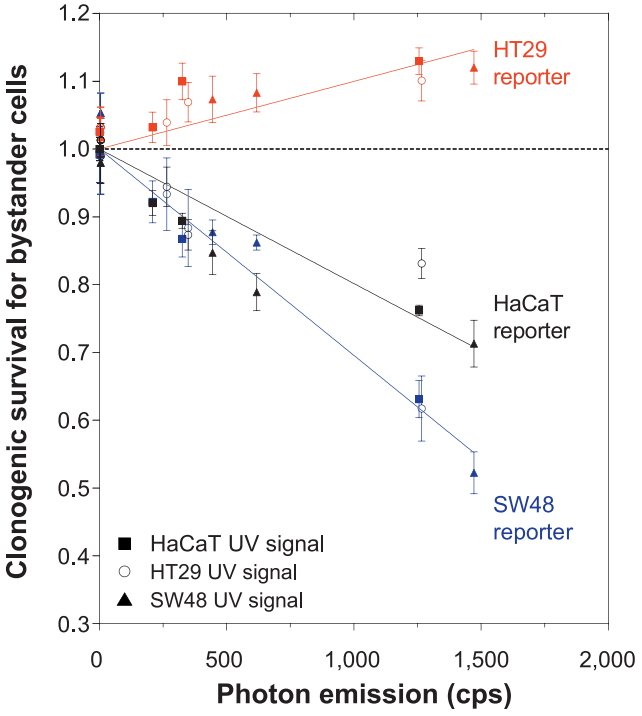

FIG. 2. HaCaT (black), HT29 (red) and SW48 (blue) reporter cell survival in response to signals from tritium-irradiated HaCaT (square), HT29 (open circle) and SW48 (triangle) cells. Error bars represent SEM where n = 3 for three independent experiments (total n = 9). Two-way ANOVA, post hoc: Tukey's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

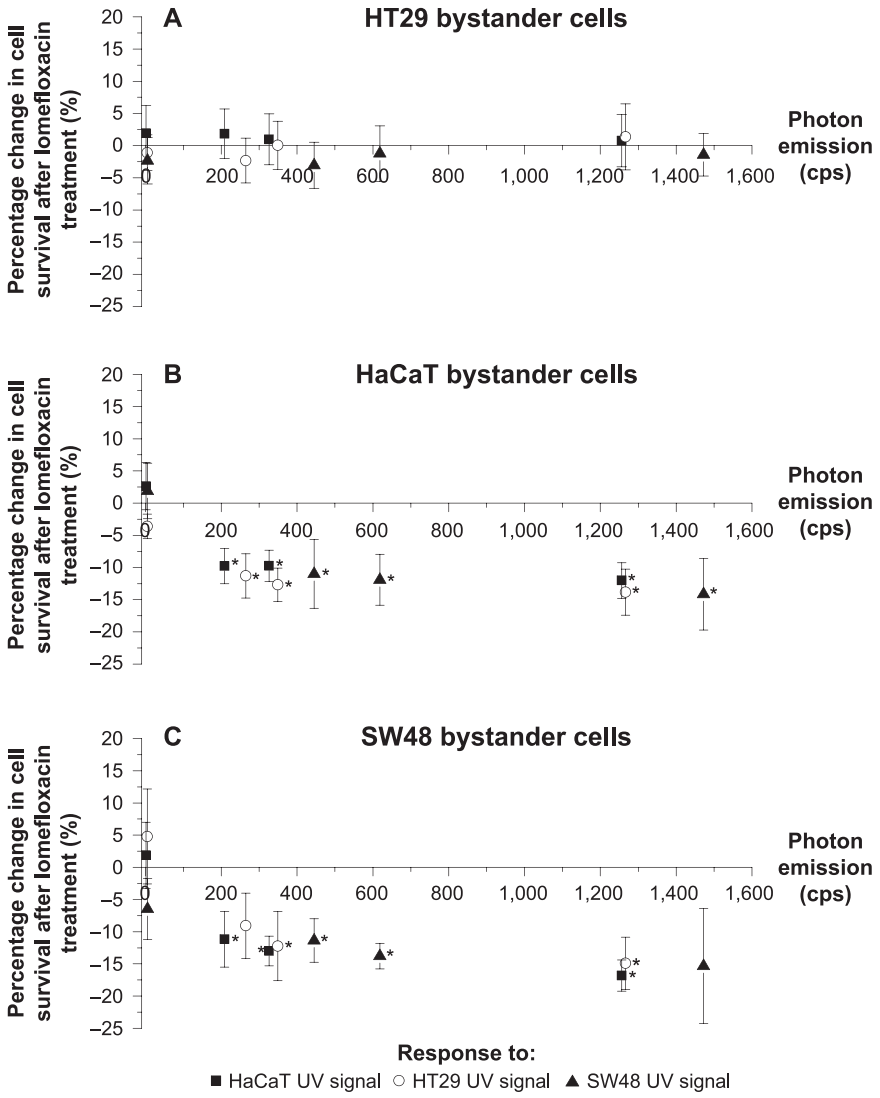

FIG. 3. Percentage change in HT29 (panel A), HaCaT (panel B) and SW48 (panel C) reporter cell survival when reporter cells were treated with lomefloxacin and concurrently exposed to cell-emitted UV signals, compared to bystander cell survival after UV exposure alone. Error bars represent SEM for n = 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

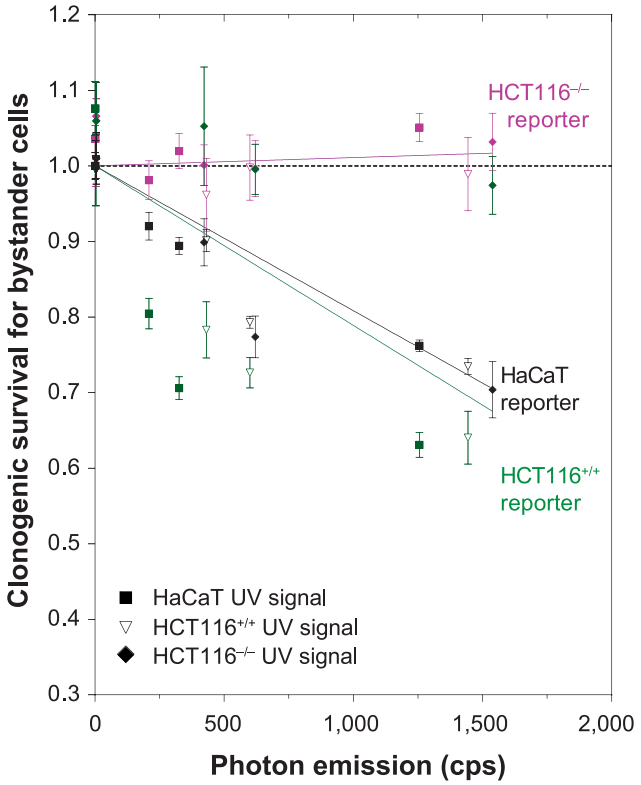

FIG. 4. HaCaT (black), HCT116$^{+/+}$ (green) and HCT116$^{-/-}$ (purple) reporter cell survival in response to signals from tritium-irradiated HaCaT (square), HCT116$^{+/+}$ (open inverted triangle) and HCT116$^{-/-}$ (diamond) cells. Error bars represent SEM for n = 3 for three independent experiments (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$^{+/+}$

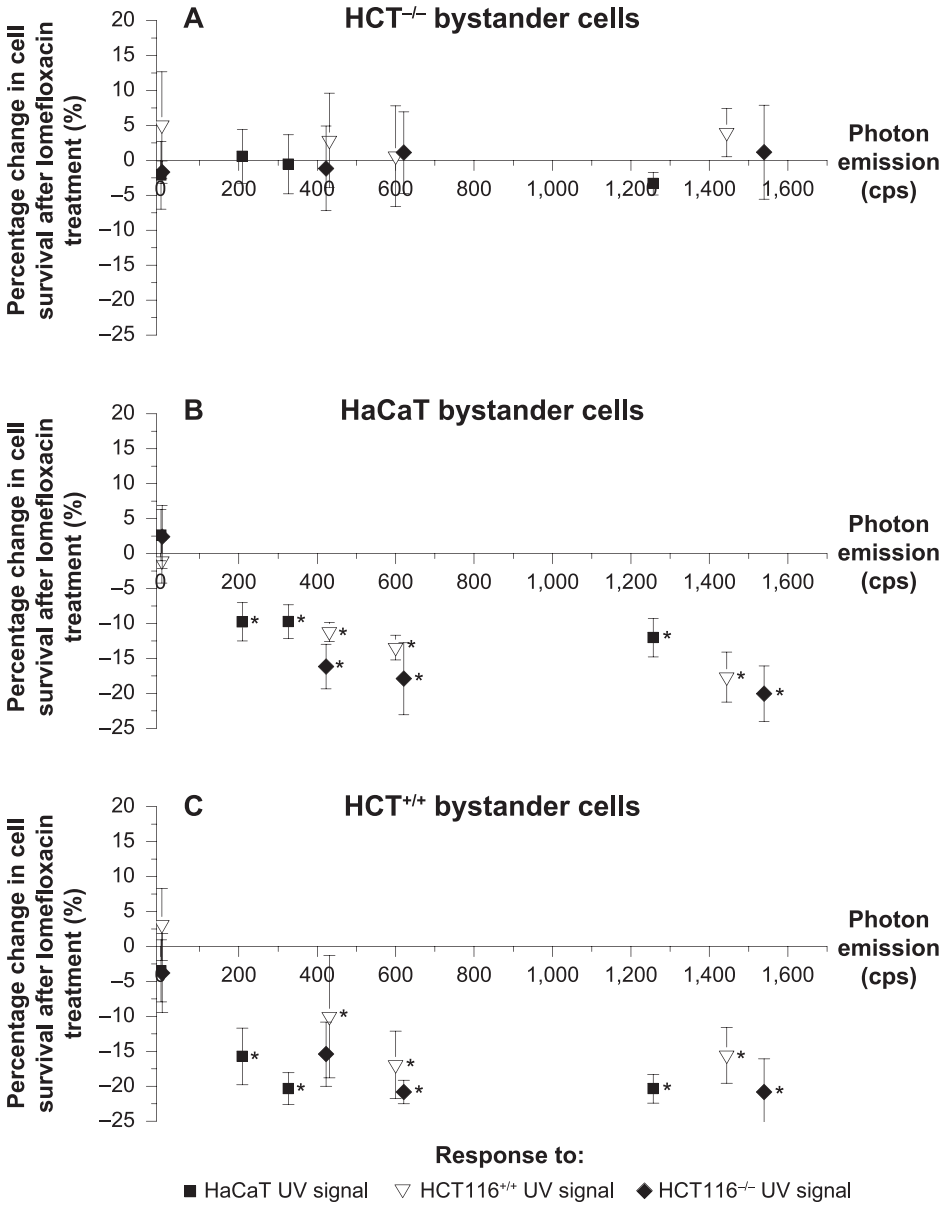

FIG. 5. Percentage change in HCT116$^{-/-}$ (panel A), HaCaT (panel B) and HCT116$^{+/+}$ (panel C) reporter cell survival when reporter cells were treated with lomefloxacin and concurrently exposed to cell-emitted UV signals, compared to bystander cell survival after UV exposure alone. Error bars represent 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

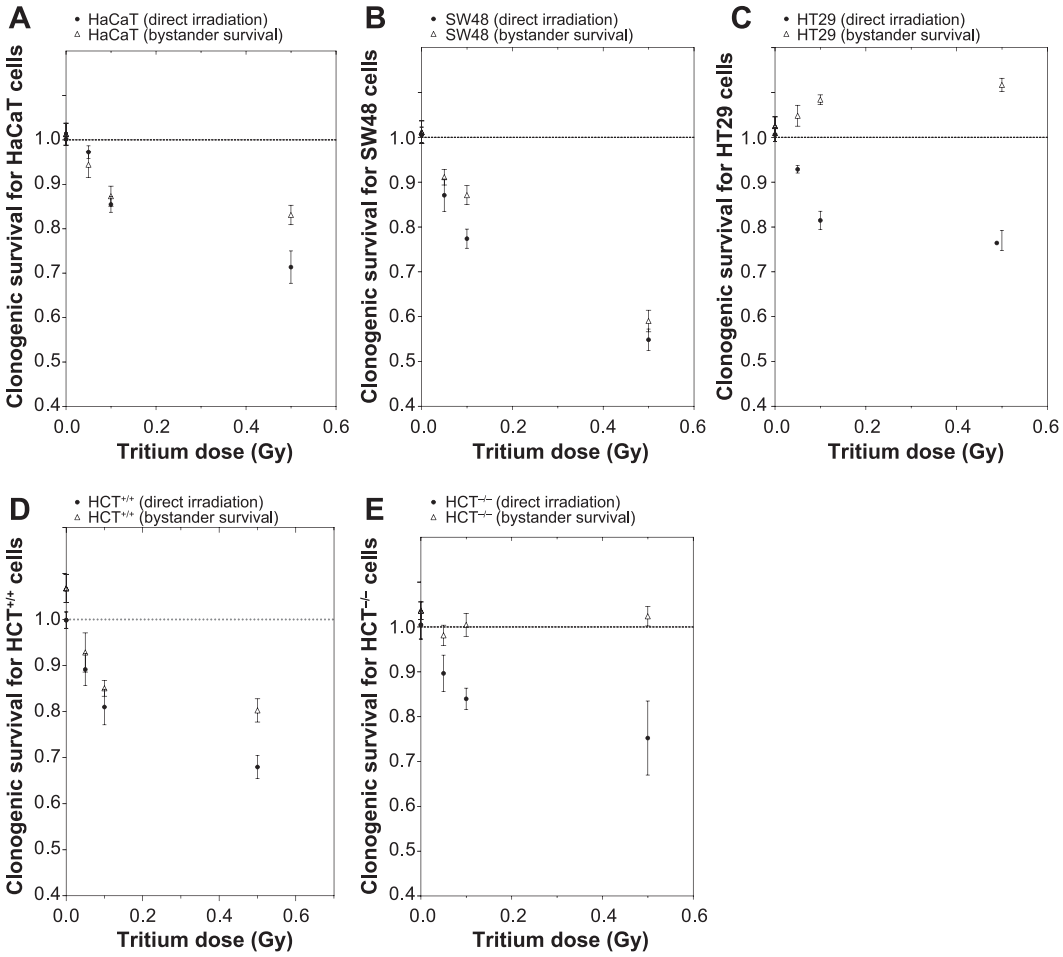

FIG. 6. Clonogenic surviving fraction for HaCaT (panel A), SW48 (panel B), HT29 (panel C), $HCT116^{+/+}$ (panel D) and $HCT116^{-/-}$ (panel E) cells in response to direct ^{3}H beta irradiation (circular markers). Bystander cell survival subsequent to exposure to UV photons emitted from beta-irradiated 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

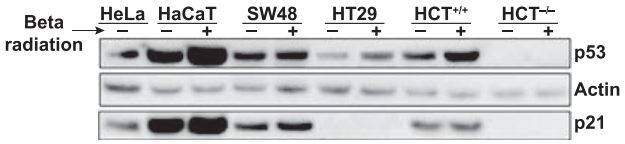

FIG. 7. Expression for p53 (53 kDa), actin (42 kDa) and p21 (21 kDa) proteins by HeLa (positive control), HaCaT, SW48, HT29, HCT116$^{+/+}$ and HCT116$^{-/-}$ cells exposed to either no radiation or 0.5 Gy ^{3}H beta radiation.

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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Chapter 6

Exosomes are Released by Bystander Cells Exposed to Radiation-Induced Biophoton Signals: Reconciling the Mechanisms Mediating the Bystander Effect

Michelle Le, Cristian Fernandez-Palomo, Fiona E. McNeill, Colin B. Seymour, Andrew J. Rainbow, Carmel E. Mothersill

The exploration of a possible relationship between exosomes and biophotons in the mediation of the bystander effect was first proposed by Dr. Carmel Mothersill. The experimental methodology was developed by the first author, Dr. Cristian Fernandez-Palomo, Dr. Carmel Mothersill, Dr. Colin Seymour, Dr. Fiona McNeill and Dr. Andrew Rainbow. For the assessment of mitochondrial membrane potential, fluorescence microscopy and the first trial of microplate experiments were conducted by Dr. Cristian Fernandez-Palomo. The remaining mitochondrial membrane potential experiments employing the microplate method were conducted by the first author. The first author also carried out the clonogenic survival experiments, western blot assays, and acquisition of transmission electron micrographs. Statistical analysis and

graphing of data were conducted by the first author with help from Dr. Cristian Fernandez-Palomo. The first draft of the manuscript was written by the first author with feedback and comments from Dr. Carmel Mothersill, Dr. Colin Seymour, and Dr. Fiona McNeill. All authors contributed to the revision of the manuscript following receipt of reviewer comments from the journal.

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RESEARCH ARTICLE

Exosomes are released by bystander cells exposed to radiation-induced biophoton signals: Reconciling the mechanisms mediating the bystander effect

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Abstract

Objective

The objective of our study was to explore a possible molecular mechanism by which ultraviolet (UV) biophotons could elicit bystander responses in reporter cells and resolve the problem of seemingly mutually exclusive mechanisms of a physical UV signal & a soluble factor-mediated bystander signal.

Methods

The human colon carcinoma cell line, HCT116 p53 +/+, was directly irradiated with 0.5 Gy tritium beta particles to induce ultraviolet biophoton emission. Bystander cells were not directly irradiated but were exposed to the emitted UV biophotons. Medium was subsequently harvested from UV-exposed bystander cells. The exosomes extracted from this medium were incubated with reporter cell populations. These reporter cells were then assayed for clonogenic survival and mitochondrial membrane potential with and without prior treatment of the exosomes with RNase.

Results

Clonogenic cell survival was significantly reduced in reporter cells incubated with exosomes extracted from cells exposed to secondarily-emitted UV. These exosomes also induced significant mitochondrial membrane depolarization in receiving reporter cells. Conversely, exosomes extracted from non-UV-exposed cells did not produce bystander effects in reporter cells. The treatment of exosomes with RNase prior to their incubation with reporter cells effectively abolished bystander effects in reporter cells and this suggests a role for RNA in mediating the bystander response elicited by UV biophotons and their produced exosomes.

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Conclusion

This study supports a role for exosomes released from UV biophoton-exposed bystander cells in eliciting bystander responses and also indicates a reconciliation between the UV-mediated bystander effect and the bystander effect which has been suggested in the literature to be mediated by soluble factors.

Introduction

Cells subjected to both non-ionizing and ionizing radiation have the capacity to generate communication signals and subsequently cause biological changes in distant non-irradiated cells [1–5]. This observed phenomenon whereby intercellular communication and biological change is initiated as a result of irradiation is referred to as the *radiation-induced bystander effect* (RIBE). The RIBE has been shown to elicit a spectrum of effects in bystander cells that reflect biological responses which are closely representative of those characterized by directly-irradiated cells. Sister chromatid exchanges, micronuclei formation, apoptosis, genomic instability, and mitochondrial dysfunction have all been demonstrated in bystander cells subsequent to the receipt of signals by directly-irradiated cell populations [6–8].

The communication of bystander signals between directly-irradiated and bystander cells can be accomplished via various mechanisms including the facilitation of molecular exchange between adjacent cells via gap junctions [3], the communication between distant cells via the transfer of soluble factors [2], the exchange of volatile components between physically separated cell populations [9, 10], and the transmission of electromagnetic signals from irradiated cells to distant recipient cells [11–13]. In the study of bystander effects signalled via the exchange of soluble factors, a role has been identified for a variety of signalling molecules such as reactive oxygen species [14], cytokines [15, 16], and exosomes [17] in the generation of bystander responses. The propagation of this bystander mechanism requires either direct physical contact between cells, the exchange of biological fluids, such as blood serum or cell culture media, between the directly-irradiated cells and the non-irradiated bystander cells, or an open system so as to facilitate the exchange of volatile components between two separate organisms or cell populations. In an alternative bystander mechanism, the role of electromagnetic radiation in the ultraviolet (UV) wavelength range has been identified [11–13]. This novel bystander mechanism has been referred to as the *UV-mediated bystander effect* whereby the communication of signals via light fields does not require physical contact between directly-irradiated and bystander cell populations [13].

Cellular communication mediated by electromagnetic radiation occurs as a result of *biophoton* emission by one population of cells and the receipt of those signals by another cell population. Biophotons are characterized by UV and visible wavelength range photons which are emitted from biological materials via processes alternative to conventional chemiluminescence [18]. While the mechanisms for biophoton emission are still unclear, the excitation of various intracellular molecules is a strong candidate mechanism [19, 20]. The initiation of biophoton emission by biological systems has been observed subsequent to stress induction by ionizing radiation [21–24], viral infection [11], and mechanical disruption [25]. While the observed rates of biophoton emission are typically quite low (0.01 photons per second per cell; 100 photons measured per 10^4 plated cells [26], 10^4 photons detected per 10^6 plated cells [13, 23]), and thus the dose delivered to cells may not be considered significant enough to induce visible effects, there is evidence to suggest that biophotons act as coherent information-encoding

signals, similar to binary-encoded data, to exchange information between biological systems [18, 19].

The bystander system which the current study has employed to investigate the UV-mediated bystander effect is characterized by the incubation of two separate cell populations in UV-transmitting vessels in order to achieve successful biophoton signal transduction [13]. Briefly, cells in one culture were directly irradiated with beta-emitter, tritium, to induce UV biophoton emission. The biophotons emitted from the tritium-irradiated cells were measured using a photomultiplier tube fitted with interference-type band pass filters and were found to exhibit emission in each of the UV-A (340 ± 5), UV-B (300 ± 5) and UV-C (280 ± 5) wavelength ranges. UV-A photon rates reached 1200 counts per second per 10^5 cells whereas the UV-B and UV-C wavelengths exhibited weaker photon emission rates following the same given activity of beta radiation [13]. A bystander cell culture was incubated 1.5 cm superior to the directly-irradiated cell monolayer for 24 hours to accommodate biophoton signal receipt. Upon analyzing the clonongenic survival data of the bystander cells which received the UV biophoton signals, it was found, using a Pearson's correlation test, that 95% of the cell killing observed in the bystander cell population shared a relationship with the measured UV-A biophoton flux. The role of the detected UV biophotons in eliciting the observed bystander responses was further confirmed when the placement of a polyethylene terephthalate UV-absorbing filter between the directly-irradiated and the bystander cell populations effectively abolished cell killing in the bystander population [13]. The UV-mediated bystander effect has since been investigated in the human keratinocyte cell line, HaCaT [13], and human colon carcinoma cell lines, SW48, HT29, HCT116 p53 +/+, and HCT116 p53 -/- [27]. The work of Kaznacheev and colleagues also supports the idea of intercellular communication by electromagnetic means as they demonstrated the ability of virally-infected cell populations to elicit stress responses in non-infected populations only when the two populations were separated by UV-transmitting materials [11]. Although they did not describe this observation using the term "bystander", the communication between stress-induced cells and nearby reporter cells certainly fits within what we now call the bystander effect. Despite the demonstrated involvement of electromagnetic radiation in the generation of bystander effects in response to various stressors [11–13], the molecular aspects by which the UV-bystander signal exerts its effects upon bystander cells remains unclear and requires further investigation.

This study thus sets out to investigate a molecular mechanism by which UV bystander signals may potentially elicit biological effects in bystander cells. Recent evidence has brought to light the ability of ultraviolet radiation to modulate the function of exosomes emitted from human keratinocyte cells [28]. Cicero *et al*. showed that the exosomes extracted from UV-B-irradiated keratinocyte cells were able to induce greater melanin production by melanocyte cells. While the study by Cicero investigated UV-B radiation, the interactions expected from UV-A photons, as investigated in the current study, are similar to those observed subsequent to UV-B exposure due to their similarity in wavelength and photon energy. Knowledge of UV's modulatory effect upon exosome function is promising as it may provide a point of reconciliation between the UV-mediated bystander effect and the previously discussed soluble factor-mediated bystander effect. To elaborate, exosomes are extracellular vesicles derived from pinched off sections of the endosomal membrane [29]. These 50–150 nm membrane-bound vesicles [30] encapsulate cytoplasmic contents such as RNA and protein during formation and are subsequently released into the extracellular space. The contents of exosomes can exert their effects upon bystander cells as a result of exosome migration through the extracellular space to distant cells and subsequent internalization of those exosomes by endocytosis. Their ability to efficiently transport essential biological molecules from cell to cell through the intercellular environment emphasizes their significant contribution to soluble-factor-mediated

intercellular signalling. Published literature has demonstrated exosomes' ability to induce carcinogenic behaviour (tumour cell promotion and migration in cells receiving exosomes from gastric tumour cells [31]) and to induce DNA damage in bystander cells receiving exosome-encapsulated RNA from x-irradiated breast cancer cells [17].

The evident role of exosomes in intercellular signalling therefore justifies the consideration of their role in inducing the bystander response. While studies demonstrating the involvement of exosomes in the RIBE already do exist in the published literature [17, 29, 32–34], the investigation of exosomes as they pertain to secondary UV biophotons, is a previously unexplored and novel concept. The rationale for investigating biophotons in relation to exosomes is based upon the hypothesis that UV biophotons may act to elicit the release of a variety of soluble factors that are commonly involved in the RIBE. While there are many soluble factor candidates that could have been selected for investigation, particular focus upon exosomes was chosen because protocols for clean exosome isolation from culture have been well established in the literature. Furthermore, the vesicular nature of exosomes facilitates opportunities for further comprehensive investigation extending beyond the investigations undertaken in the current study. This research aims to assess the potential relationship between UV biophotons and the release of soluble factors in the study of bystander signalling.

The current study investigates the relationship between cellular UV biophoton exposure and the release of exosomes in response to that exposure. The system for investigating bystander effects used in our previous research [13] suggests that soluble factors, including exosomes, cannot be the only signal from directly-irradiated cells driving the bystander effect. Our system did not facilitate any medium transfer or cell-cell contact between the directly-irradiated and bystander populations, yet significant bystander effects could still be observed. With this in mind, we can concede two solutions; either there are two mutually exclusive mechanisms by which the bystander effect can be induced, or the UV signal is able to trigger the release of soluble factors from bystander cells. We hypothesized that exposure of cells to UV biophotons will trigger the release of soluble factors that are subsequently capable of eliciting bystander responses. That is, we believe that the UV biophotons emitted from cells as a result of direct beta-particle irradiation, is an intermediate signal that is responsible for triggering the release of bystander-eliciting soluble factors. The current study has confirmed this relationship via the assessment of clonogenic survival and mitochondrial membrane potential in bystander cells receiving exosomes extracted from UV-exposed cells.

Materials and methods

Cell culture

HCT116 p53 +/+ human colon carcinoma cells, received as a gift from Dr Robert Bristow (University of Toronto) and Dr Bert Vogelstein [35], were cultured in RPMI1640 supplemented to a final concentration of 10.5% fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulphate. Cells were routinely cultured in 75 cm^2 flasks (BD Falcon), given medium exchanges every 2 to 3 days, and passaged with 3 mL of 0.25% trypsin-EDTA solution when cells reached 80–90% confluence. Neutralization of the trypsinization process was accomplished by adding 7 mL of complete growth medium to the trypsinized cell suspension. Cell cultures were routinely incubated at 37˚C, 95% humidity and 5% CO_2. All reagents used were from Gibco unless otherwise stated.

Cells were given full volume medium renewals 24 hours prior to an experiment using RPMI1640 supplemented with exosome-depleted FBS (Gibco cat no. A2720801) in place of the FBS used for routine subcultivation. For all cell culture activities carried out through the experimental process, the exosome-depleted growth medium was used. For cells intended to

receive direct irradiation and for cells destined to receive ultraviolet (UV) photon signals from beta(β)-irradiated cells, 25 cm^2 flasks containing a total volume of 5 mL complete growth medium were seeded with 2×10^5 cells each. For reporter cells destined to receive either cell-conditioned medium harvested from UV biophoton-exposed cells (UV-ICCM), control cell-conditioned medium (CCCM) or exosomes isolated from either UV-ICCM or CCCM, cells were seeded into 25 cm^2 flasks at clonogenic densities (500 cells per flask, 5 mL total volume). Use of the term *UV-ICCM* throughout the text refers to cell culture medium that has been conditioned by cells that have been exposed to the *UV biophotons* emitted by β-irradiated cells; it does not refer to the cells which have been directly exposed to β-particles.

Direct beta-irradiation and bystander protocol

Beta (β)-irradiation of cell cultures containing 2×10^5 cells was accomplished by adding tritiated water directly into the cell culture medium. 857.5 μCi of pure β-emitter, tritium (^3H), was added into cell culture medium and retained in the medium for 24 hours to achieve a total dose of 0.5 Gy. The (^3H) dose was determined using Eq 1 where D represents the dose in Joules/kilogram (Gy), $N_0\lambda_R$ is the ^3H activity in disintegrations per second (Becquerel), $\bar{E}_\beta$ is the average tritium beta particle energy, t is the duration of the irradiation in seconds, and m represents the mass of the irradiated object. During the 24 hour irradiation period (at 37˚C, 95% humidity, 5% CO_2), 25 cm^2 bystander flasks each containing 2×10^5 cells were placed directly superior to the petri dishes containing the directly-irradiated cultures such that the bystander cells were in the field of the ultraviolet (UV) photon emissions generated by the directly-irradiated cells but were not directly irradiated by the beta particles from the tritium. The monolayer of directly-irradiated cells was separated from the bystander cell monolayer by a distance of approximately 1.5 cm. The two cultures were incubated together in a partitioned light-tight box to eliminate potential effects from ambient light during the opening of the incubator door and from cross-interference of UV biophoton signals from other directly-irradiated cultures within the light tight box. Controls for the β-irradiation trials included bystander flasks placed superior to non-β-irradiated (sham) cells and irradiated cell culture medium (without cells).

Immediately following 24 hour irradiation, UV-ICCM and CCCM from bystander flasks were harvested, filtered (0.2 μm pore filter, Pall Corporation), and either transferred to flasks containing clonogenic reporter cells or transferred to polycarbonate ultracentrifuge tubes for exosome extraction.

$$D = \frac{N_0 \lambda_R \bar{E}_\beta t}{m} \quad (1)$$

Exosome isolation

Exosomes were isolated via ultracentrifugation of UV-ICCM or CCCM at 100,000 xg for 90 minutes using a Thermo Scientific WX90 Sorvall Ultracentrifuge with a F50L-8x39mL fixed angle rotor. For the exosome experiments, an additional control was added whereby exosomes were extracted from complete growth medium which was not irradiated nor conditioned by cells to ensure that any observed effects were not attributed to the culture medium itself. The samples were kept at 4˚C for the duration of the ultracentrifugation process. Following ultracentrifugation, the supernatant was aspirated and the exosome pellet was resuspended in 250 μL Dulbecco's Phosphate Buffered Saline (DPBS). Exosome isolates were transported from the lab housing the ultracentrifuge to our cell culture lab on ice and immediately added into the cell culture medium of the reporter cells. These reporter cells were cultured in 5 mL of growth

medium supplemented with exosome-depleted fetal bovine serum. The elapsed time from exosome resuspension to addition of exosomes to reporter cells was approximately 20 minutes. The reporter cells were plated at clonogenic densities to assess for survival or plated onto 96-well plates to assess for mitochondrial membrane potential using the JC-1 assay. Remaining exosome fractions were stored at -20˚C for future validation of exosome-enriched proteins using the western blot assay.

Clonogenic survival in reporter cells

The clonogenic survival assay was used to assess the survival of bystander cells which received UV-ICCM, CCCM, or exosomes extracted from UV-ICCM and CCCM. For reporter cells which directly received UV-ICCM or CCCM, the medium that was originally used to culture the reporter cells was discarded and replaced by the full volume of UV-ICCM or CCCM that was harvested. For reporter cells receiving exosome fractions, the cell culture medium originally used to culture the cells was retained and 250 μL of exosome fraction (exosomes extracted from the UV-ICCM of 2×10^5 cells) was added to the existing medium. An additional experiment was also conducted whereby UV-ICCM and CCCM was ultracentrifuged and the supernatant (free of exosomes) was harvested and subsequently placed onto reporter cells.

In the permutation whereby the role of RNA-carrying exosomes was being assessed, RNase was added to and incubated at 37˚C for 30 minutes with the volume of UV-ICCM, CCCM or exosome fraction prior to their transfer into the reporter cell culture.

UV-ICCM, CCCM, or exosome fractions were incubated with the reporter cells for approximately 9 days at 37˚C, 95% humidity, and 5% CO_2 to facilitate the growth of single cells into colonies. Reporter flasks were then stained and the quantity of cells which developed into colonies (>50 cells) were scored. The number of colonies formed in the treatment and control flasks were normalized to six plating efficiency flasks for each trial. The plating efficiency flasks were seeded with 500 cells per flask where three were plated at the beginning of the seeding process and three were plated at the end. The average plating efficiency among all three trials (18 plating efficiency flasks) was 33.5% ± 2.5% (standard error of the mean).

Mitochondrial membrane potential in reporter cells

Mitochondrial membrane potential was assessed in this study to determine the role of exosomes generated as a result of cellular exposure to UV bystander signals in the initiation of apoptosis in reporter cells exposed to those exosomes. For each experimental sample, a 1 mL suspension of 2×10^6 HCT116 p53 +/+ cells was first incubated with 250 μL exosome fraction, 1 μL carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (membrane depolarization positive control), or 1 μL dimethyl sulfoxide (DMSO) (membrane depolarization negative control) for 1 hour at 37˚C. Following incubation with treatment samples and subsequent elimination of treatment samples from the cell suspension by 5-minute centrifugation at 1000 rpm and resuspension in complete growth medium, 3.83 μM MitoPT JC-1 reagent from the MitoPT JC-1 mitochondrial permeability assay kit (ImmunoChemistry Technologies, cat no. 924) was incubated with the cell suspension for 15 minutes at 37˚C. Following incubation, the cells were washed with DPBS and subsequently pelleted at 1000 rpm for 5 minutes to remove the supernatant containing residual JC-1 stain. The cell pellet was resuspended in 1 mL DPBS and 100 μL of the suspension was subsequently pipetted into the well of a black glass-bottom 96-well plate (BD Falcon) to achieve a total of 2×10^5 cells in each well. Each treatment sample was pipetted into 6 wells such that there were 6 replicates of each sample on a given 96-well plate.

Fluorescence spectroscopy was accomplished using a Tecan Infinite M200 Pro plate reader and i-control software where excitation was set to 488 nm and emission (measurement)

wavelengths were set to 590 nm (red) and 527 nm (green). Mitochondrial membrane potential was assessed by taking the ratio of red to green fluorescence indicative of the relative ratio of aggregates to monomers in the cell culture. The concentration of JC-1 aggregates and monomers indicate JC-1 accumulation within the mitochondria of healthy non-apopototic cells and distribution of JC-1 dye in the cytosol in mitochondrial membrane potential-compromised cells, respectively.

Fluorescence microscopy was also conducted in order to visualize the relative quantity of aggregate (red) fluorescence and monomer (green) fluorescence exhibited by the treated samples and the control samples.

Ribonuclease A treatment

The experiments previously described whereby clonogenic survival and mitochondrial membrane potential were assessed following treatment with exosomes or UV-ICCM extracted from cells exposed to the UV bystander signal, were conducted in another permutation whereby exosomes, UV-ICCM, or UV-ICCM depleted of exosomes were treated with Ribonuclease A (RNase A) subsequent to UV-exposure and prior to the addition of the exosome fraction or UV-ICCM to clonogenic and/or mitochondrial membrane potential reporter cells.

Lyophilized RNase A was purchased from Sigma-Aldrich (Sigma-Aldrich, R6513) and reconstituted in sterile distilled water to a stock concentration of 10 mg/mL upon receipt. Working concentrations of 10 μg/mL were diluted from the stocks and frozen at -20˚C for future use. For RNase destined for incubation with pure UV-ICCM or with exosome fractions, the working concentration of RNase was added to a given volume of UV-ICCM or exosome fraction to produce a final concentration of 2 μg/mL.

For ICCM, RNase was added to the UV-ICCM following 24 hour UV irradiation and after the UV-ICCM had been filtered through a 0.2 μm pore filter. The UV-ICCM was incubated with RNase for 1 hour at 37˚C prior to the addition of the ICCM-RNase solution into clonogenic reporter flasks. For exosome fractions, RNase was added to the exosome fraction following ultracentrifugation and resuspension in DPBS. The RNase was incubated with the exosome fraction for 1 hour at 37˚C prior to the addition of the exosome-RNase solution into either flasks containing clonogenic reporter cells & 5 mL culture medium or into cell suspensions destined for mitochondrial membrane potential assessment.

Western blot to validate exosome isolation

Western blots were conducted using protein extracted from both exosome fractions and from HCT116 p53+/+ whole cell lysate which had been exposed to UV photons emitted from non-irradiated or 0.5 Gy β-irradiated HCT116 p53 +/+ cells.

Proteins of interest included actin (42 kDa) and exosome-associated proteins, CD63 (non-glycosylated: 25 kDa, glycosylated: 30–70 kDa) and TSG101 (49 kDa). 10 μg of protein was loaded into each well of a 10-well 12% bis-tris gel (Life Technologies) where the total volume in each well was 25 μL. Proteins were transferred onto a 0.2 μm nitrocellulose membrane (GE Health Sciences) and the membrane was blocked with 5% skim milk-TBST at room temperature for 60 minutes. The membrane was incubated with the primary antibody overnight at 4˚C (anti-Actin rabbit polyclonal: Sigma-Aldrich A5060, 1:1000 in 5% milk-TBST; anti-CD63 rabbit polyclonal: Abcam ab68418, 1:1000 in 5% milk-TBST; anti-TSG101 mouse monoclonal: Abcam ab83, 1:1000 in 5% milk-TBST) followed by the secondary antibody for 60 minutes at room temperature (anti-rabbit, GE Amersham 45000679; anti-mouse, GE Amersham 45000682, 1:5000 in 5% milk-TBST). Following antibody incubation, blots were treated with enhanced chemiluminescence substrate (Thermo Scientific) prior to image acquisition

(BioRad ChemiDoc MP, Image Lab 4.1 software). Protein band densities were quantified using image processing software, ImageJ. Protein from HepG2 and HeLa whole cell lysates were used as positive controls for CD63 and TSG101 protein expression, respectively. These whole cell lysates were chosen as positive controls since CD63 and TSG101 expression by HepG2 and HeLa cells had been validated by the manufacturer and thus their use as positive controls were recommended in the product data sheets supplied [36, 37].

Transmission electron microscopy to validate exosome isolation

For visualization of samples using Transmission Electron Microscopy (TEM), exosomes were isolated in the same manner as described previously and subsequently resuspended in distilled H_2O. Exosome suspensions were prepared on formvar-coated copper-palladium grids and negatively stained with uranyl acetate. Image acquisition was conducted using a JEOL 1200EX TEMSCAN electron microscope at the Health Sciences Centre Electron Microscopy Facility (McMaster University).

Statistical analysis

Statistical differences among the clonogenic survival of cells subsequent to different treatments were assessed using a 1-way analysis of variance (ANOVA) test. Post-hoc analysis was conducted using Tukey's honestly significant difference (HSD) test. A 1-way ANOVA was also employed to assess the statistical differences among the degree of mitochondrial membrane depolarization induced by various treatments. Tukey's HSD test was employed for post-hoc analysis. Statistical analyses were conducted using GraphPad Prism 6 and SPSS Statistics 17.0.

Results

ICCM and exosomes from UV-exposed bystander cells

Reporter cells were subjected to cell conditioned medium or exosomes harvested from cells that were exposed to secondary UV biophotons to determine whether the UV signal emitted from β-irradiated cells could prompt a release of exosomes capable of eliciting a bystander response. It is emphasized that the term *UV-ICCM* throughout the text refers to culture medium conditioned by bystander cells which have been exposed to the *UV biophotons* emitted by β-irradiated cells.

Clonogenic survival following UV-ICCM transfer. Upon transfer of ICCM from UV-exposed bystander cells to clonogenic-density reporter cells, a reduction in survival to 85.7% ± 3.0% was observed (Fig 1A). This reduction was significant when compared to the survival elicited subsequent to the transfer of medium from control cells not exposed to secondary UV biophotons and from cell-free cultures (UV-exposed medium only) to reporter cells ($p<0.001$).

Clonogenic survival following exosome transfer. The experiment was taken a step beyond that described in the previous section by extracting the exosomes from the UV-ICCM following irradiation and placing the exosome isolates, as opposed to the UV-ICCM, onto reporter cells. Fig 1B illustrates the ability of exosomes extracted from UV-ICCM to elicit a significant reduction in the clonogenic survival of HCT116 p53 +/+ reporter cells when compared to reporter cells which received control exosomes ($p<0.012$). The clonogenic survival of the UV-ICCM exosome-treated reporter cells was 81.2% ± 2.3%, whereas the survival of reporter cells receiving non-irradiated cell control exosomes, irradiated no-cell control exosomes, and medium only exosomes were 101.0% ± 1.5%, 103.2% ± 6.0% and 102.8% ± 3.1%, respectively.

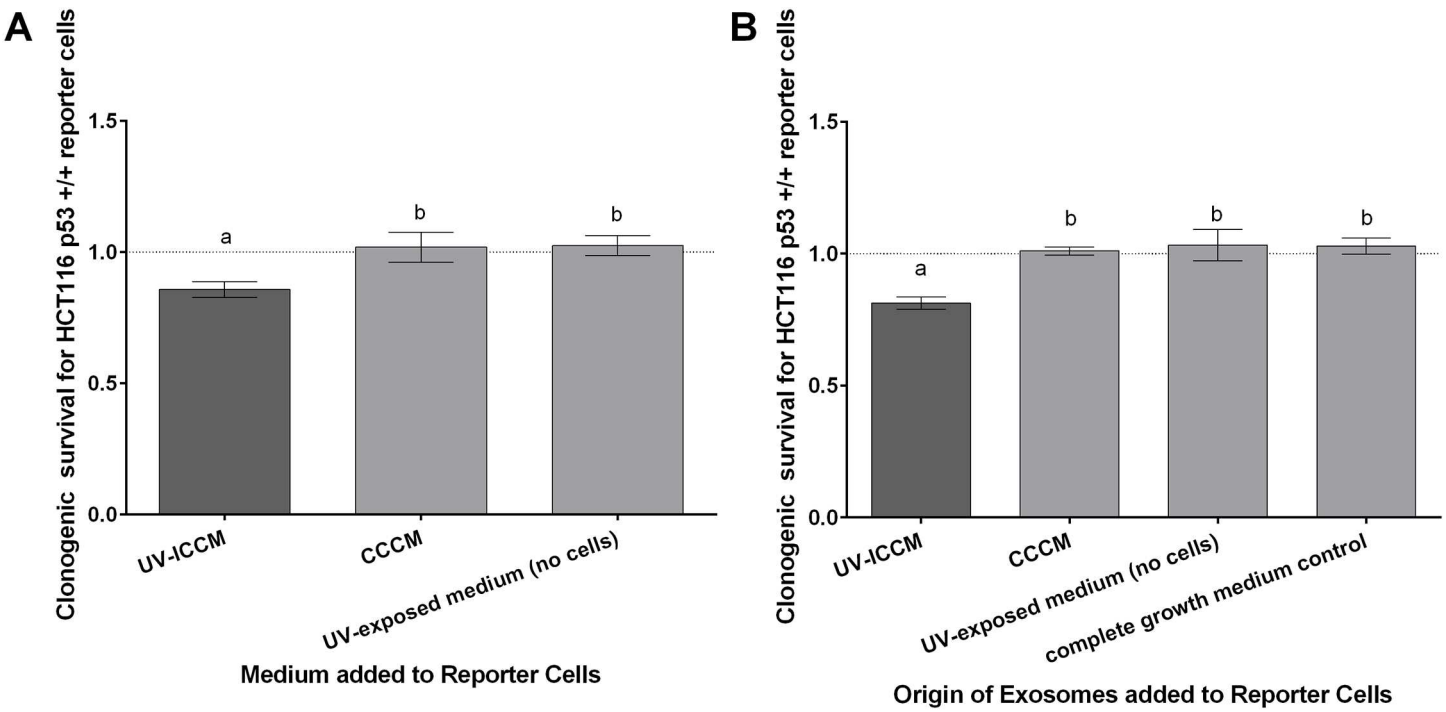

Fig 1. Reporter cells subjected to exosomes or conditioned culture medium from UV-exposed bystander cells (UV emitted from beta-irradiated cells). (A) Surviving fraction of HCT116 p53 +/+ cells cultured in UV-exposed ICCM or CCCM. Error bars represent SEM for 18 replicates (3 replicates for each of 6 independent experiments) for the UV-ICCM treatment and the CCCM control, and 9 replicates (3 replicates for 3 independent experiments) for the UV-exposed medium (no cell) control. **(B)** Surviving fraction of HCT116 p53 +/+ cells cultured in exosomes extracted from UV-exposed ICCM or CCCM. Error bars represent SEM for 18 replicates (3 replicates for each of 6 independent experiments) for the UV-ICCM exosome treatment and CCCM exosome control, and 9 replicates (3 replicates for 3 independent experiments) for the no cell control & medium only control. Letters (a,b,c) indicate significant differences between samples as assessed by means of 1-way ANOVA, 95% confidence level.

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When clonogenic survival of reporter cells receiving UV-ICCM and reporter cells receiving isolated exosomes are compared, it is found that the levels of cell killing induced by each of these treatments are comparable such that they are not significantly different ($p = 0.493$). This lack of difference in effect induced by UV-ICCM and exosomes may suggest that the cell killing effects observed as a result of UV-ICCM transfer are most likely due to the effect of exosomes since there does not appear to be an induced effect that is not accounted for by the exosomes.

Clonogenic survival following exosome-depleted CCM transfer. An additional experimental permutation was conducted whereby UV-ICCM and control CCM was ultracentrifuged to pellet and subsequently remove exosomes from the medium. The exosome-depleted UV-ICCM or CCCM was then placed onto reporter cells to determine the effect of exosome-free UV-ICCM and CCCM. The treatment of reporter cells with exosome-depleted UV-ICCM proved to induce significant ($p<0.0001$) cell killing in treated reporter cells to 80.1 ±3.0%. In contrast, the reporter cells treated with CCCM did not exhibit significant reductions in survival (100 ± 2.68%) when compared to the survival of reporter cells which received CCCM that was not depleted of exosomes ($p = 0.78$). We suggest that the effects observed here may be attributed the action of other soluble factors present in the UV-ICCM. During the UV exposure period (24 hour incubation where bystander cells were being exposed to UV), the exosomes released from the UV-exposed cells could very possibly act upon the same population of UV-exposed cells to prompt the release of cytokines, nitric oxides, and other soluble factors prior to isolation of exosomes from the UV-ICCM. This is suggested since the magnitudes of

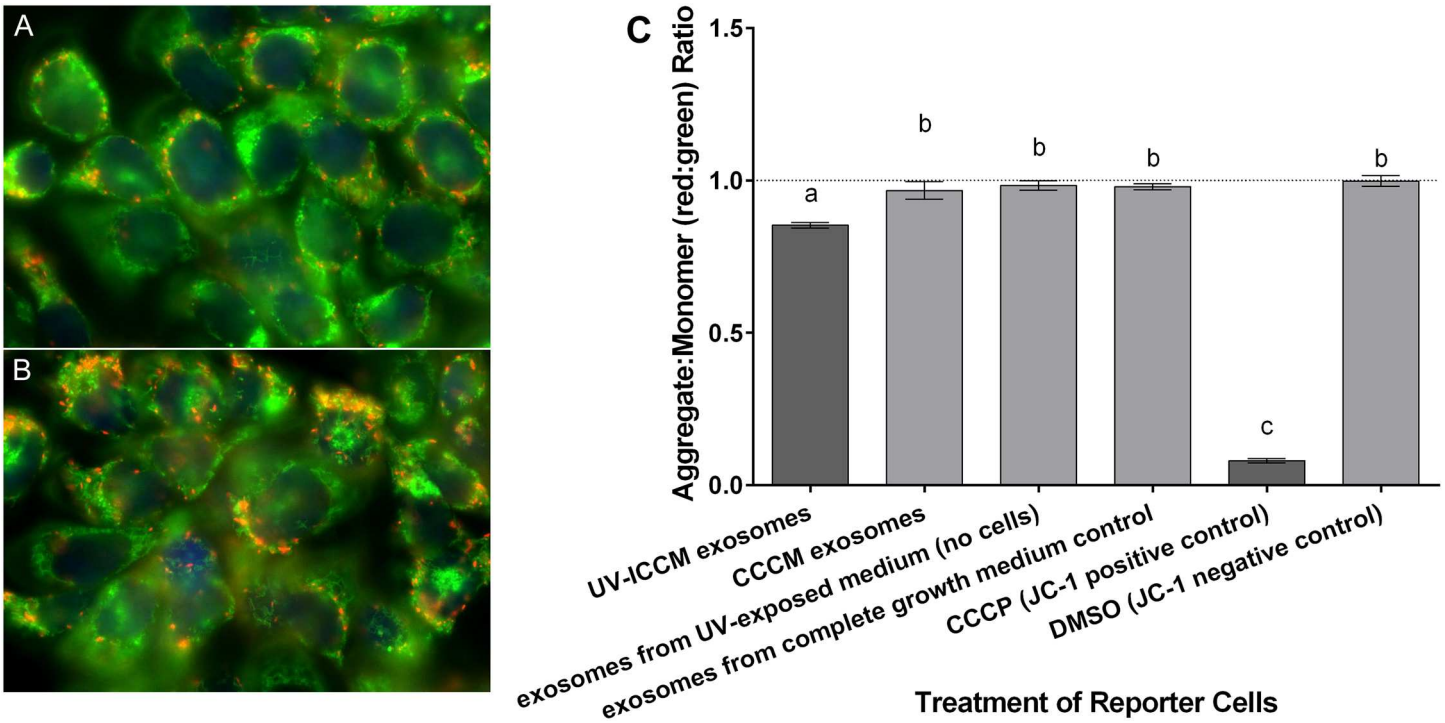

Fig 2. Fluorescence of JC-1 dye incubated with HCT116 p53 +/+ reporter cells which received. (A) exosomes extracted from ICCM treated with cell-emitted UV biophotons and **(B)** exosomes extracted from CCCM which did not receive UV biophoton irradiation. Fluorescence microscopy images were acquired using an Olympus IX81 microscope and Image Pro AMS 5.1 software. **(C)** Mitochondrial membrane potential observed in HCT116 p53+/+ cells following the receipt of exosome fractions extracted from UV-exposed bystander cells. The UV was emitted from directly-irradiated cells that were exposed to 0.5 Gy ^{3}H β-radiation. Error bars represent SEM for a total of 18 replicates (6 replicates for each of 3 independent experiments). Fluorescence ratios were normalized to the DMSO negative control.

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cell killing induced by UV-ICCM (with exosomes present) and that induced by exosomes extracted from UV-ICCM are comparable and thus do not support the idea that the exosomes and the other soluble factors present in UV-ICCM are acting in an additive manner, rather it is possible that one may lead to another. Despite these preliminary suggestions, further investigation will be required to properly interpret the implications of these results.

Mitochondrial membrane potential. The effect of exosomes extracted from UV-ICCM upon the mitochondrial membrane potential of reporter cells was also assessed to determine the possibility for apoptosis induction in the exosome-treated reporter cells. Treatment of HCT116 p53 +/+ cells with exosomes extracted from UV-ICCM proved to induced a marked depolarization in the mitochondrial membrane of reporter cells (Aggregate to monomer ratio (AMR): 0.852 ± 0.009 (standard error of the mean)). The loss of mitochondrial membrane integrity in this population is illustrated in Fig 2A as a predominance of green monomer fluorescence. The appearance of red fluorescence in the UV-ICCM exosome-treated population was evidently diminished when compared to the red fluorescence demonstrated in the cell population treated with control exosomes (extracted from cells that were not exposed to UV) (Fig 2B). Fig 2C shows that the depolarization induced by the exosomes extracted from UV-ICCM was significantly different when compared to experimental controls and the assay negative control (DMSO) ($p<0.0001$). The membrane depolarization induced by the assay's positive control (CCCP) (AMR: 0.080 ± 0.007) was significantly greater than that induced by the UV-ICCM exosomes ($p<0.0001$). However, the depolarization induced by exosomes extracted from UV-ICCM was still significant compared to negative controls, thus indicating

that the treatment of reporter cells with exosomes may be able to induce apoptosis in a significant proportion of cells within the reporter population, albeit it is not able to generate as great of a response as other treatments such as CCCP.

RNase-treated ICCM and exosomes from UV-exposed bystander cells

RNase was added into UV-ICCM or exosome fractions to confirm that the effects observed in response to reporter cell treatment with ultracentrifuged pellets were indeed induced by exosomes. More specifically, the intention was to determine the role of RNA-carrying exosomes in eliciting the responses observed.

Clonogenic survival following UV-ICCM transfer. Clonogenic survival was assessed in HCT116 p53 reporter +/+ cells that received UV-ICCM treated with RNase, UV-ICCM that was not treated with RNase, and CCCM which was not subjected to any exposure from secondarily-emitted UV. Fig 3A illustrates that RNase is effective in abolishing any negative cell killing effects that manifested in cells which received ICCM harvested from cells exposed to secondary UV radiation. Clonogenic survival in the RNase-treated population was not significantly different from that exhibited by the control cells receiving medium from non-UV-exposed cells (CCCM) ($p = 0.972$). In contrast, the receipt of UV-ICCM not treated with RNase was proven effective in reducing clonogenic survival significantly below the level of CCCM controls ($p<0.0001$).

Clonogenic survival following exosome transfer. Clonogenic survival of HCT116 p53 +/+ reporter cells was assessed following addition of exosome fractions to the reporter cell cultures. Exosome fractions were either extracted from UV-ICCM and subsequently treated with RNase, extracted from UV-ICCM and not treated with RNase (positive control), or extracted from conditioned medium of cells that were not exposed to UV biophotons (negative control). Fig 3B indicates the effectiveness of RNase in preventing a reduction in clonogenic cell survival for those exosome fractions which were extracted from UV-ICCM. The RNase treatment of the UV-ICCM exosome fraction was found to produce a statistically similar survival level to the level observed in the non-UV-exposed control (exosomes from CCCM) ($p = 0.840$). RNase treatment of the UV-ICCM exosomes proved to significantly assuage the proportion of cells killed when compared to the exosomes extracted from UV-ICCM that did not receive RNase treatment ($p<0.0001$).

In order to confirm that the effects observed in the reporter cells were indeed attributed to the action of RNase upon exosomes and not the direct action of the RNase upon the cells, controls were conducted whereby RNase was added into a non-irradiated cell population and a cell population which was directly-exposed with tritium. The results of these controls showed that RNase did not abolish nor assuage the cell killing observed in cells that were directly exposed to beta radiation (Cells exposed to 857.5 μCi ^{3}H: 51.9 ± 7.7%, cells exposed to 857.5 μCi ^{3}H + RNase: 45.4 ± 5.8%; $p = 0.76$). Furthermore, the addition of only RNase into cells resulted in a survival rate of 93.3 ± 9.7%. Thus, RNase treatment of these non-irradiated cells did not affect cell survival significantly when compared to the survival of non-irradiated cells that were not treated with RNase ($p = 0.80$).

Clonogenic survival following exosome-depleted CCM transfer. UV-ICCM, control CCM, and complete growth medium that was not conditioned with cells was ultracentrifuged to pellet and remove exosomes from the medium. Subsequently, the exosome-depleted UV-ICCM, CCCM and complete growth medium were treated with RNase prior to incubation with clonogenic reporter cells. The purpose of this control was to determine whether the RNA accounting for the observed bystander effects originated from the surface of the exosomes (contained within the medium) or from within the exosomes. After treating the clonogenic

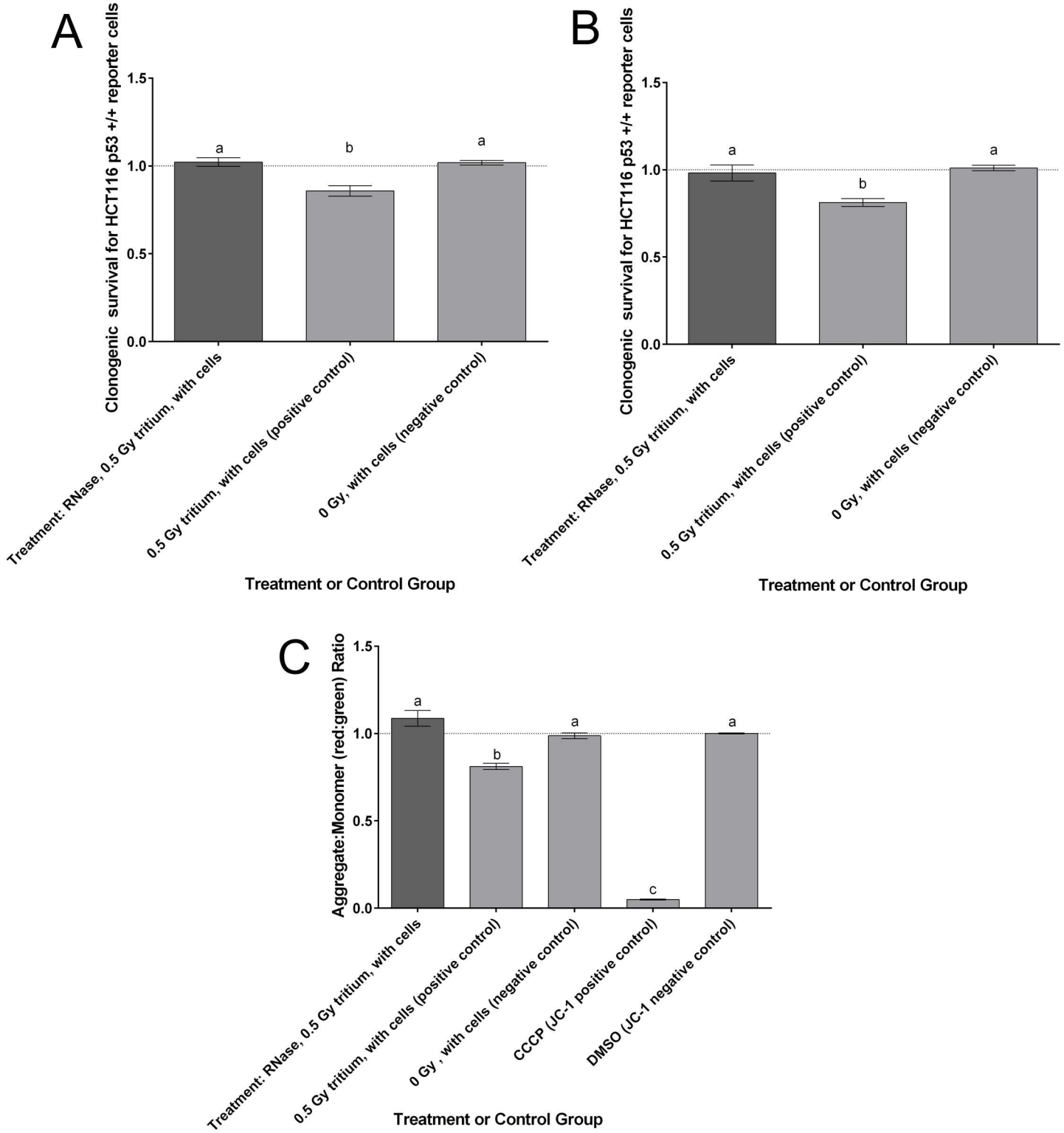

Fig 3. Reporter cells subjected to RNase-treated ICCM or exosomes derived from UV-exposed bystander cells. (A) Clonogenic survival of HCT116 p53 +/+ reporter cells receiving RNase-treated UV-ICCM, UV-ICCM, or CCCM. Error bars represent SEM for a total of 18 replicates (3 replicates for each of 6 independent experiments) for the 0.5 Gy positive control and 0 Gy negative control, and 9 replicates (3 replicates for 3 independent experiments) for the RNase-treated 0.5 Gy group. **(B)** Clonogenic survival of HCT116 p53 +/+ reporter cells following treatment of the reporter cells with RNase-treated UV-exposed exosomes, UV-exposed exosome fractions (no RNase treatment), or non-exposed exosome fractions. Error bars represent SEM for a total of 18

replicates (3 replicates for each of 6 independent experiments) for the 0.5 Gy positive control and 0 Gy negative control, and 9 replicates (3 replicates for 3 independent experiments) for the RNase-treated 0.5 Gy group. **(C)** Mitochondrial membrane potential (assessed via the incubation of cells with JC-1 mitochondrial potential dye) of HCT116 p53 +/+ reporter cells receiving RNase-treated or non-RNase-treated exosome isolates extracted from ICCM or CCCM. Error bars represent standard error of the mean for 6 replicates tested for each of three independent experiments (18 replicates total). Letters (a,b,c) represent significant differences between treatments as assessed by 1-way analysis of variance; post-hoc testing assessed using Tukey's HSD test at the 95% confidence level.

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reporter cells with RNase-treated UV-ICCM depleted of exosomes, the survival observed was 84.6% ± 2.5%. Although the magnitude of cell death was slightly assuaged, the effect was not significantly different ($p = 0.275$) from that observed following the treatment of reporter cells with UV-ICCM depleted of exosomes that was not treated with RNase (80.1% ± 3.0%). While we cannot rule out a role for RNA present outside of the exosomes in eliciting part of the bystander response, the results suggest that the RNA-attributed effects observed in these experiments are predominantly driven by the RNA found within the exosomes. When reporter cells were exposed to RNase-treated CCCM depleted of exosomes and RNase-treated complete growth medium (not cell conditioned and depleted of exosomes), the resultant surviving fractions were 100.7% ± 3.2% and 100.1% ± 2.0%, respectively.

Mitochondrial membrane potential. Mitochondrial membrane potential in HCT116 p53 +/+ reporter cells was assessed following the treatment of reporter cells with RNase-treated exosome fractions or control exosome fractions. Thereafter, JC-1 dye was added to the reporters to identify the ratio of J-aggregates and monomers in reporter cells resultant to exosome treatment (Fig 3C). The treatment of reporter cells with RNase-treated exosomes isolated from UV-ICCM conferred a lack of significant change in the reporter cells' mitochondrial membrane potential (AMR: 1.087 ± 0.045 (standard error of the mean)) when compared to the reporters which were treated with the assay's negative control, DMSO (AMR: 1.00 ± 0.003) ($p = 0.330$) and when the RNase-treated group was compared to the non-irradiated control (AMR: 0.987 ± 0.016) ($p = 0.163$). In contrast, treatment of the reporter cells with exosomes isolated from UV-ICCM which were not treated with RNase proved to induce significant mitochondrial membrane depolarization (AMR: 0.812 ± 0.018) when compared to both of the negative controls ($p<0.0001$). The capability of RNase to abolish significant mitochondrial membrane depolarization, therefore, suggests that RNA, a factor which exosomes have been shown to carry [38], could be a factor that is responsible for eliciting a bystander response in reporter cells (those treated with exosomes extracted from UV-exposed cells).

Validation of exosome isolation

To validate that the exosome extraction technique used in the current study was successful in isolating exosomes, western blots were conducted to identify exosome-associated proteins and transmission electron microscopy (TEM) was conducted to confirm the presence of and size of the microvesicles extracted by means of ultracentrifugation.

Exosome-associated transmembrane protein, CD63, was expressed in its glycosylated form in exosome samples whereas whole cell lysates for the positive control (HepG2 cells) and for HCT116 p53+/+ cells expressed non-glycosylated and partially-glycosylated CD63, respectively (Fig 4A). The whole cell lysates extracted from UV-exposed cells appeared to undergo glycosylation to a greater extent than those which were extracted from non-UV-exposed cells. The observed expression of fully glycosylated CD63 was expected for exosome samples as found previously by Jelonek et al [39]. Furthermore, the absence of non-glycosylated or partially-glycosylated CD63 in the exosome isolates could be suggestive of a lack of contamination by cellular material in the exosome sample.

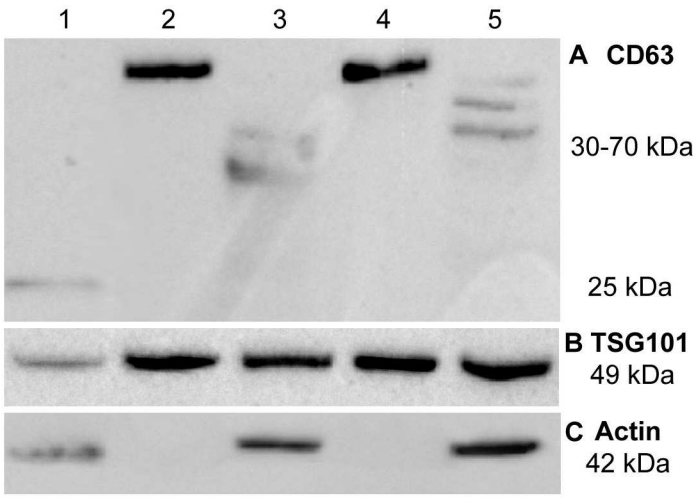

Fig 4. Protein bands acquired using western blots for expression of. (A) CD63 (glycosylated form: 30–70 kDa, non-glycosylated form: 25 kDa), **(B)** TSG101 (49 kDa), and **(C)** Actin (42 kDa). Lane 1: positive control (10 μg protein from HepG2 whole cell lysate for CD63 and actin antibodies; 10 μg protein from HeLa whole cell lysate for TSG101 antibody). Lane 2: exosomes extracted from HCT116 p53 +/+ CCCM. Lane 3: HCT116 p53 +/+ whole cell lysate not exposed to UV. Lane 4: exosomes extracted from HCT116 p53 +/+ UV-ICCM. Lane 5: HCT116 p53 +/+ whole cell lysate exposed to UV. All lanes contain 10 μg protein each. The lack of actin expression demonstrated by lanes 2 and 4 indicate the absence of actin in exosome samples. Because actin is not required for exosome transport, the absence of actin in exosome samples is expected and indicates a lack of contamination by cellular debris in the exosome isolates.

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Upon assessing protein expression in exosome samples extracted from UV biophoton-exposed and non-exposed cells, it was shown that CD63 expression in exosomes from UV biophoton-exposed cells was significantly greater than that expressed in control exosomes ($p = 0.028$). The area under the curve, representative of band density as assessed by ImageJ, produced a normalized value of 9.8 ± 0.5 for exosomes extracted from UV biophoton-exposed cells and a value of 8.7 ± 0.1 for control exosomes (normalized to expression of HepG2 positive control). From these results, it can be suggested that the exposure of cells to UV biophotons may be responsible for initiating a release of exosomes from UV biophoton-exposed cells which is greater than the quantity of exosomes that would be secreted from non-UV-exposed cells. It is noted, however, that *whole cell lysates* subjected to secondary UV biophotons did not express CD63 to a degree that was significantly different from non-UV-exposed HCT116 cells ($p = 0.779$, normalized protein expression value: 1.64 ± 0.06 and 1.76 ± 0.29, respectively). Thus, the suggestion that UV biophoton exposure triggers the release of more exosomes requires further investigation before a sound conclusion can be drawn.

The second exosome-associated protein assessed in the current study, TSG101, is a cytosolic protein that is a component of the endosomal sorting complex required for transport and is involved in the generation of exosomes [40]. TSG101 expression was evident in both exosome samples and whole cell lysates for HCT116 p53 +/+ cells (Fig 4B). Upon comparing TSG101 expression between UV biophoton-exposed and non-exposed cells, the degree of protein expression did not differ among exosome isolates ($p = 0.685$, normalized protein expression: 3.9 ± 0.38 and 3.7 ± 0.44, respectively) nor among whole cell lysates ($p = 0.182$, normalized protein expression: 3.2 ± 0.25 and 2.6 ± 0.13, respectively). The lack of difference in TSG101 protein expression between exosome samples extracted from UV biophoton-exposed cells and control exosome samples casts doubt upon the idea that UV biophotons trigger the release of more exosomes from exposed cells. Rather, the difference in mitochondrial membrane

potential and clonogenic survival induced by the exosomes from UV-ICCM may be explained, not by a greater quantity of exosomes, but a difference in the contents of the exosomes released by the UV-exposed cells and the control cells. This suggestion would of course require further investigation that is beyond the scope of this study at the present time.

Actin served as a negative control for protein expression in exosome samples such that its expression was not expected in exosome isolates but was expected in whole cell lysates [41, 42]. The results conferred in the current study agree with the aforementioned hypothesis since actin expression was present in HCT116 p53 +/+ whole cell lysates but not in exosome samples (Fig 4C).

Microvesicles possessing a diameter of approximately 100 μm or less were readily visualized when TEM was employed to scrutinize the samples extracted via ultracentrifugation of ICCM and CCCM (Fig 5). The sizes of the visualized vesicles were within the range characteristic of

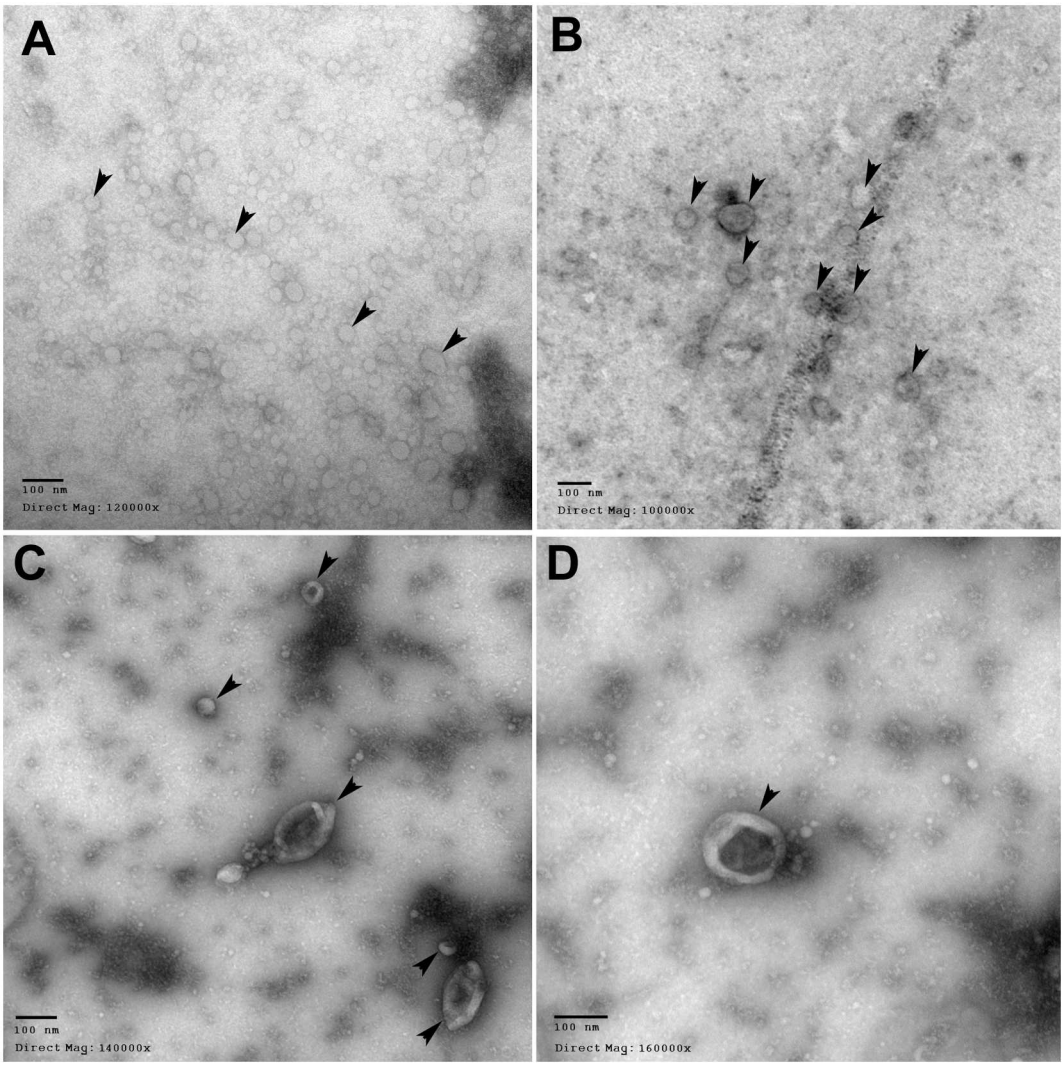

Fig 5. Transmission electron microscopy images illustrating the exosomes that were extracted from HCT116 p53 +/+ cells via ultracentrifugation. Exosomes are indicated by arrowheads. Scale bars in each of the four panels represent 100 nm. **(A)** Exosomes extracted from UV-exposed cells, direct magnification: 120 000x. **(B)** Exosomes extracted from non-UV-exposed control cells, direct magnification: 100 000x. **(C)** Exosomes from UV-exposed cells, direct magnification: 140 000x. **(D)** Exosome from UV-exposed cells, direct magnification: 160 000x.

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exosomes (50–150 nm). From the observations made using TEM images and western blot analysis of exosome-associated proteins, it is possible to confirm with confidence that the exosomes isolation method used in the current study (ICCM and CCCM ultracentrifugation) was successful.

Discussion

This study demonstrates novel evidence of a link between radiation-induced UV biophotons and exosomes (chosen for assessment to represent all soluble factors). To our knowledge, the current study is the first to suggest the reconciliation of these two bystander effect mediators. We previously demonstrated the modulation of the cell survival response in bystander cells exposed to UV biopohotons [13] whereby the directly-irradiated cell culture and the bystander culture were physically separated to the extent where there was no transfer of medium nor cell-cell contact at any point during the experiment. Even in the absence of medium transfer, co-culture, and direct cell-cell contact, the bystander effect was still elicited in the bystander cells receiving secondary UV signals. This observation thus introduced the idea that soluble factor release by directly-irradiated cells could not be the only mechanism driving the bystander response. Rather, there are either at least two separate bystander mechanisms (soluble molecules and biophotons), or soluble factors were being released by bystander cells subjected to UV signals emitted from directly-irradiated cells. In an effort to rationalize the observed bystander effects, we hypothesized the possibility of a link between UV biophotons and the release of exosomes from UV-exposed cells due to the literature that has recently emerged on the subject of exosome-mediated radiation bystander effects [17, 29, 32–34] and the demonstrated capacity for UV radiation to modulate exosome functions [28]. The observations made in the current study strongly support the existence of a bystander mechanism whereby the UV biophotons generated by directly-irradiated cells interact with bystander cells to induce the release of response-eliciting exosomes. That is not to say that soluble factors and exosomes are not released in response to direct cellular exposure to ionizing radiation. The results conferred in the current study simply illustrate that transfer of medium and direct cell-to-cell contact are not always required to elicit bystander responses in non-irradiated cells. We acknowledge that there are many soluble signalling factors that are involved in communicating the bystander effect and do not seek to invalidate those well-established mechanisms in any respect, we simply propose a plausible solution for situations in which bystander effects can be elicited in the absence of medium transfer and direct cell-cell contact. Alternatively, medium transfer and cell-cell contact are not required in another situation whereby volatile components from biological system can affect a nearby biological system [9, 10]. However, in the case of the current study, the two cell populations were physically separated such that even volatile species could not be shared or transferred.

β-irradiation of HCT116 p53 +/+ cells induced UV biophoton emission which was then subjected to bystander cells. The exosomes released from these UV-exposed bystander cells were subsequently isolated and used to assess downstream effects in reporter cells. These experiments show that the exosomes isolated from cells exposed to radiation-induced UV biophotons are capable of modulating the biological endpoints of cell death and mitochondrial membrane potential in reporter cells. Because exosomes are extensively diverse in regard to their content & abundance, and furthermore because their intravesicular contents can be modulated in response to various environmental conditions, it is difficult to establish the exact factors which are responsible for the effects that are observed in this particular case. It is suggested that a modulation of the RNA and protein cargo are likely to be influential in eliciting a bystander effect in cells receiving UV-exosomes compared to controls. However, we also

did not rule out the idea that the UV biophotons could be responsible for initiating the release of exosomes from UV-exposed cells such that the samples extracted from UV-exposed ICCM would exhibit greater quantities of exosomes than the non-exposed controls.

Ribonucleic acid (RNA), particularly microRNA (miRNA), are carried within exosomes and have been proven to play a role in the radiation-induced bystander effect when x-rays have been used as the primary radiation source [17, 32]. Irradiation of a given cell can trigger the upregulation of specific miRNAs such as those involved in DNA damage response functions [32]. Through packaging of these miRNAs within vesicles such as exosomes, the miRNAs are easily exchanged intercelluarly and can subsequently elicit bystander effects in recipient cells. The current study shows that the induction of bystander effects in cells receiving exosomes extracted from UV-ICCM is quite possibly attributed to the action of the exosomes' RNA contents. When the exosome pellet extracted from UV-ICCM were treated with RNase and subsequently incubated with reporter cells, reporter cells demonstrated a lack of stimulation represented by an absent cell death response and an abolished response in terms of mitochondrial membrane depolarization. These findings contrast significantly with those conferred following treatment of reporter cells with exosomes extracted from UV-ICCM that did not receive RNase treatment. Although we were not able to directly assess the permeability of exosomes to RNase, we conducted an experiment whereby exosome-depleted UV-ICCM was treated with RNase to determine whether the RNA accounting for the bystander effect originated from outside or within the exosomes. Our results suggest that RNA on the outside of exosomes may contribute to a portion of the effect. However, the effect appears to be attributed mainly to RNA contained within the exosomes.

It is possible that the mitchondrial membrane depolarization and cell death observed in reporter cells treated with exosomes extracted from UV-ICCM is the result of an upregulation of miRNAs detrimental to mitochondrial function or a downregulation of mito-protective miRNAs. To gain an accurate representation of the miRNAs that are involved in mediating the UV bystander effect, profiling of miRNA will be required in exosomes derived from both non-UV-exposed and UV-exposed media. It is possible, however, to confirm that the degradation of RNA was influential in abolishing significant bystander responses that are induced by the isolated exosome pellet under normal experimental conditions. This study has demonstrated the first evidence of RNA's involvement in the UV-mediated bystander response.

This study used TEM to characterize the size of the vesicles isolated from ICCM by means of ultracentrifugation. TEM imaging was able to confirm that the size of the vesicles isolated in our experiments were characteristic of exosomes (50–150 nm). Furthermore, the expression of two exosome-associated proteins was assessed as per the guidelines recommended by Lotvall *et al.* [43]. Positive protein expression results were conferred for exosome associated proteins, CD63 and TSG101. Semi-quantitative assessment of CD63 protein expression suggested a possibility that UV biophoton exposure of reporter cells could trigger the release of more exosomes compared to non-exposed controls. This phenomenon of increased exosome abundance has also been observed by another research group using nanoparticle tracking analysis in glioma cell lines following exposure to x-radiation exposure [39]. However, the consistent expression of TSG101 protein across all exosome samples (both those isolated from UV-exposed cells and control cells) contrasts with the previous hypothesis and introduces the idea that cellular exposure to UV biophotons may induce a change in the contents carried by the exosomes as opposed to triggering the release of a greater quantity of exosomes. Although this suggestion has not yet been investigated in the current study which employs UV biophotons as the trigger, the published literature is supportive of the idea that radiation insult is capable of affecting the contents of excreted exosomes. Arscott and colleagues conducted molecular profiling of exosomes isolated from x-irradiated and non-irradiated U87MG cells to find that

exosomes originating from x-irradiated cells exhibited 1308 and 209 mRNA changes 24 and 48 hours post-irradiation when compared to the mRNA sequences of non-irradiated cells [44]. Analysis of the protein contents of x-radiation-derived exosomes by Jelonek and colleagues revealed the presence of 236 proteins that were not detected in exosomes derived from non-irradiated FaDu cells. Among the proteins that were expressed in response to irradiation, the functions that predominated were those involved in cell division, transcription, and cell signalling [39]. The available literature which reports on exosomes derived from UV-exposed cells is limited. However, Cicero *et al*. investigated the exosome expression following direct UV-B irradiation of human keratinocytes and similarly concluded that the UV-B exposure did not affect the number of exosomes and rather hypothesized that ultraviolet radiation propagates its effects by altering the exosome composition [28]. Based upon obeservations made by previous investigators, it is not unreasonable to suggest that a stressor, such as secondarily-emitted UV biophotons, could initiate a change in the contents of exosomes released from UV-exposed cells. While this hypothesis has yet to be addressed, we consider the investigation of this inquiry an important future endeavour as it will provide valuable insight into the findings of the current work.

Although the work conducted in this study is restricted to *in vitro* investigations, it has generated results that have the potential to be expanded upon to elucidate the clinical relevance associated with exosomes isolated from UV-ICCM. The observation that exosomes extracted from UV-ICCM are capable of eliciting significant mitochondrial membrane depolarization can be considered an important first step in explaining a molecular mechanism for the radiation-related chronic fatigue and immune dysfunction syndrome (CFIDS). Mitochondrial membrane depolarization can be indicative of compromised ATP generation and subsequently manifest as the symptoms which characterize CFIDS [45]. The exertion of a systemic effect by exosomes, following even a targeted event such as an irradiation, makes plausible the suggested relationship between radiation exposure and CFIDS [46]. It will be crucial to explore this relationship further since the characterization and analysis of exosomes extracted from biological fluids may eventually be used as a predictor of many disease processes, including CFIDS.

Limitations

A limitation of the current study involves the inconsistency in the variables between each of the endpoints investigated. The ratio of exosomes to reporter cells and the co-incubation times were different between the two assays used in the current study such that 9-day incubation with exosomes and 500 reporter cells were used in the clonogenic survival assay, while 1 hour exosome co-incubation and 2×10^6 reporter cells were used in the mitochondrial membrane potential assay. Some of these differences were inevitable due to restrictions associated with assay-specific requirements and subsequently, these discrepancies between assay protocols result in the inability to conduct a valid and meaningful comparison of the magnitude of effect that the exosomes had upon each of the two endpoints assessed in the study. Despite the discrepancy, it is important to note that two widely different assays produced results that agree with each other when exposed to the same given treatment. This finding is important because we can be certain that the exosomes produced compatible effects, even under variable conditions.

Conclusion

This paper was focused upon reconciling two apparently opposing bystander mechanisms. However, it was not meant to discount any other bystander mechanisms. These experiments

show that exosomes capable of eliciting bystander effects are released from cells in response to exposure to non-ionizing UV signals emitted from directly-irradiated cells rather than being released as a direct result of the primary beta-irradiation itself. The exosomes extracted from UV-ICCM are effective in modulating clonogenic survival and mitochondrial membrane potential in bystander cells to a significant degree compared to exosomes extracted from CCCM harvested from non-UV-exposed cells. These effects could be abolished by the treatment of the exosome pellet with RNase. RNA is therefore considered influential in mediating the observed bystander effects. Similar expression of exosome-associated proteins among UV-ICCM-derived exosomes and control exosomes suggests that UV may not affect the quantity of exosomes released, rather it may elicit a modification of the cargo carried by the exosomes; it will be very important to investigate this hypothesis further. Most importantly, the study is the first to demonstrate a relationship between the radiation-induced bystander effect mediated by UV biophotons and exosomes. The significance of this result indicates that the transfer of medium is not always required for bystander signals to be communicated. Effect-eliciting soluble factors may still be generated in a bystander population which is subjected to the UV biophoton signals emitted from a directly-irradiated population.

Supporting information

S1 File. Raw Data File. This file contains the data corresponding to the results presented in the current manuscript.
(XLSX)

Acknowledgments

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Chapter 7

Modulation of Electron Transport Chain Activity by Radiation-Induced Biophotons

Michelle Le, Fiona E. McNeill, Colin B. Seymour, Andrew J. Rainbow, James Murphy, Kevin Diamond, Carmel E. Mothersill

The investigation of the effects of biophoton signalling upon the mitochondrial electron transport chain were initially proposed by Dr. Fiona McNeill and Dr. Carmel Mothersill. Conceptualization of endpoints of interest were developed by the first author, Dr. Carmel Mothersill, and Dr. Fiona McNeill. The protocol for assessing enzyme activity of each of the mitochondrial complexes was developed by the first author with guidance from Dr. James Murphy. Spectrometer equipment was provided by Dr. Kevin Diamond. Experiments and statistics were performed by the first author and interpretation of results were collectively discussed among the first author, Dr. Carmel Mothersill, Dr. Fiona McNeill, Dr. Colin Seymour, and Dr. Andrew Rainbow. The first draft of the manuscript was prepared by the first author and all authors subsequently provided feedback for the written presentation of the research.

Abstract

Radiation-induced biophotons are an electromagnetic form of bystander signalling. In human cells, biophoton signalling is capable of eliciting effects in non-irradiated bystander cells. However, the mechanisms by which the biophotons interact and act upon the bystander cells are not clearly elicudated. To address this question, we have investigated the spectral biophoton emission from 200-1100 nm and investigated the effect of biophoton emission upon the function of the complexes of the electron transport chain (ETC). The exposure of bystander HCT116 p53 +/+ cells to biophoton signals emitted from β-irradiated HCT116 p53 +/+ cells proved to induce significant modifications in the activity of Complex I (NADH dehydrogenase or NADH:ubiquinone oxidoreductase) such that the activity was severely diminished compared to non-irradiated controls. The enzymatic assay showed that the efficiency of NADH oxidation to NAD+ was severely compromised. It is suspected that this impairment may be linked to the photoabsorption of biophotons in the blue wavelength range. The photobiomodulation to Complex I was suspected to contribute greatly to the inefficiency of ATP synthase function since it resulted in a lower quantity of H^+ ions to be available for use in the process of chemiosmosis. The spectral characterization experiments demonstrated a positive relationship between ^3H activity and photon emission across the whole range of UV, visible, and IR wavelengths measured by the detection system. This result validates the use of photon quantification from a narrow wavelength window as a representative marker of overall photon emission across the UV, visible and IR portions of the electromagnetic spectrum. Overall, these results provide evidence for a link between biophoton emission and biomodulation of the mitochondrial ATP synthesis process. However, there are many aspects of biological modulation by radiation-induced biophotons which will require further elucidation.

7.1 Introduction

The biological effects of low-dose ionizing radiation is an area of research which is currently very widely studied due to its relevance in every day practice in occupational and clinical settings. Yet, there is still much uncertainty surrounding the biological implications of low doses as there are many conflicting effects which have been observed in the low dose realm, whether they are observations of hyper-radiosensitivity [1] or

hormetic responses [2, 3]. With that being said, the linear non-threshold model has been challenged in the scientific research community due to the suggestion that it does not accurately represent the risk of biological effects at low doses [4, 5]. Among the phenomena which have contributed to the challenging of the LNT at low doses is the radiation-induced bystander effect (RIBE). The RIBE is a phenomenon whereby cells which have not been directly exposed to ionizing radiation, but which have received bystander signals from cells that have been directly irradiated, exhibit characteristics resembling the effects of having been irradiated. RIBEs manifest as a result of intercellular signalling via the transfer of soluble factors through gap-junction intercellular communication (GJIC) channels [6] and medium transfer experiments [7], or via a physical mechanism characterized by electromagnetic signalling [8, 9]. The latter mechanism of bystander effect mediation (biophoton-mediated bystander effects) will be the focus of the present study.

The emission of weak photon fluences from biological material (ranging from 10 to 10^3 photons cm^{-1} s^{-1} [10]) is referred to as biophoton emission and is a well established phenomenon in the field of biophotonic research [11, 12]. Emission occurs both spontaneously [13] and as a result of exogenous perturbation by triggers such as visible light [14], UV light [15, 16], chemical stress [11, 17], and mechanical stress [18–20]. Moreover, investigation of the properties of biophoton emission using sensitizers and quenching agents have revealed excited species involved in cellular metabolism as a potential source of biophotons [21]. The quantification of biophoton emission from whole organisms and tissues has since been established as a method of non-invasively characterising the oxidative status of a given system [22–24]. The action of biophotons as a means of intercellular communication was identified in 1980 when Kaznacheev demonstrated the induction of significant adverse effects in a fibroblast cell population that was optically-coupled, but not chemically associated, with a fibroblast culture which was treated with the Coxsackie A13 virus [25]. This effect has since been corroborated by multiple supporting studies citing evidence for intercellular communication via a signal that is electromagnetic in nature [26–29].

We have recently investigated this mechanism of communication following exposure of *in vitro* cell cultures to ionizing β-radiation. Our investigations have demonstrated that radiation-induced biophoton signalling is able to induce clonogenic cell death in bystander cells and have proven that the magnitude of the effect is dependent upon the function of the bystander cells' p53 proteins [30]. Intercellular biophoton

signalling also has a profound effect upon mitochondria as the signals were effective in inducing mitochondrial membrane depolarization [31]. The ability of the biophoton signal to modulate mitochondrial membrane potential leads to the suggestion that the mechanism for electromagnetic signals in driving bystander responses could be linked to mitochondrial function. Mitochondria have been identified as an intergral participant in the RIBE whether as an extra-nuclear target of direct irradiation [32] or as a recipient of bystander signals [33, 34]. The modification of mitochondrial physiology following chemically-mediated bystander signalling has been documented [34–36]. However, it has not previously been studied in the realm of biophoton-mediated bystander effects. The interest in assessing mitochondrial function following receipt of biophoton bystander signals stems from the involvement of redox reactions in regulating the mitochondrial functions responsible for cellular metabolism. It is hypothesized that the input of energy carried by biophotons into the mitochondrial electron transport chain (ETC) may act to either drive or inhibit the redox reactions involved in electron shuttling. We anticipate that both upregulation or downregulation of electron transport chain activities leading to a modulation in ATP production could have profound effects upon cellular response. In this study, we aimed to address this inquiry by assessing the activity of the electron transport chain (ETC) complexes along with ATP synthase in response to biophoton exposure.

This study also aims to tackle a limitation identified in our previous biophoton work whereby the biophoton measurements were restricted to discrete wavelength bands confined to the UV wavelength range [9]. This limitation stemmed from a lack of access to appropriate equipment required for the detection of biophotons across a large wavelength range. The current study addresses this limitation by extending our investigation across the UV, visible, and IR wavelength ranges with the hope that the spectroscopic biophoton data acquired will help to characterize the effects that biophotons may be able to exert upon biological systems.

These investigations encompass an analysis of enzymatic activity of the various complexes of the ETC in HCT116 p53 +/+ bystander cells in response to biophoton exposure. Spectroscopy in the UV and visible wavelength ranges are also employed to characterize the biophoton emission resultant to direct cellular irradiation with β-emitter, tritium (^{3}H). The primary objective of this work is to further clarify the mechanism by which biophotons induce modifications in cells that are recipients of the bystander signal.

7.1.1 Background: the Electron Transport Chain

The electron chain and chemiosmosis together constitute the process of oxidative phosphorylation. The electron transport chain (ETC) carries electrons between molecules in a series of redox reactions to produce energy. This energy is subsequently used to power proton pumps to generate an electrochemical gradient across the inner mitochondrial membrane. The electrochemical gradient is required to drive chemiosmosis, the process of synthesizing ATP [37].

At the beginning of the ETC, electron carriers NADH and $FADH_2$ produced as a result of glycolysis and the citric acid cycle arrive at Complexes I and II, respectively, and transfer their electrons to molecules (in the case of Complex I, the electrons are accepted by electron transfer flavoprotein (ETF) [38]), oxidizing NADH and $FADH_2$ to NAD^+ and FAD. In Complex I, the electrons are shuttled by ETF to flavin mononucleotide (FMN), from FMN to iron-sulfur clusters, and subsequently to ubiquinone (Q) whereby Q is reduced to ubiquinol (QH_2) [39]. The energy generated as a result of the electron-shuttling redox reactions is used by Complex I to pump protons across the inner mitochondrial membrane. $FADH_2$ contributes synonymously to Complex II as NADH does to Complex I. The electrons from $FADH_2$ are transferred to Fe-S units within Complex II to arrive at ubiquinone. In contrast with Complex I, Complex II is not a proton pump since $FADH_2$ is not as efficient of an electron donor as NADH [40].

The reduced electron carrier, ubiquinol, delivers the electrons from Complexes I and II to Complex III. The movement of electrons through Complex III to the oxidized form of electron carrier, cytochrome c, elicits the same proton-pumping function as described for Complex I. The electrons then get shuttled to Complex IV by reduced cytochrome c where electrons are passed through the complex to dioxygen ($^3O_2$) again leading to protons getting pumped from the matrix into the intermembrane space. The transfer of electrons to O_2 results in the generation of water molecules following acceptance of H^+ ions present in the matrix [37].

The result of electron shuttling down the ETC is the generation of an electrochemical gradient or proton gradient across the inner mitochondrial membrane in order to facilitate subsequent function of ATP synthase in the process of chemiosmosis. Chemiosmosis describes the movement of H^+ ions across the inner mitochondrial membrane via the hydrophilic transmembrane channel, ATP synthase. The movement of H^+ across ATP synthase turns a biological turbine referred to as F_o in the clockwise

direction at approximately 6000 rotations per minute (rpm) [41]. The mechanical energy generated from this movement drives a series of conformational modifications in the head proteins of ATP synthase (collectively referred to as F_1-ATPase) to catalyze the production of ATP via the addition of ADP and phosphate (P_i).

7.2 Materials & Methods

7.2.1 Cell Culture

Human colon carcinoma cell line, HCT116 p53 +/+, was routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin sulphate. Cultures were incubated at 37°C at 95% humidity and 5% CO_2. Cells were cultured in 75 cm^2 flasks and passaged when the cells reached 70-80% confluence. Cells were dissociated from the flask substrate by incubating them with 3 mL of a 1:1 solution of 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) for 3 minutes. The trypsinzed cells in solution were then neutralized with 7 mL of complete growth medium and subsequently plated into new flasks. 24 hours prior to an experiment, the cells received full-volume medium changes with complete growth medium. All reagents used for cell culture were obtained from Gibco/Life Technologies unless otherwise specified.

For experimental set-up in the case of enzymatic assay experiments, cells destined to receive electromagnetic bystander signals were seeded in 100 mm diameter petri dishes containing a 5 mL volume of growth medium, at a density of 2.1×10^6 cells per flask (2.8×10^4 cells/cm^2), and allowed to grow for 72 hours before being harvested for mitochondrial isolation. Cells destined to receive direct irradiation from beta(β)-particles, cells were seeded at a density of 2000 cells/cm^2 in 75 cm^2 flasks containing 10 mL of growth medium.

For spectroscopic experiments, HCT116 p53 +/+ cells were plated at a density of 2000 cells/cm^2 in 100mm diameter (75 cm^2) petri dishes. The cells were cultured in 10 mL of complete growth medium with RPMI 1640 free of phenol red and allowed to incubate for 6 hours to allow for cells to attach to the petri dish.

7.2.2 Cell Irradiation

Cells were either exposed directly to β-particles from tritium (^{3}H) or exposed to the electromagnetic bystander signals that were emitted from the β-particle-irradiated cells. For cells destined to receive direct irradiation from ^{3}H, 857.5 μCi of tritiated water was added to the cell culture and incubated with the cells for 24 hours to achieve a dose of 0.5 Gy. For cells destined to receive the electromagnetic bystander signals, cells in a 100 mm diameter dish were placed superior to the direct-β-irradiated cell population such that the two monolayers were 15 mm apart. The bystander cells were exposed to the electromagnetic bystander signals for 24 hours.

7.2.3 Mitochondrial Isolation

Following exposure of bystander cells to the electromagnetic bystander signals, mitochondria were isolated from the bystander cell populations following a protocol adapted from O'Dowd and colleagues [42]. Mitochondrial isolation was accomplished by first washing the confluent bystander cell monolayer with 5 mL of phosphate-buffered saline (PBS) 2 times. The washing PBS was then discarded and 10 mL of fresh PBS was added to the flask and the bystander cell monolayer was dissociated from the flask substrate using a cell scraper. The cell suspension was then centrifuged at 1000 g at 4°C for 10 minutes in a ThermoScientific Sorvall ST40R benchtop centrifuge. The supernatant was discarded and the cell pellet was resuspended in 400 μL ice-cold mitochondrial isolation buffer (adapted from O'Dowd et al. 2009). The cells were transferred to a 1.5 mL centrifuge tube and homogenized on ice using a handheld VWR homogenizer with a polybutylene terephthalate pestle. The cells were homogenized by breaking the cells with 40 strokes while the homogenizer rotational speed reached 12,000 rotations per minute (rpm). Large debris was pelleted at 2000 g at 4°C for 10 minutes in a ThermoScientific Sorvall Legend Micro21R benchtop centrifuge. The supernatant was collected and transferred to a new 1.5 mL centrifuge tube and mitochondria were pelleted at 10,000 g at 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in another 400 μL of ice-cold mitochondrial isolation buffer. The mitochondria were pelleted a second time at 10,000 g at 4°C for 10 minutes and the supernatant was discarded again. The remaining mitochondrial pellet was resuspended in 200 μL of 10% glycerol-PBS solution and the sample was frozen at -80°C until needed for future use to assess enzymatic activity.

7.2.4 Protein Quantification

To quantify the amount of mitochondrial protein in each of the isolated mitochondria samples, 15 μL of the mitochondrial sample was added to 15 μL 2% CHAPS-TBS solution and the sample was vortexed for 1 minute. The mitochondria were then centrifuged at 10,000 g for 2 minutes and the supernatant was analyzed using the ThermoScientific BCA protein assay kit (cat no: 23227).

7.2.5 Enzymatic Activity Assays for Electron Transport Chain Complexes

7.2.5.1 Complex I: NADH dehydrogenase

The activity of Complex I was assessed in mitochondria isolated from non-irradiated control cells and in mitochondria isolated from bystander cells exposed to electromagntic bystander signals. The protocol used was adapted from that developed by Spinazzi *et al.* [43]. The reaction (reaction 7.1) was initiated by adding Coenzyme Q1 (Ubiquinone 1) to a final concentration of 0.1 mM to the Complex I reaction buffer (50 mM potassium phosphate buffer pH 7.5, 3 mg/mL fatty acid-free BSA, 0.3 mM KCN, 0.1 mM NADH, 30 μg of mitochondrial protein).

$$\mathrm{NADH} + \mathrm{H^+} + \mathrm{UQ1} \xrightarrow{\mathrm{I}} \mathrm{NAD^+} + \mathrm{UQ1\,H_2} \tag{7.1}$$

The assay was conducted in a total volume of 200 μL using a glass-bottom 96-well plate (MatTek) and absorbance measurements were taken at 340 nm, at 37°C, for 2 minutes using a Tecan Infinite 200 Pro plate reader to assess the disappearance of NADH (NADH extinction coefficient: 6.2 $\mathrm{mmol^{-1}\ cm^{-1}}$). In parallel, another well was set up whereby the reaction buffer was treated with Complex I inhibitor (Rotenone) to a final concentration of 10 μM, so that the specific Complex I activity could be determined. Prior to any measurements, the Complex I reaction buffer was incubated at 37°C for 10 minutes to allow the reagents to equilibrate. All chemicals used in enzymatic assays were obtained from Sigma-Aldrich unless otherwise specified. 2-minute baseline measurements were also taken for each well whereby absorbance (340 nm) of the reaction mixture was measured prior to the initiation of the reaction by the addition of Coenzyme Q1. The baselines were subtracted from absorbance

data acquired in the presence of Coenzyme Q1 to eliminate any effects attributed to evaporation of the reaction buffer.

7.2.5.2 Complex II-III: succinate dehydrogenase, ubiquinol cytochrome c oxidoreductase

Complex II and III activity were assessed using the protocol developed by Spinazzi *et al.* [43]. The 200 μL volume of reaction buffer (20 mM potassium phosphate buffer pH 7.5, 300 μM KCN, 10 mM succinate, 20 μg of mitochondrial sample) was incubated at 37°C for 10 minutes to allow for full activation of the enzyme. Following incubation, baseline measurements were recorded for 3 minutes (measurements taken at 10-second intervals) at 550 nm using a Tecan Infinite 200 Pro plate reader. 50 μM of oxidized cytochrome c was then added to the buffer to initiate the reaction (reaction 7.2) and the absorbance at 550 nm was measured for an additional 3 minutes to assess the reduction of cytochrome c (extinction coefficient for reduced cytochrome c: 18.5 $mmol^{-1}$ cm^{-1}). In parallel, another well containing the reaction buffer was treated with 10 mM of the Complex II inhibitor, malonate, in order to assess the specific activity.

$$\text{succinate} + \text{oxidized cytochrome c} \xrightarrow{\text{II}-\text{III}} \text{malate} + \text{reduced cytochrome c} \quad (7.2)$$

7.2.5.3 Complex IV: cytochrome c oxidase

The activity of cytochrome c oxidase (Complex IV) was assessed in mitochondrial samples isolated from non-irradiated control cells and cells exposed to electromagnetic bystander signals emitted from cells directly-irradiated with tritium. The protocol used for the assay was adapted from the protocol developed by Spinazzi et al. [43] whereby 20 μg of mitochondrial sample was added into the reaction buffer containing 50 mM of potassium phosphate (KH_2PO_4) buffer (pH 7.0) and 60 μM reduced cytochrome c. The reaction mixture was incubated at 37°C for 10 minutes. Prior to the addition of the mitochondrial sample, the baseline activity was assessed by measuring the absorbance of the reaction mixture at 550 nm for 3 minutes (10-second intervals). The absorbance at 550 nm was measured for an additional 3 minutes immediately following the addition of the mitochondrial sample to determine to rate of cytochrome c oxidation (extinction coefficient for reduced cytochrome c: 18.5 $mmol^{-1}$ cm^{-1}). Reaction 7.3 was assessed for Complex IV activity.

$$\text{reduced cytochrome c} + \frac{1}{2}\,O_2 + 2\,H^+ + 2\,e^- \xrightarrow{\text{IV}} \text{oxidized cytochrome c} + H_2O \tag{7.3}$$

In parallel, the specific activity of Complex IV was investigated via the addition of 0.3 mM of the Complex IV inhibitor, potassium cyanide (KCN), into the reagent mixture prior to the initiation of the reaction.

Preparation of reduced cytochrome c was accomplished by adding sodium dithionite to a solution of oxidized cytochrome c until the colour of the solution changed from brown to an orange-pink hue. The amount of reduction was checked by measuring the ratio of absorbance of the solution at 550 nm to 565 nm.

7.2.5.4 Complex V: ATP synthase

The activity of ATP synthase (Complex V) was assessed using an assay adapted from a protocol received through personal communication with Dr. James Murphy (IT Sligo, Sligo, Ireland). 190 μL of the reaction mixture containing 50 mM Tris buffer (pH 8.0), 1 mg fatty acid-free BSA, 20 mM $MgCl_2$, 50 mM KCl, 5 μM antimycin A, 10 mM phosphoenol pyruvate (PEP), 2.5 mM MgATP, 4 units LDH, 4 units pyruvate kinase (PK), and 400 μM NADH, was first incubated at 37°C. Baseline absorbance measurements at 340 nm were taken for a 3 minute duration following incubation and thereafter, reaction 7.4 was started by the addition of 20 μg of mitochondrial protein (isolated from non-irradiated control cells or cells which were exposed to electromagnetic bystander signals). The ADP produced from the Complex V-driven reaction then interacted with PEP in the presence of pyruvate kinase (PK) to initiate reaction 7.5 producing ATP and pyruvate. The pyruvate product from the prior reaction then oxidized NADH in the presence of LDH to produce NAD^+ (reaction 7.6). The rate of NADH oxidation (NADH extinction coefficient: 6.2 $mmol^{-1}$ cm^{-1}) was subsequently determined by measuring the absorbance at 340 nm every 10 seconds for a 3 minute duration. To assess the specific activity of Complex V, 2.5 μM of the inhibitor oligomycin A was added into the reagent mixture prior to the addition of mitochondrial protein.

$$\begin{aligned} \text{ATP} &\xrightarrow{\text{V}} \text{ADP} + \text{phosphate} \\ \text{C}_{10}\text{H}_{16}\text{N}_5\text{O}_{13}\text{P}_3 &\xrightarrow{\text{V}} \text{C}_{10}\text{H}_{15}\text{N}_5\text{O}_{10}\text{P}_2 + \text{P} \end{aligned} \tag{7.4}$$

$$\text{ADP} + \text{phosphoenol pyruvate} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate} \tag{7.5}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+ \tag{7.6}$$

7.2.5.5 Citrate Synthase

The activity of citrate synthase, a 51.7 kDa mitochondrial matrix enzyme, was measured and used as a marker representative of overall mitochondrial mass in a sample. We measured citrate synthase activity as a control (similar to the way that actin is used as a loading control in western blots) to ensure that the mitochondrial mass was similar between control and treatment samples. The ability of citrate synthase (CS) to catalyze the reaction between Acetyl CoA (AcCoA) and oxaloacetic acid (reaction 7.7) was assessed by first measuring the baseline absorbance of the reaction buffer (100 mM Tris Triton X-100 buffer pH 8.0, 0.1 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), 0.3 mM Ac CoA, 20 μg of mitochondrial sample) at 37°C and 412 nm for 3 minutes. The reaction was then initiated by adding oxaloacetic acid to the reaction mixture to a final concentration of 0.5 mM. Thereafter, the absorbance at 412 nm was measured for an additional 3 minutes. The extinction coefficient of DTNB is 13.6 mmol^{-1} cm^{-1}.

$$\text{AcCoA} + \text{oxaloacetic acid} + \text{H}_2\text{O} \xrightarrow{\text{CS}} \text{citrate} + \text{CoA}{-}\text{SH} \tag{7.7}$$

7.2.6 Statistical Analysis for Enzymatic Assays

Samples were assessed using three biological replicates which were assessed via three technical repeats to achieve a final sum of 9 data points per experimental permuation. Calculated enzyme activity values were compared using a 2-way analysis of variance (ANOVA).

Quantitative assessment of enzymatic activity was determined using equation 7.8 adapted from Spinazzi and colleagues [43] where the substrates used and their corresponding extinction coefficients are specified in table 7.1. In equation 7.8, v_A defines the rate of consumption of a reactant and v_z defines the rate of formation of a product in units of nmol min^{-1} mg^{-1}, $\frac{\Delta A}{t}$ is the change in absorbance per minute, ε is the extinction coefficient of the substrate specified in table 7.1, V is the volume of the mitochondrial sample in mL, and C is the concentration of mitochondrial protein in the sample in mg mL^{-1}.

Specific activity or the degree of activity that is certainly attributed to complex function was calculated by subtracting the activity of the complex with inhibitor from the activity of the complex without the inhibitor. From this, sensitivity of the assay for a given complex can be determined by taking the ratio of the specific activity and the activity of the complex without the inhibitor.

$$v_A \, or \, v_z = \frac{\frac{\Delta A}{t} \cdot 1000}{\varepsilon \cdot V \cdot C} \tag{7.8}$$

Table 7.1: Substrates Measured in the Investigation of Enzymatic Activity

System Assessed	**Substrate**	**Concentration [μM]**	**Extinction coefficient [mmol^{-1} cm^{-1}]**
Complex I	NADH	100	6.2
Complex II-III	Cytochrome c	50	18.5
Complex IV	Cytochrome c	60	18.5
Complex V	NADH	400	6.2
Citrate Synthase	DTNB	100	13.6

7.2.7 Spectroscopy: Characterizing Spectral Emission from ^{3}H-irradiated Cells

HCT116 p53 +/+ cells were seeded at a density of 2000 cells/cm^2 in a 100x15 mm petri dish and were allowed to incubate for 6 hours to allow for cell attachment to the dish substrate. Following 6 hour incubation, the complete growth medium was discarded and replaced with 5 mL of colourless phosphate buffered saline. 0, 85.7, 171.5, or 857.5 μCi of tritium was then added into the cell culture dish and the lidless

dish was subsequently placed into a wooden light-tight box lined with black paper and electrical tape and covered with a light-protecting fabric. Measurements were started within one minute following placement of the dish into the light-tight box.

Emission of photons covering the UV, visible and IR wavelength range was measured using an Ocean Optics HR4000 spectrometer. The optical resolution ranges from 0.02 to 8.4 nm at full width half maximum (FWHM) and the signal to noise ratio is 300:1. The spectrometer is sensitive to photons between 200 and 1100 nm. A UV-vis 1000 μm fibre optic patchcord (Edmund Optics, cat no: 58458) was connected at one end to the light-sensitive port of the spectrometer via a SMA (SubMiniature version A) connector, while the other end was fed through an aperture in the top of the light-tight box to detect the photon signals emitted by the cells within the box.

Spectrometer read out and acquisition settings were available for visualization and manipulation using Ocean Optics SpectraSuite spectroscopy software. Acquisition of emission spectra was conducted over a 30 minute period where each acquisition lasted 60 seconds (maximum acquisition time possible was 65 seconds for a single acquisition). The intensity value (total number of counts over the 60 second acquisition period) for each wavelength was saved in a tab delimited file for each 60 second acquisition. Following three independent trials whereby 30 one-minute measurements were acquired, the 90 gross count values at each wavelength were averaged to give an average gross count for a given wavelength (μ_s; gross sample count).

To illustrate the spectral biophoton counts visually, the count rates upon exposure to a given activity were integrated over four different wavelength ranges: 200-400 nm (ultraviolet), 400-570 nm (violet, blue, green), 570-700 nm (yellow, orange, red), and 700-1100 nm (infrared). The count rates were then normalized such that they would represent emission over a 200 nm bandwidth and presented in counts per second (cps). The photon counts measured from non-irradiated cells were also subtracted from each of the measurements when radiation were present so that the data presented was a net difference count rate as opposed to a gross count rate. This was done to account for differences in detection efficiency among different wavelength ranges.

7.3 Results

7.3.1 Electron Transport Chain Enzymatic Activity

7.3.1.1 Complex I Activity

The ability of Complex I to oxidize NADH to NAD+ was assessed in mitochondrial isolates extracted from non-irradiated controls cells and bystander cells exposed to electromagnetic bystander signals (figure 7.1). The specific activity of Complex I was assessed by treating the reaction mixture with the Complex I inhibitor, rotenone, such that any NADH oxidation observed that was non-specific to Complex I could be identified. For the experimental permutation containing mitochondria from non-irradiated (control) cells and absent of rotenone, the concentration of NADH decreased over the 2-minute measurement at a rate of 40.80 ± 14.50 nmol min^{-1} mg^{-1} of mitochondrial protein. In contrast, the reaction whereby rotenone was added into the mixture containing mitochondria from control cells demonstrated an enzymatic activity rate of 5.36 ± 1.30 min^{-1} mg^{-1} of mitochondrial protein. The presence of the Complex I inhibitor demonstrates a significant reduction in enzyme activity elicited by the control mitochondria ($p<0.0001$). From analyzing the degree of oxidation of NADH upon Complex I inhibition with that observed in the uninhibited sample, the specific activity of Complex I can be reported as 35.44 ± 13.63 nmol min^{-1} mg^{-1} protein and the sensitivity of Complex I is 81.22 ± 11.76%.

When Complex I in bystander cell-extracted mitochondria was assessed in the absence of rotenone, the enzyme activity was 7.56 ± 2.67 nmol min^{-1} mg^{-1} protein. When enzyme activity was assessed in bystander cell-extracted mitochondria in the presence of the inhibitor rotenone, the enzyme activity was 3.88 ± 1.15 nmol min^{-1} mg^{-1} protein. It is apparent that the function of Complex I has been compromised in mitochondria extracted from the cells which were exposed to the electromagnetic bystander signals to the extent that the enzyme activity exhibited in these samples does not differ significantly from the permutation in which Complex I function was inhibited (bystander with rotenone $p=0.929$; control with rotenone $p=0.983$). Most importantly, the enzyme activity observed in the bystander cell mitochondria (no inhibitor) was significantly weaker than that observed in the control cell mitochondria (no inhibitor) ($p<0.0001$). This result suggests that the bystander signal was effective in eliciting a modification in the activity of Complex I such that it is less efficient

at receiving electrons from NADH. From this observation, it can be deduced that Complex I in the bystander mitochondria are less effective at moving electrons and subsequently pumping protons into the intermembrane space.

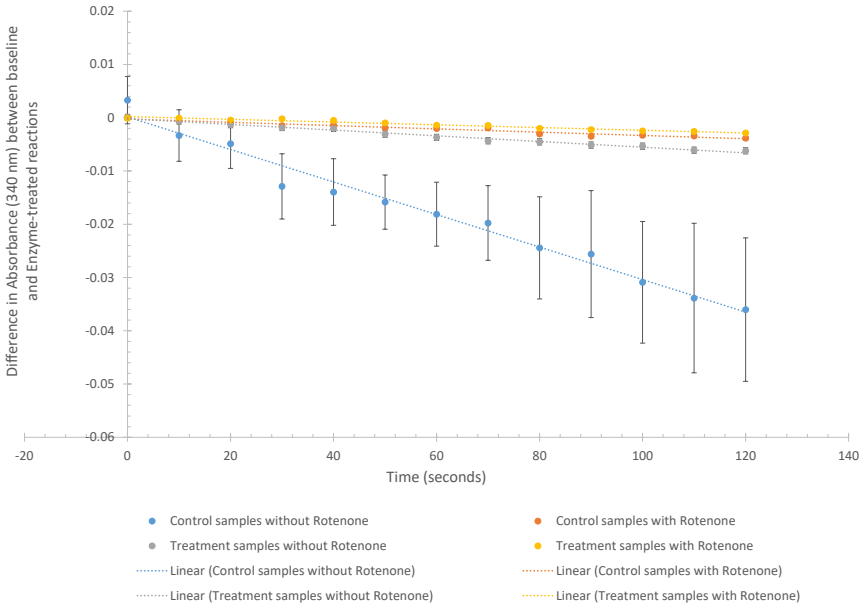

Figure 7.1: Complex I activity. Oxidation of NADH demonstrated by a decrease in absorbance at 340 nm over a 2 minute duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) tested in triplicate (3 technical replicates). Errors bars represent standard error for n=9.

7.3.1.2 Complex II-III Activity

The activity of succinate dehydrogenase and ubiquinol cytochrome c oxidoreductase were assessed in mitochondrial samples that were isolated from non-irradiated control cells and bystander cells exposed to electromagnetic bystander signals (figure 7.2). When non-irradiated control mitochondria were assessed for their Complex II-III activity, it was observed that the oxidized cytochrome c was reduced at a rate of 71.64 $\pm$ 14.64 nmol min^{-1} mg^{-1} mitochondrial protein. In parallel, treatment of the reaction mixture with malonate was used to determine the specific activity of Complex II-III such that any reduction of cytochrome c observed in its presence could be attributed to factors extraneous to Complex II-III themselves. When malonate was added

to the reaction mixture containing non-irradiated control mitochondria, the rate of cytochrome c reduction was 10.16 ± 2.76 nmol min^{-1} mg^{-1} protein. The addition of the competitive inhibitor, malonate, was effective in significantly diminishing the observed enzyme activity (p=0.001). This diminished activity shows that the Specific Activity of Complex II-III in control mitochondria is 61.48 ± 13.23 nmol min^{-1} mg^{-1} and the sensitivity of Complex II-III is 85.48 ± 2.82%. This suggests that approximately 14% of the enzyme activity that was observed is not attributed to Complex II-III.

Enzyme activity was also assessed in mitochondria isolated from electromagnetic-irradiated cells. These mitochondrial samples expressed cytochrome c reduction at a rate of 89.66 ± 31.73 nmol min^{-1} mg^{-1} mitochondrial protein. When comparing the mitochondrial enzyme activity of the biophoton-irradiated samples to the control samples, we find that there is no significant difference among the two populations (p=0.599). That is to say that the exposure of cells to the electromagnetic bystander signal did not alter the enzyme activity of Complexes II and III significantly compared to controls which were not exposed to the bystander signal. For the mitochondria extracted from bystander signal-exposed cells, we also assessed the enzyme activity following incubation with the inhibitor, malonate, in order to confirm that most of the activity observed was indeed attributed to Complex II and III. In the presence of the inhibitor (malonate), cytochrome c was reduced at a rate of 12.83 ± 3.81 nmol min^{-1} mg^{-1} mitochondrial protein. The sensitivity of Complex II and III activity in the bystander signal-exposed samples was therefore 82.46 ± 7.4%.

7.3.1.3 Complex IV Activity

The activity of cytochrome c oxidase was measured in non-irradiated (control) mitochondrial samples and in mitochondrial samples isolated from cells exposed to electromagnetic bystander signals (figure 7.3). The activity found in control mitochondrial samples was 22.75 ± 7.22 nmol min^{-1} mg^{-1}. The activity in control samples was not significantly different from the activity in mitochondrial samples exposed to the bystander signal (20.58 ± 4.50 nmol min^{-1} mg^{-1}, p=0.926). The lack of statistical difference between the complex activity between treatment and control samples suggests that the bystander signal was not effective in altering the ability of Complex IV to oxidize cytochrome c.

When the Complex IV inhibitor, potassium cyanide (KCN), was added into the reaction mixture and it was found that the activity for control samples and treatment

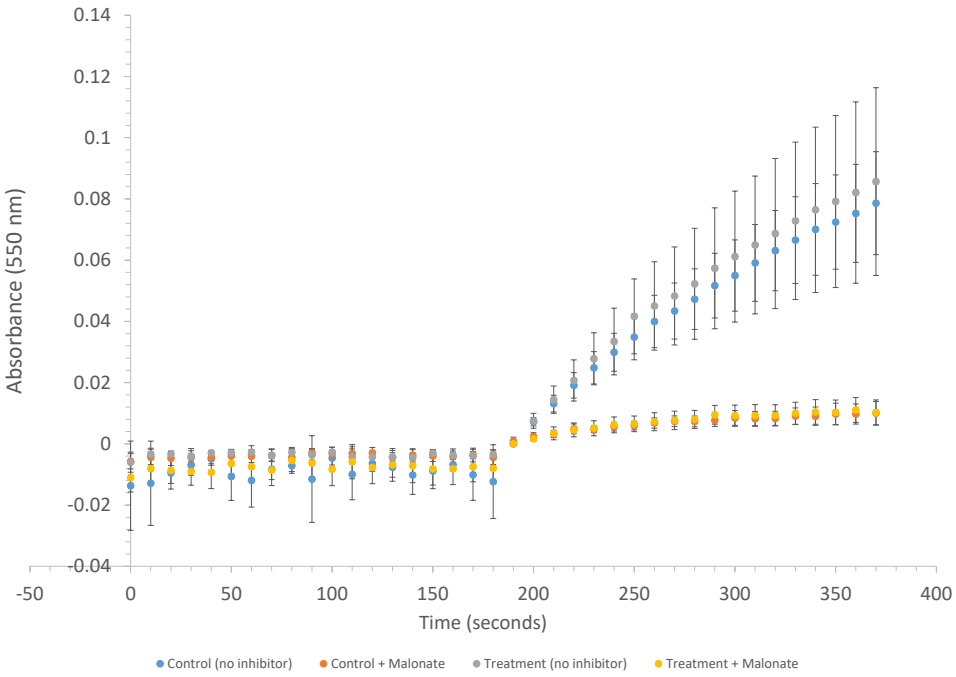

Figure 7.2: Complex II-III activity. Reduction of cytochrome c demonstrated by an increase in absorbance at 550 nm over a 3 minute duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) tested in triplicate (3 technical replicates). Errors bars represent standard error for n=9.

samples were 4.73 ± 0.55 nmol min^{-1} mg^{-1} and 4.84 ± 0.99 nmol min^{-1} mg^{-1}, respectively. From the inhibitor-treated samples it can be concluded that the specific activity of Complex IV in the control samples was 18.01 ± 7.22 nmol min^{-1} mg^{-1} and 15.75 ± 3.76 nmol min^{-1} mg^{-1} in the treatment samples. The sensitivity of Complex IV was therefore found to be 74.05 ± 7.27% for the control samples and 75.90 ± 3.34% for the treatment samples.

7.3.1.4 Complex V Activity

The activity of ATP synthase was determined by measuring the rate of NADH oxidation via tracking absorbance at 340 nm (figure 7.4). When mitochondria from non-irradiated control cells were assayed, the activity of Complex V was found to be 96.70 ± 26.07 nmol min^{-1} mg^{-1} of mitochondrial protein. In contrast, the activity of Complex V that was exhibited by mitochondria isolated from bystander signal-exposed cells was

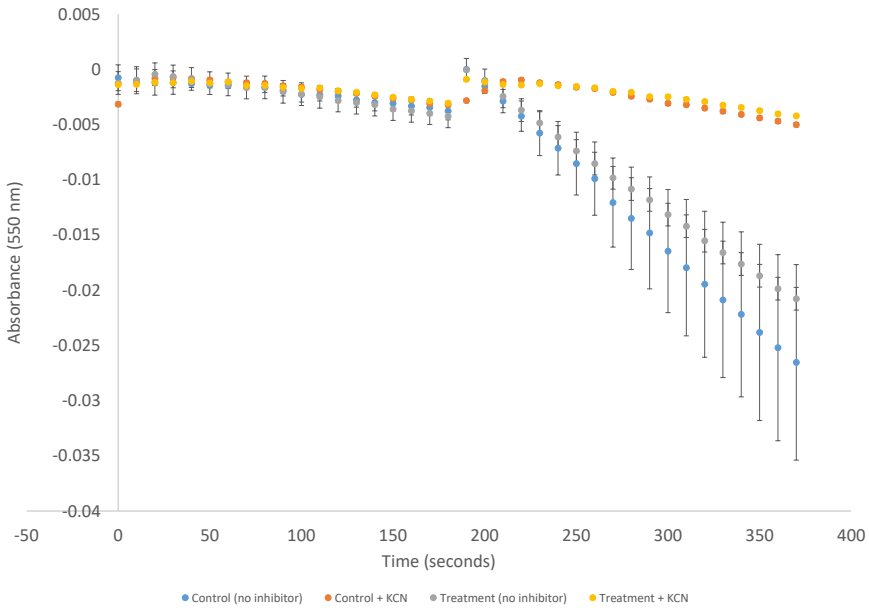

Figure 7.3: Complex IV activity. Oxidation of cytochrome c demonstrated by a decrease in absorbance at 550 nm over a 3 minute duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) tested in triplicate (3 technical replicates). Errors bars represent standard error for n=9.

significantly lower ($p<0.0001$) at 19.03 ± 6.33 nmol min^{-1} mg^{-1}. The activity expressed by the bystander mitochondrial was similar to the activity that was found upon incubation of the reaction mixture with the Complex V inhibitor, Oligomycin A (activity of control samples with inhibitor: 11.85 ± 6.39 nmol min^{-1} mg^{-1}, $p=0.920$; activity of bystander-exposed samples with inhibitor: 8.59 ± 2.00 nmol min^{-1} mg^{-1}, $p=0.792$). With the use of the inhibitor, we can see that the sensitivity of Complex V's activity was 87.20 ± 4.30% and that the rest of the NADH oxidation being observed was background activity that is not attributed to the action of Complex V. The results illustrated here also suggest that the electromagnetic bystander signal is effective in compromising mitochondrial Complex V activity given that the activity observed in the bystander samples was considerably lower than that observed in the control samples. It can be suggested from these observations that the bystander signal could be responsible for compromised ATP and energy production by the mitochondria.

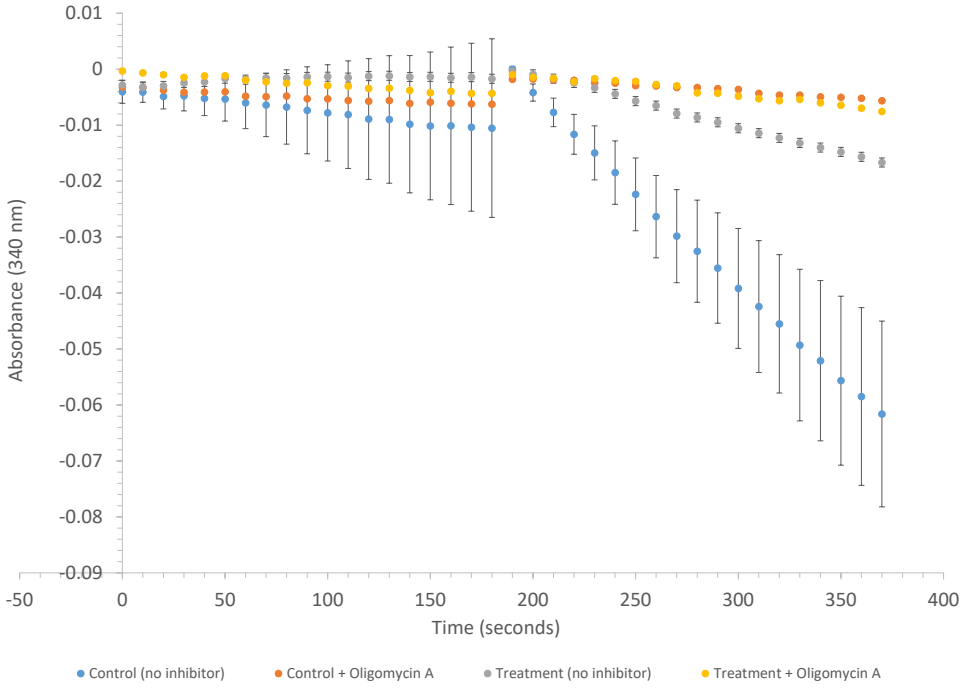

Figure 7.4: Complex V activity. Oxidation of NADH demonstrated by a decrease in absorbance at 340 nm over a 3 minute duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) tested in triplicate (3 technical replicates). Errors bars represent standard error for n=9.

7.3.1.5 Citrate Synthase Activity

The citrate synthase activity found for control samples was 672.74 ± 76.58 nmol min^{-1} mg^{-1} and 682.92 ± 32.04 nmol min^{-1} mg^{-1} for treatment samples. The activity between these samples was not significantly different (p=0.834). Because the citrate synthase activities between control and treatment samples were similar, we can be confident that the two different types of samples did not differ in terms of the mitochondrial content. Thus, any differences in activity observed in Complexes I through V are attributed to modifications induced by the treatment itself, as opposed to differences in mitochondrial quantity. Citrate Synthase activity for control and treatment samples is illustrated in figure 7.5.

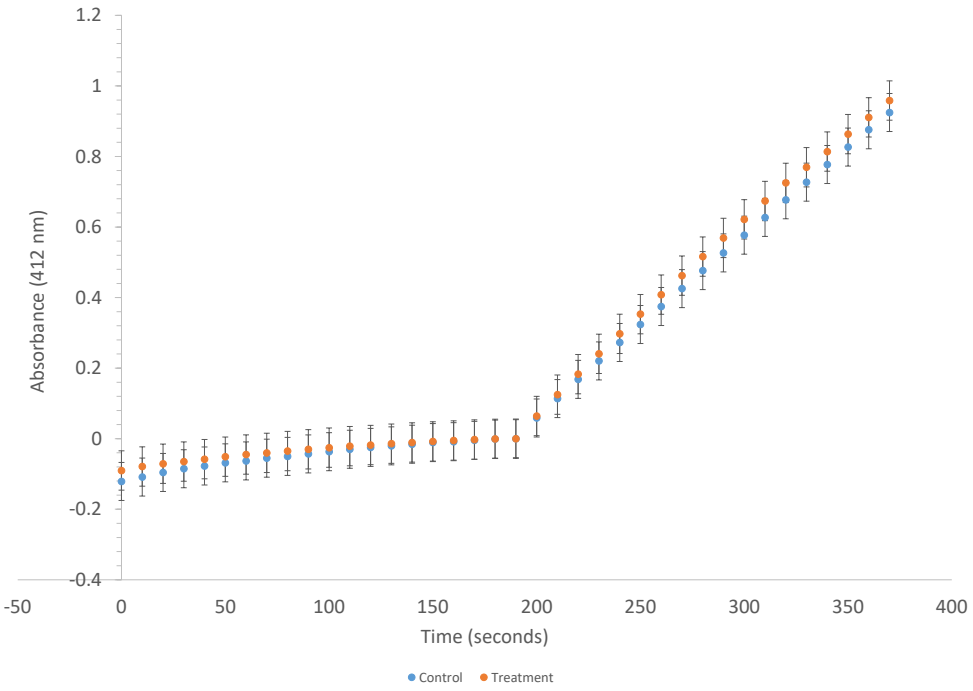

Figure 7.5: Citrate Synthase activity. The formation of CoA-SH demonstrated by an increase in absorbance at 412 nm over a 3 minute duration. Each data point represents data acquired from three different mitochondrial protein samples (biological replicates) tested in triplicate (3 technical replicates). Errors bars represent standard error for n=9.

7.3.2 Spectroscopy

The pattern of photon emission with increasing activity was similar among all four of the wavelength ranges assessed [200-400 nm (UV), 400-570 nm (violet, blue, green), 570-700 nm (yellow, orange, red), and 700-1100 nm (infrared)]. The photon emission across the entire UV-visible-IR wavelength range expresses a positive relationship with radiation activity (figure 7.6). For each of the wavelength domains assessed, the photon emission rate at a given activity was seen to increase significantly compared to the emission rate exhibited by the lower activity assessed. The photon count rate at 85.7 μCi was significantly greater than the count rate at 0 μCi ($p<0.001$), the count rate at 171.5 μCi was significantly greater than that at 85.7 μCi ($p<0.001$), and similarly for the cells exposed to 857.5 and 171.5 μCi, the count rates were significantly different ($p<0.001$). This observation was true for each of the four wavelength ranges investigated. Interestingly, the photon emission rate at 857.5 μCi was not as great as expected based upon previous photon quantification experiments using a single-photon counting system to detect discrete 10 nm wavelength windows in the UV range [30]. The photon emission in the current system appears to exhibit a greater asymptote with increasing activity compared to previous quantification experiments that were performed using different single photon counting instrumentation of a photocathode, PMT, and a $\pm$ 5 nm band pass filter. Nonetheless, the photon count rate still did exhibit increasing intensity with increasing radioactivity, agreeing with our previously reported findings.

7.4 Discussion

The biophoton emission from ^{3}H-irradiated HCT116 p53+/+ cells was measured using a spectrometer sensitive to UV, visible, and infrared photons in an effort to expand the characterization of radiation-induced biophoton emission beyond the isolated measurement of UV wavelengths we had carried out previously [44]. Upon assessing the relationship between ^{3}H activity and biophoton count rate, a positive relationship between ^{3}H activity and photon intensity at all wavelengths from UV to IR was observed. However, the emission observed during irradiation with 857.5 μCi ^{3}H was not as great as expected based upon previous measurements that were taken using a single-photon counting photomultiplier tube and a band-pass interference

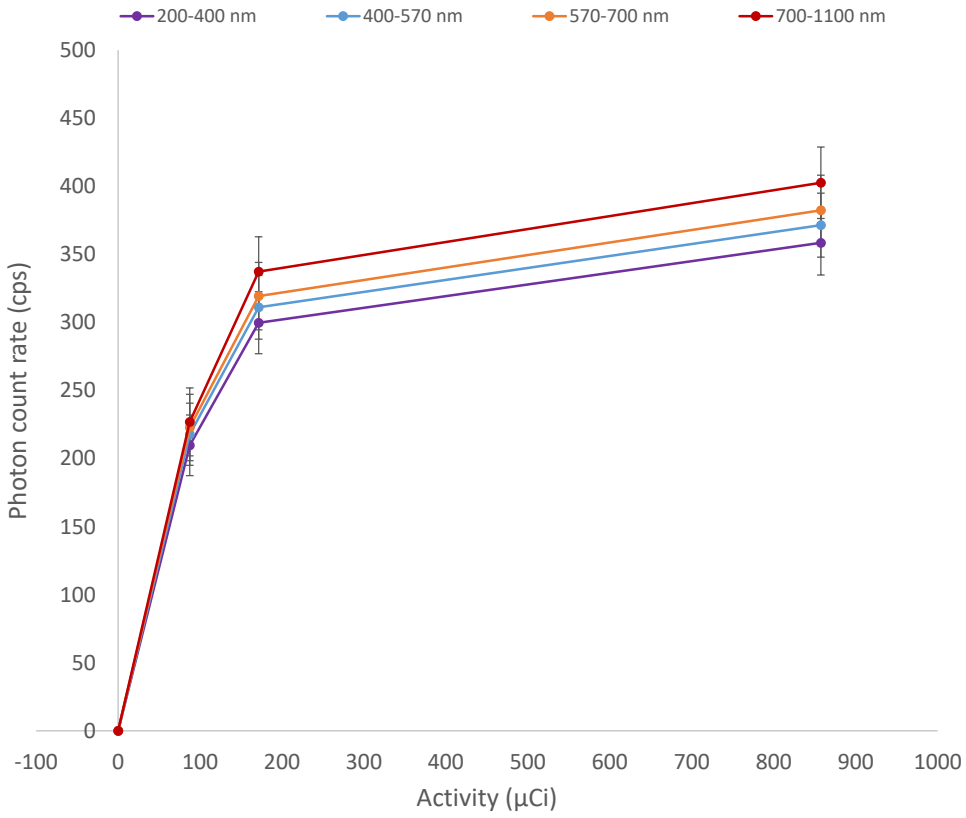

Figure 7.6: Biophoton counts integrated over UV (200-400 nm), visible (violet, blue, green: 400-570 nm; yellow, orange, red: 570-700 nm), and infrared (700-1100 nm) wavelength ranges. Count rates are illustrated for irradiation of HCT116 p53 +/+ cells with 85.7 μCi, 171.5 μCi, and 857.5 μCi ^{3}H. Each data point represents data acquired from three petri dishes measured for thirty 1-minute counts each. Errors bars represent standard deviation for n=3.

filter centered in the UV range [30]. The biophoton emission observed in the current study demonstrated an effect resembling a saturation upon irradiation with 857.5 μCi whereas previous quantification revealed a response that was more resemblant of a linear dose-response relationship. This discrepancy may be due to differences in the photon measurement methodology. Using this spectrometer data we integrated over four sets of 200 nm wavelength ranges, whereas previous measurements were of a very narrow band of $\pm$ 5 nm at 340 nm. However, there may also be uncertainties in the positioning of the fibre optic cable (which is more sensitive to angle than the previous filter and PMT measurement set-up) which are not fully taken into account in the presented uncertainties. For this reason we argue that despite the observation of this saturation effect, the photon emission strength at 857.5 μCi still proved to be significantly greater than that emitted as a result of 171.5 μCi irradiation, and the emission resulting from 171.5 μCi irradiation was greater than that manifesting after 85.7 μCi. Such an observation indicates that the photon emission increases across all wavelengths in the UV, visible and infrared domains with increasing radioactivity. This finding supports the previous use of a 10 nm window of UV biophoton emission as a marker representative of overall biophoton emission from a given population of irradiated cells [9, 30].

The enzymatic activity experiments demonstrated an impairment in the function of Complex I, also known as NADH dehydrogenase or NADH: ubiquinone oxidoreductase, such that NADH exhibited a significantly lower ability to become oxidized into NAD+. It is possible that blue light (450-495 nm) emitted by the ^{3}H-irradiated HCT116 p53 +/+ cells triggered a modification in the biological activity of the electron transfer flavoprotein (ETF) responsible for shuttling the 2 electrons from NADH to the flavin mononucleotide (FMN) [50]. When light induces chromophore isomerization in proteins which posess optical actuators, such as flavoproteins, the protein undergoes a conformational or chemical change resulting in the modification of the protein's activity whereby the modification in activity can manifest as an activation or deactivation [51]. It is suggested that the absorption of biophotons in the blue wavelength range emitted from ^{3}H-irradiated cells is able to induce a modification in ETF such that its ability to accept electrons from NADH is impaired thus leading to a low rate of NADH oxidation into NAD+. This is possible since photons are known to reduce flavoproteins [52]; that being said, the photoreduction of ETF would render it unable to accept electrons from NADH since it already carries an electron

or 2 electrons as a result of the photoreduction. The relative inefficiency in electron acceptance by ETF will lead to fewer electrons being donated to FMN and subsequently, fewer electrons being sent downstream to iron-sulfur clusters (Fe-S) and finally to ubiquinone (Q) to become ubiquinol (QH_2). Ultimately, the diminished ability for electron transport through Complex I will result in an overall lower rate of proton (H^+) pumping into the intermembrane space. In addition to ETF, electron transfer flavoprotein:ubiquinone oxidireductase (ETF-QO) was also initially considered as a candidate to explain the effects observed in Complex I since its activity is also triggered or diminished by blue light wavelengths. However, its role in the observed reduction in activity cannot be confirmed because the endpoint for measuring Complex I activity was the oxidation of NADH. Since ETF-QO participates in the shuttling of electrons through the series of Fe-S clusters to ubiquinone, we did not have a direct means of measuring its role as we did not measure the activity of this component of Complex I.

Complex IV (cytochrome c oxidase) activity in the cells exposed to radiation-induced biophotons was increased slightly above controls, however, the difference was not significant. It is possible that Complex IV was not influenced by the biophotons at all, but it is more likely that the observed effect resulted from the interaction of a few different biophoton wavelengths to produce the net effect observed. It is well document that red and infrared light acts as a photomodulator upon cytochrome c oxidase to ultimately stimulate an increase in ATP production [53–55]. Photons ranging from 600-850 nm are used to stimulate cytochrome c oxidase activity by taking advantage of the absorbance profiles of 4 redox centres (Cu_A, Cu_B, heme a, heme a_3) [54] belonging to cytochrome c oxidase. These redox centres are responsible for transferring electrons from cytochrome c to $^3O_2$ (reducing to water), to drive the proton pumping function of Complex IV. The acceptance of incoming red photons has been shown to accelerate the process of electron transfer by the redox centres [55, 56]. Photons in this range can act to stimulate both Cu_A and Cu_B activity [54] contributing to what would be observed as an increase in Complex IV activity above that exhibited by the control. However, while Complex IV demonstrated an increase in activity in the current study, it was not significantly different from the activity of Complex IV in control cells. It is suggested that the stimulatory effect induced by the incidence of light at red wavelengths must compete with the absorption of blue photons by cytochrome c oxidase. Peak absorption by cytochrome c oxidase occurs between 400

and 440 nm [57, 58] which induces photochemical destruction to heme a_3 (also known as cytochrome a_3) resulting in an impairment of the overall function of cytochrome c oxidase [59, 60]. Considering both the action of activity-stimulating red light and activity-downregulating blue light, it is noted that cytochrome c oxidase absorbs photons from 400-440 nm (blue) much more strongly than it absorbs photons from 500-650 nm (red) (approximately 4 times more absorption of blue light than red) [61]. Therefore, even though the net photon counts observed in the red light range in our spectroscopy experiments were greater than the net photon counts detected at blue wavelengths, the greater red photon fluence may have been outweighed by the relative strength of blue light absorption by cytochrome c oxidase resulting in a net effect that was slightly but not significantly lower in activity than the control.

In the current study, Complex V, or ATP synthase, activity demonstrated significantly diminished activity in cells exposed to biophotons compared to control cells. Such an effect means that overall ATP production by biophoton-exposed cells is compromised. This effect can be explained by a reduction in the strength of the electrochemical gradient across the inner mitochondrial membrane. Since fewer electrons were able to be shuttled from NADH to Ubiquinone in Complex I, the proton pumping function of Complex I did not have as much energy available to carry out its proton pumping function. This results in the presence of fewer H^+ ions in the intermembrane space and consequently a weaker electrochemical gradient. The electrochemical gradient is quite strongly dependent upon the function of Complex I because NADH (involved in Complex I) is better at donating electrons than is $FADH_2$ (involved in Complex II). Because of this, Complex I actually pumps protons across the membrane whereas Complex II does not. Subsequent to this step, the electrons from both Complexes I and II are carried to Complex III by ubiquinol. Subsequently, the electrons from Complex III are carried to Complex IV by cytochrome c. While there may be a relatively lower quantity of electrons being input into Complexes III and IV, their actual functional capacities are not affected by upstream impairments. To elaborate, the rate at which they can oxidize or reduce cytochrome c remains unchanged. The result of the input of fewer electrons into the system is simply a lower overall quantity of oxidized or reduced cytochrome c molecules. In contrast, the function of ATP synthase is mediated by the concentration of H^+ ions in the intermembrane space because H^+ ions act to turn the ATP synthase "turbine" in the process of chemiosmosis. Due to the dependence of

chemiosmosis upon the H^+ ion concentration, ATP synthase function is affected by upstream impairments that result in compromised proton pumping. The impairment of NADH's electron donor efficiency results in a reduction in the intermembrane space's proton concentration by up to 63%. This is because transfer of NADH electrons along the ETC leads to the pumping of approximately 10 protons from the matrix to the intermembrane space, whereas transport of $FADH_2$ electrons along the ETC drives the pumping of only approximately 6 protons [62]). Thus, in the case where the oxidation of NADH is completely inhibited, there will be 63% fewer protons than expected in the intermembrane space available for use in ATP production by ATP synthase.

Compromised ATP synthesis characterizes a state of mitochondrial dysfunction which can lead to impairments in biological function. On a cellular level, when a cell is severely deficient in ATP to the extent where it does not have sufficient energy to sustain processes required for viability, the cell can undergo apoptotic death or necrotic cell death which does not require regulation via the input of energy [63, 64]. On the whole-organism level, mitochondrial dysfunction has been shown to exhibit a strong correlation with the severity of fatigue in humans [65, 66]. In a study by Myhill *et al.*, venous blood samples were taken from patients experiencing fatigue and from healthy volunteers to show that ATP concentration in neutrophils and the efficiency of oxidative phosphorylation was significantly diminished in the participants experiencing fatigue compared to healthy controls [65]. Moreover, the assessment of mitochondrial enzyme activity in fatigue patients with known mitochondrial disorders found reductions in Complex I, III and IV activities compared to controls [67]. This literature in concert with the current study's findings provide support for a possible role of biophoton bystander signalling in the induction of fatigue.

7.5 Conclusions

Biophotons emitted from human cell lines exposed to ionizing radiation possess the capability of modulating the activity of the electron transport chain to ultimately modify the mitochondrial ATP production process. In the particular cell line investigated in the current study (HCT116 p53 +/+), the biophotons emitted as a result of β-irradiation were effective in reducing the activity of Complex I which consequently affected the ability of ATP synthase to produce ATP due to a deficiency of H^+ in the intermembrane

space. The impairment of ATP synthesis by radiation-induced biophoton signalling suggests a possible etiological role for radiation in driving fatigue in whole organisms. The overall magnitude of UV-vis-IR biophoton emission from ^{3}H-irradiated cells proved to escalate with increasing radiation activity. The positive relationship between biophoton signal strength and radioactivity validates the measurement of a small wavelength window (for example, a 10 nm width) as a representative marker of overall photon emission across the UV, visible, and IR spectra. While these results provide evidence to support the ability of biophotons in modulating biological functions, the biophoton spectrum of emission is very complex and is likely not isolated to eliciting effects upon a single biological system. Further investigation will be required to further elucidate the effects that biophotons can elicit upon bystander populations.

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Chapter 8

Discussion and Conclusions

The research conducted in this thesis sought to explore the phenomenon of biophoton emission upon exposure to ionizing β-radiation and further, to investigate the possibility of a role for biophotons in the RIBE. This chapter will include discussions on major findings of the current research, the relevance of these findings, limitations of the work, and recommendations for future investigations.

8.1 Biophoton emission is governed by both physical and biological factors

There is substantial research in the field of biophotonics evidencing the emission of weak photon fluences from cells and organisms [1–4]. Biophoton emission has been observed both spontaneously [1] and following perturbation by various stressors [2–4]. However, until recently, biophoton emission following exposure to ionizing radiation had not yet been explored. Our colleague, Dr. Bilal Ahmad was the first to show that ionizing radiation could induce significant UV photon emission from biological materials and cells, although his investigations were restricted only to dead cells due to administrative/regulatory constraints [5, 6]. These observations motivated a follow-up examination of his findings in an effort to further elucidate the characteristics and nuances of biophoton emission triggered by ionizing radiation.

The work presented in chapter 3 demonstrates the ability for ionizing radiation exposure (^{90}Y β-radiation) to stimulate marked UV biophoton emission in living cells. Furthermore, radiation activity, cell density of the directly-irradiated culture,

and cell viability were revealed to play roles in modulating the biophoton emission strength elicited by irradiation. Further characterization of the biophoton signal was investigated in chapter 5 where irradiation with ^{3}H and subsequent measurement of UV biophoton emission from cell lines expressing a range of p53 functionality, elucidated a modulation of photon emission intensity in cells expressing mutated p53. Finally, biophoton quantification encompassing UV, visible, and IR wavelengths from 200 to 1100 nm upon ^{3}H-irradiation was assessed in experiments presented in chapter 7. These measurements revealed that the photon emission strength upon exposure to a given activity of radiation was similar across the entire wavelength spectrum. Accordingly, biophoton emission magnitude increased at all wavelengths with increasing radiation activity. This result validates the acceptability of employing the measurement of a narrow wavelength band as a marker representative of biophoton emission strength across a broader range of the electromagnetic spectrum. The observation that biophoton emission increases with increasing radioactivity also suggests that the generation of biophotons upon exposure to ionizing radiation is logically attributed to the input of energy into molecules leading to excitation and subsequent radiative emission. In this respect, the origin of biophotons is explained purely by the classical theory of physical energy transitions. Interestingly, our assessment of biophoton emission from β-irradiated dead cells and living cells challenged the idea that biophotons strictly originate from simple physical transitions following the input of energy by radiation.

The very first endeavour of this project was to clarify the influence of cell viability upon the ability for radiation to stimulate biophoton emission. The results following exposure of both dead and living HaCaT human keratinocyte cells to β-radiation demonstrated greater photon emission from dead cells compared to living cells up to an applied activity of approximately 200 μCi ^{90}Y. However, following irradiation with activities greater than this from 200 to 700 μCi ^{90}Y, the photon signals detected from the dead and living cells proved to be comparable. While differences were not noted at higher activities (and thus doses), the discrepancy between signal magnitude at lower doses strongly suggests a contribution by metabolic processes to the modulation of the overall biophoton signal observed from living cells. It is conceivable that biophoton emission can be observed following irradiation of both living and dead cells since the production of excited species and subsequent photon-generating de-excitation can proceed in living and dead cells following initiation of radical chain reactions by irradiation. It is only living cells, however, that possess the ability to elicit antioxidant

defences to quench the reactive and excited species responsible for fluorescence and phosphorescence resultant to transitions from excited states to ground states. The lower photon fluence exhibited by living cells at lower radiation activities is suggested to be a product of the quenching of excited species, whereas the expression of greater photon emission at higher doses by living cells can be attributed to greater accumulation of ROS leading to a diminished ability for the antioxidant defences to suppress ROS-driven photon emission [7]. Overall, these results strongly suggest an influence of cellular metabolism in the mediation of biophoton signal strength.

While we have reported the detection of biophoton emission upon irradiation of dead cells, research conducted in existing biophoton literature presents contrary reports citing the inability to detect photon emission from dead cells [8, 9]. One critical difference between these experiments and those presented in the current thesis manifests as the absence and presence of an external stimulus, respectively. It is logical to expect a lack of photon emission from dead cells in an unperturbed system because there are no intracellular processes driving energy exchange within the cell, nor is there any input of energy from an exogenous source to induce excitation of molecules present within the system. In contrast, we would expect some degree of biophoton emission from an unperturbed living biological system or cell because living systems undergo oxidative metabolism involving redox reactions which contribute to an inherent production of ROS and excited species in the absence of an external stimulant. This assumption is validated by the observation of low-level photon emission upon quantification in a population of post-irradiation live cells described in chapter 3. Even in the absence of energy input by ionizing β-radiation, photon emission that was significantly greater than background levels, persisted in these living cells for at least 90 minutes post-irradiation. Despite that these unperturbed emissions were expressed at a magnitude approximately 10^4 times lower than those observed during irradiation, they are evidence that energy exchange is occurring within the cells to maintain some level of photon production. We had previously attempted to explain this observation by ascribing to the fact that phosphorescence produced as a result of intersystem crossing can occur following excitation, thereby resulting in the observation of latent photon emission at some time following the initial excitation event or irradiation. While this is certainly possible and we don't doubt that some of the post-irradiation emission is contributed by transitions of this nature, it is also suggested that a dynamic interaction between ongoing intracellular metabolism and antioxidant activities are

also responsible for the signal that is observed in a cell population that is not being subjected to stress during the time of photon quantification.

Taking the observations from this thesis into consideration, the net biophoton signal can be attributed to a complex interaction between two factors: 1) energy input into biological molecules by exogenous sources to induce excitation of biological molecules and 2) biologcal processes that inherently involve energy exchange to result in either the excitation of intracellular species or the deactivation of those excited states. The mechanism becomes complex when considering that exogenous energy is capable of inducing purely physical transitions of molecules to an excited state thereafter leading to radiative transitions, or it can act indirectly to modulate cellular subunits and induce modifications to oxidative metabolism. These functional modifications can in turn result in the production of electronically excited states via redox reactions and thereby lead to another means by which photon emission can be elicited. These contributions can also then be challenged by the opposing actions of the antioxidant system. Overall, the results conferred in this thesis strongly support the interaction of both physical and biological processes in governing the emission of biophotons as a result of exposure to ionizing radiation. The involvement of a metabolic component in biophoton generation is particularly promising since it raises the possibility of biophoton detection as a method by which we can identify the extent of oxidative processes in a cell population caused by ionizing radiation. This is clnically relevant as it provides a possible technique for non-invasively estimating the localized and distant effects of targeted irradiation in the body. This practice is currently employed in the field of biophotonic research and has proven effective in identifying pathologies characterized by non-homeostatic oxidative levels such as rheumatoid arthritis in mice [10], oxidative stress in the rat brain [11], and tumour growth [12]. It is hoped that further elucidation of cellular metabolism's influence upon radiation-induced biophoton emission may lead to an analogous use of this technique for radiobiological applications.

The research conducted in the current thesis is restricted to the characterization of biophoton emission only as a result of irradiation with low-LET β-particles. However, it will be important to determine whether biophoton emission is also inducible following exposure with other types of ionizing radiation such as x- or γ-rays and ionizing radiations that possess high-LET, such as α-particles, that are more likely to elicit direct actions upon their biological targets as opposed to generating reactive species

via indirect pathways as low-LET radiation does. When the use of an alternative source of radiation was considered for the current thesis, the obstacle faced was the difficulty that was encountered in finding a suitable primary radiation source that could be employed concurrent to photon quantification. ^{3}H was selected for our experiments because it was a pure β-emitter with no associated γ-ray emissions during its decay to a stable isotope. Furthermore, it was a portable source that could be placed easily within the light-tight apparatus containing the PMT detector. When we considered other electromagnetic radiation types, there were geometric obstacles associated with concurrent measurement of biophoton emission because x-ray tubes and high activity γ sources were fixed to shielding and containment set-ups due to the inherent design of the system or security concerns, respectively. In this case, the physical constraint would pose the inability to irradiate the cells and quantify photon emission simultaneously. In the consideration of α sources, the physical size was not a concern since an open or portable source could be used. But the challenge for concurrent irradiation and quantification in this case, manifested as the potential for interference by other electromagnetic radiations in the effort to quantify only the biophotons. Most of the radioisotopes decaying by α-emission are either associated with γ emission or at least one of their daughter products are [13]. Therefore, the prospect of discriminating between a true biophoton signal and a false positive γ-ray when employing an α-emitting radioisotope was considered fairly poor.

Because interference by other electromagnetic radiations is unavoidable and physical constraints do not allow for simultaneous irradiation and quantification in a light-tight environment, it is proposed that quantification of biophoton emission in α, γ or x-irradiated cells be tested post-irradiation. We have demonstrated the detection of low-level, yet significant, biophoton emission from β-irradiated cells and suggest that these post-irradiation emissions are attributed to both phosphorescence and ongoing oxidative reactions occurring as a result of cellular metabolism. The biophoton signal strength, if any, is expected to be quite low. Therefore the experimenter should be careful to ensure that the containment apparatus is light-tight so as to keep the background noise to a minimum. If biophoton emission can be observed post-irradiation from cells exposed to other types of ionizing radiation alternative to β, this will provide further support for the hypothesized production of biophoton emission as a metabolic process and not only as a result of transitions in the excitation-deexcitation framework of classical physics. This would have significant implications for the use of biophoton

detection as a means of characterizing and identifying oxidative activity within a cell population and possibly the human body.

8.2 Biophotons are effective in communicating bystander signals to non-irradiated cells

The second line of inquiry posed by this thesis investigated the possibility for radiation-induced biophotons to elicit effects in non-irradiated bystander cell populations. The research conducted towards answering this question confirms the ability for radiation-induced biophotons to elicit reductions in cell survival (chapters 4 & 5), the release of soluble factors (chapter 6), mitochondrial membrane depolarization (chapter 6), and modifications in ETC activity (chapter 7) in bystander cells. This work also revealed a role for p53 status of the recipient bystander cells in modulating the response to the biophoton signals. This was particularly interesting since p53 function did not appear to influence the ability of the directly-irradiated cells to produce the signal, thereby suggesting that p53 status plays a pivotal role in bystander response kinetics. While intercellular communication driven by biophotons is not a novel concept [14–23], this is the first incidence of its demonstration following ionizing radiation exposure and thus the first known report of its involvement in the RIBE.

We sought to identify the biophoton wavelength(s) responsible for eliciting the observed bystander effects by employing both physical and chemical interventions in the investigation of biophoton communication between directly-irradiated and bystander cell populations. The application of a UV-absorbing polyethylene terephthalate filter between the directly-irradiated and bystander cell cultures was effective in almost completely abolishing the bystander response expressed by recipient cells. Moreover, incubation of the bystander cells with lomefloxacin, a photosensitizer effective particularly at UV-A wavelengths, and melanin, a photoprotector, resulted in upregulation and downregulation of the bystander response, respectively. These results together with the observation that the bystander response was strongly correlated with the biophoton signal strength measured at 340 ± 5 nm led to an initial suggestion that the wavelengths responsible for eliciting the bystander effect belonged to the UV range. This perspective changed upon further characterization of photon emission across the UV, visible and IR spectra which led to the observation that the magnitude of

photon emission increased similarly with radioactivity for all wavelengths within the characterized spectrum. The observation that activity could modulate the photon emission signal in a similar manner across the whole spectrum leads us to believe that the biophoton bystander effect is most likely mediated by more than one photon frequency. Following this hypothesis, our filter and sensitizer results may be explained by multiple wavelengths acting coherently upon the recipient cells in order to elicit an observable effect. In this respect, interference of even a portion of the responsible spectrum could result in a lack of response or at least a modulation in expression. This hypothesis aligns with results conferred by Fels in his study of optically-coupled paramecium using materials which were effective in transmitting either UV-vis or only visible light [21]. Differences were observed in the division rate of paramecia exposed to UV & visible light together compared to those exposed to visible light alone. Together with this supporting observation from the literature, the results conferred in the current thesis suggest that cells use two or more wavelengths in order to communicate information intercellularly.

While a role for multiple frequencies in driving the bystander response is suggested based upon the current research, the work conducted in this thesis did not have the opportunity to sufficiently explore this idea to the extent where detailed conclusions could be drawn. Currently, the critical wavelengths responsible for eliciting bystander effects in recipient cells have not yet been identified. A proposal for future work is therefore the characterization of the biophoton wavelengths that are most susceptible to absorption by the bystander cell population. Identification of these frequencies will help to determine the important wavelengths involved in triggering biological processes and effects in bystander cells. In a review of biophoton communication by Laager, he suggested that chromophores governing separate biochemical pathways may be responsible for receiving biophoton signals and that only the initiation of multiple pathways will lead to the expression of an observable effect in recipient cells [24]. This suggested model can be considered comparable to a biological coincidence detector; that is, by coupling two or more detectors (in this case, chromophores within the cell), the cell has a greater capability of differentiating between ambient noise and the actual biophoton signal that contains information. Considering that biophoton communication has been demonstrated to persist under ambient light conditions (i.e. experiments not performed in light-tight apparatuses) [22], the hypothesis proposed by Laager

is certainly a possible method to explain how biophotons may effectively transmit information in the complex and chaotic intercellular environment. In order to test the influence of different photon wavelengths upon the bystander effect, it is recommended that the use of different container materials which permit the transmission of different wavelengths be employed. These investigations could extend to the use of quartz, glass, and plastic vials or dishes. Alternatively, filters which can be placed between cell cultures could be used to selectively attenuate specific bands of wavelengths across the UV and visible spectrum.

As it pertains to the experimental set-up for assessing optical intercellular communication, a limitation of the current studies is that the assessment of biophoton communication was only tested for one fixed distance between the directly-irradiated and bystander cell populations (15 mm). This has been realized as a possible limitation following the observation that Rossi and colleagues found an influence of different distances upon the magnitude of response exhibited by the bystander cells assessed in their study [23]. In mouse fibroblast populations, cell viability proved to exhibit a greater reduction when the populations were separated by a 4 mm distance when compared to an 11 mm distance. However, in human endothelial cells, it was apparent that cell viability in the bystander cells was diminished to a greater extent when placed at 11 mm from the biophoton-emitting cell culture as opposed to 4 mm [23]. It is apparent that different cell types may respond to given experimental conditions in different ways, therefore a need for further investigation is evident. Optimization of the optical communication distance is proposed via the use of quartz vials to maximize signal transmission and to allow the experimenter to place the two cultures in close proximity to each other without the restriction posed by petri dish height. This experimental methodology was not initially tested because the author was concerned about how leaving adherent cells in suspension for relatively prolonged periods of time would affect the health of the cells. The hesitation to employ this method was attributed to a concern over introducing confounding factors that could affect both the signal generated by the directly-irradiated cells and the response expressed by the non-irradiated bystander cells. However, it has recently come to the author's attention that the use of microcarrier beads can be considered to address this concern. Microcarrier beads are 10 μm to 5 mm spheres upon which adherent cells may grow as monolayers [25]. Their use could facilitate the culture of adherent cells in a system resembling a suspension culture, without the concern of compromised cellular function.

Although additional work is required to further clarify the means by which biophotons communicate effects to bystander cells, the results conferred in the current studies point to the ability for radiation-induced biophotons to elicit responses in recipient cells. This elucidates an additional mechanism by which RIBEs can be communicated and thus infers that cells are not required to be in direct contact nor share biological fluids with each other to send and receive signals triggering effects resembling those of their directly-irradiated neighbours. As it pertains to the current bystander literature, the results from this thesis provide an alternative means by which to explain the inter-animal effects observed in non-irradiated neighbours of irradiated fish [26], mice [27] and rats [28]. This also has clinical implications when we consider individuals who have undergone diagnostic PET procedures and have still retained radioactive tracers within their systems after completion of the exam, or occupational radiation workers who have internalized tritium into their bodies. This raises an awareness that communication of radiation effects could be occurring between the irradiated individual and caregivers or family members who may be in close contact.

8.3 Intercellular communication via biophoton signalling and soluble factor exchange are interrelated

The finding that biophoton communication is involved in RIBE signalling prompted a motivation to investigate its relation, if any, to RIBE signalling mediating by soluble factor exchange. The main purpose of this endeavour was to determine whether the biophoton-mediated bystander effect was a completely independent and alternative means by which bystander effects could be communicated or whether it was somehow related to soluble factor bystander communication. The studies described in chapter 6 worked towards elucidating either a possible relationship or a mutual exclusivity between biophoton and soluble factor bystander signalling by exposing bystander cells to the radiation-induced biophoton signal, extracting exosomes from those biophoton-exposed cells, and subsequently placing the exosomes onto secondary reporter cells in which various endpoints were assessed. The data from these experiments demonstrated an ability for radiation-induced biophotons to result in the release of exosomes which

are capable of inducing reductions in clonogenic survival and mitochondrial membrane depolarization in downstream non-irradiated reporter cells. These results reveal that biophoton signalling produced by β-irradiated cells is associated with modifying the soluble factor bystander signal. Thus it can be suggested that these two forms of bystander signalling are not mutually exclusive.

The results presented in this thesis suggest that biophoton signalling is not a completely alternative mechanism for intercellular communication that adds to the overall magnitude of the bystander effect. Rather, it appears to be a physical signal which can trigger release of soluble factors which thereafter elicit the downstream bystander effects that are observed as a result of soluble factor signalling. In this respect, intercellular communication via biophoton signalling appears to serve as a form of biological redundancy, acting to ensure that bystander effects are communicated, even in some cases where exchange of biological fluids or cell-to-cell communication are not available. Functional redundancy is present in many biological systems and has proven to be a ubiquitous characteristic in biology to ensure that every effort is made to carry out essential functions in the face of biological modifications leading to aberrations in signalling pathways. DNA damage repair is an example of one system that shows the highly adaptive nature of biological systems. Cell cycle arrest to facilitate effective DNA repair can be achieved through any of the three following pathways: p21 inactivation of cyclin E-Cdk2, Cdc25A or Cdc25C phosphatase dephosphorylation of cyclin E-Cdk2 [29]. Furthermore, the mutiplicity of the pathways available to achieve DNA repair is also exemplified by the capacity for all of ATM, ATR, DN-PKcs and Chk2 to phosphorylate the same set of enzymes [29]. Biological redundancy, the case where two or more entities can carry out analogous functions, is thought to be the cell's way of ensuring contingencies are in place for situations where the machinery responsible for carrying out important functions have failed or are not available [30].

As it pertains to the RIBE, the results presented in chapter 6 provide evidence to suggest that bystander communication is particularly robust. The resilience of bystander signalling leads the author to suggest that the expression of radiation-induced bystander effects must be important, otherwise, biology would not express multiple pathways for its induction. By proxy, this raises the widely discussed topic of whether bystander effects are beneficial or detrimental [31]. Cell survival is an endpoint that is often assessed in the investigation of RIBEs whereby bystander signalling often results in the induction of diminished cell survival. While a prima facie analysis of

this response could lead to the perception that the RIBE is harmful, it can in fact be interpreted as a protective effect which acts to eliminate mutated and aberrantly-functioning cells from the population [31]. This perspective aligns with the expression of redundancy in RIBE signalling to suggest that bystander effects are beneficial to the cell population, tissue, or organism as a whole. The presence of built-in redundancies would otherwise not be sensible if RIBEs were intended to be harmful.

8.4 Mitochondria are involved in the response of bystander cells to biophoton signalling

The work presented in chapters 6 and 7 confirm the involvement of mitochondria in the bystander response to radiation-induced biophoton signalling. In chapter 6, experiments assessing mitochondrial membrane potential in a set of secondary non-irradiated reporter cells treated with biophoton-induced exosomes confirmed the presence of an indirect means by which biophoton signalling could elicit mitochondrial membrane depolarization. The ability for biophotons to induce mitochondrial modulation provided a rationale for further investigation. In chapter 7, the activities of the mitochondrial ETC complexes were subsequently investigated in response to β-radiation-induced biophoton signals. Since ETC function is governed by redox reactions [32], we hypothesized that energy input by biophotons could be effective in driving or inhibiting the progression of redox reactions. Biophoton signals emitted from β-irradiated cells significantly reduced the activity of Complex I and ATP synthase, while it was not effective in modulating the activity of Complexes II-III, and IV.

The reduction in ATP synthase activity is logically attributed to the biophoton-induced modification in Complex I activity since a compromised ability for Complex I to efficiently pump H^+ ions across the inner mitochondrial membrane will lead to the production of a weak electrochemical gradient and subsequently inefficient chemiosmosis [33]. Moreover, it is not surprising that mitochondria demonstrate a role in mediating the bystander response triggered by biophotons since they have already shown an involvement in the response to soluble factor bystander signals [34–36]. The reduction in ATP synthesis capacity observed in biophoton-exposed cells provides a possible means by which to explain the reduction in cell survival detected in the same biophoton-exposed bystander cells (data presented in chapters 4 and 5). Under

conditions of ATP deficiency, cells can undergo cell death due to the inability to initiate processes required to sustain cell proliferation [37]. These results therefore suggest a possible mechanism by which significant cell death may be expressed in biophoton-exposed bystander cells.

Clinically, the observation that mitochondrial ATP production is deficient in biophoton-exposed cells may theorize a link between ionizing radiation exposure and pathologies that are characterized by ATP-deficiency. Although this suggestion is speculation at the present time, the current research provides a preliminary indication that biophoton bystander signalling may play a role in contributing to radiation-induced fatigue. The identification of assuaged ATP synthesis by radiation-induced bystander signals provides a possible mechanism for ionizing radiation in inducing pathological conditions related to *systemic* expression of energy deficiency [38, 39]. The proposed hypothesis may be possible because abscopal effects are simply bystander effects that are expressed *in vivo*, therefore the dissemination of signals throughout the body via bystander signalling can certainly lead to the expression of bystander responses in tissues and cells which are distant from the original site of direct irradiation [40]. Currently, there is anecdotal evidence to support the possibility of a relationship between low doses of ionizing radiation and the *in vivo* expression of fatigue based upon the observation of symptoms in atomic war veterans [41–43]. The ability for low doses of ionizing radiation to induce significant cognitive and fatigue-related effects were not recognized until more recently, thus the cohorts of war veterans exposed to low doses of radiation were not studied thoroughly immediately following the exposure events [44]. Presently, the age of the individuals in the affected cohorts are too great for systematic studies to be conducted upon them [44]. Evidence for a link between low dose ionizing radiation and the expression of chronic fatigue therefore motivates investigations to determine whether radiation exposure could lead to functional modifications attributed to the expression of fatigue. Follow-up studies are recommended whereby quantification of intracellular ATP levels in bystander cells is carried out to determine whether the induction of fatigue attributed to ATP-deficiency may even be plausible. Intracellular ATP quantification is an important next step because studies have shown that the initiation of alternative ATP production mechanisms are enacted when mitochondrial ATP generation is compromised [45]. Compensatory ATP production by other systems may therefore negate the radiation-induced fatigue hypothesis suggested here.

8.5 Conclusion

The current thesis presents novel work supporting a relationship between biophoton communication and radiation-induced bystander effects. While intercellular communication via the exchange of information by biophotons has previously been exhibited in the scientific literature, this is the first investigation, to our knowledge, that demonstrates intercellular communication via biophotons induced by ionizing radiation.

One of the contributions of this work to the current knowledge in the field is the elucidation of the characteristics of biophotonic emission. Because significant biophoton fluences were differentially expressed in dead and viable cells following identical irradiation conditions, the signal is thought to be affected by an intracellular metabolic process. Moreover, the positive relationship between photon emission strength and radiation activity (and consequently dose), leads to a strong belief that the production of biophotons occurs as a result of de-excitation transitions by excited state species generated by direct energy input by the ionizing radiation, itself, or during radiation-induced oxidative stress. To this end, the measurement of biophoton emission may be a useful and non-invasive tool to identify oxidative stress in an *in vitro* system or in the body.

This work also elucidates the capability for radiation-induced biophotons to induce responses in non-irradiated bystander cells. This mechanism shows that it is possible for irradiated cells to exchange information with its neighbours without the requirement for cell-to-cell communication or exchange of biological fluids therefore providing an alternative explanation for the observation of effects resembling irradiation in non-irradiated animals co-habitating with irradiated animals. Consequently, this also raises concern for the possibility that radiation effects can be communicated from person to person in cases where individuals are exposed to and retain radioactivity within their bodies following medical, occupational or environmental exposure to ionizing radiation.

This thesis has also elucidated a relationship between biophotons and soluble factor exchange in mediating the bystander effect. The finding that biophoton signals can lead to the release of soluble factors effective in eliciting bystander responses indicates that these two bystander mechanisms do not act in a mutually exclusive manner. Rather, it is much more likely that the biophoton signal is an intermediate step leading to the release of response-eliciting soluble factors such that it acts as a measure of

redundancy to ensure that bystander responses are communicated to non-irradiated populations. These results are a prime example of the robust nature of biological systems when it pertains to executing important functions.

A final contribution to the current knowledge is the finding that deficient ATP synthesis by the mitochondrion manifests in non-irradiated cell populations following the receipt of bystander signals. These results elucidate an important role for mitochondria in the mediation of a response to bystander biophoton signalling. In terms of clinical relevance, downregulatory modification of ATP synthesis processes in the mitochondria could lead to pathological conditions in the whole organism that are attributed to intracellular ATP deficiencies. Further investigation will be important in determining whether this hypothesis will lead to evidence providing reasonable support for a link between ionizing radiation exposure and pathologies characterized by fatigue.

Collectively, the studies presented in this thesis provide insight into an additional mechanism by which the radiation-induced bystander effect may be communicated. While it has shed some new light onto the biological processes that occur following exposure to ionizing radiation, it also identifies a link between radiation research and the phenomenon of intercellular information exchange via electromagnetic (light) signalling, formally referred to as the field of biophotonic research. Both RIBE and biophotonic fields of study have co-existed and grown substantially as independent entities for numerous years. It has now become apparent that there may be a convergence between these two streams of research that warrants further investigation. The established use of biophoton emission to identify oxidative stress by biophotonics researchers may similarly be used to detect the extent and localization of oxidative damage induced by radiation. Moreover, further elucidation of the photon wavelengths involved in the response of cells to biophotons may provide insight into which cellular pathways are triggered in bystander cells based upon the knowledge on optically-triggered biological processes that is already very well established in the field of biomedical optics. Overall, the potential for developing the applications proposed in this chapter provides substantial motivation for continued exploration of the role for biophotons in the field of radiation biology.

8.6 References

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Appendix A

Supplementary Information

A.1 Supplementary Figures and Tables

A.1.1 Chapter 4

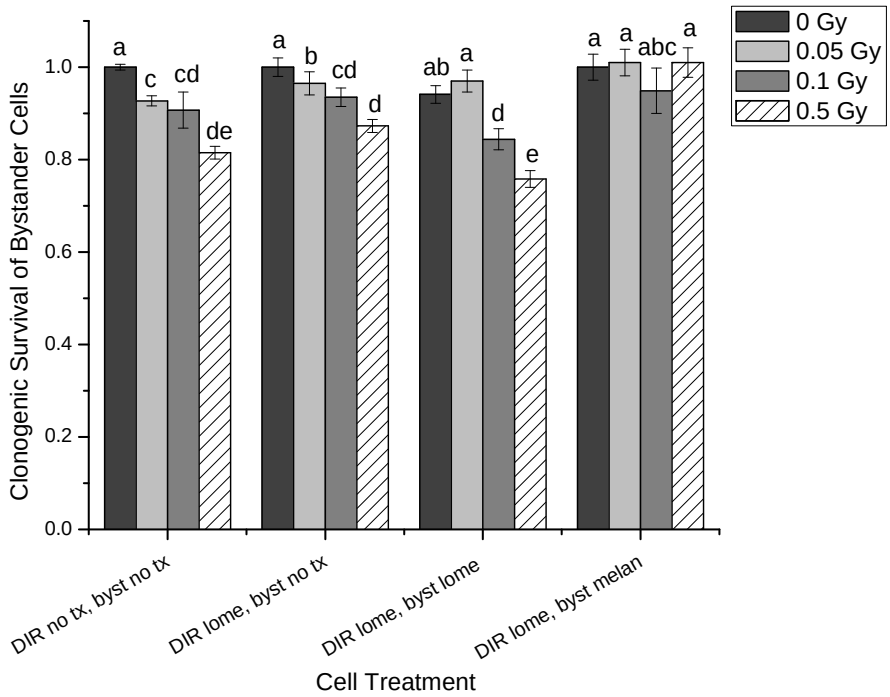

Figure A.1: (Supplementary Figure S1) Clonogenic survival of HaCaT reporter cells treated with no drug, lomefloxacin, or melanin while receiving bystander signals from directly-irradiated HaCaT cells treated with lomefloxacin.

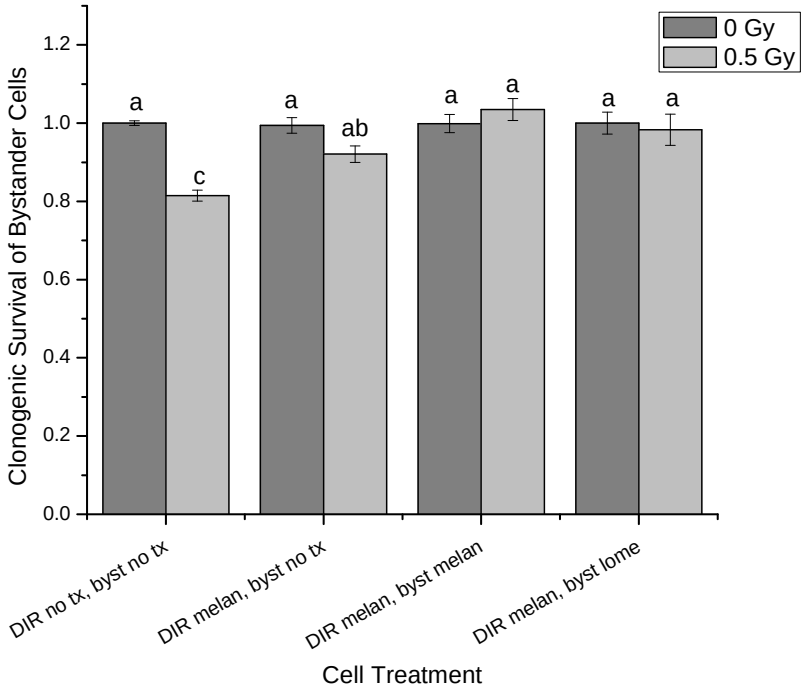

Figure A.2: (Supplementary Figure S2) Clonogenic survival of HaCaT reporter cells treated with no drug, lomefloxacin, or melanin while receiving bystander signals from directly-irradiated HaCaT cells treated with melanin.

Table A.1: (Supplementary Table S1) Tritium activities, exposure duration and corresponding dosimetry

3H Activity (μCi)	**Length of Exposure (hours)**	**Dose (Gy)**
1.7	24	0.001
8.6	24	0.005
17.1	24	0.01
42.9	24	0.025
85.7	24	0.05
171.5	24	0.1
428.7	24	0.25
857.5	24	0.5

A.1.2 Chapter 5

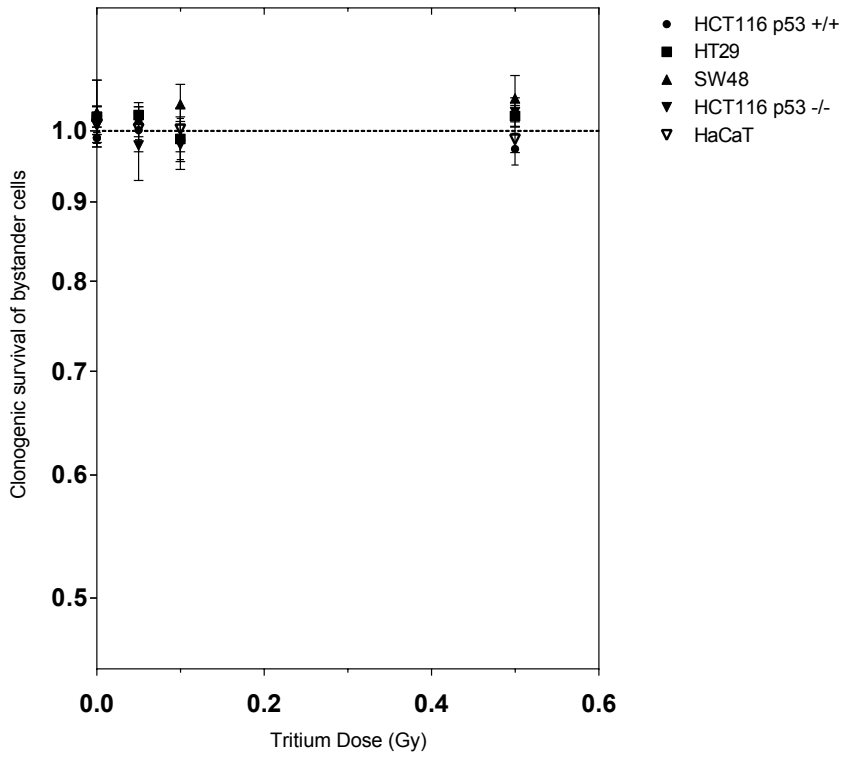

Figure A.3: (Supplementary figure S1) Bystander cell survival of HaCaT, SW48, HT29, HCT116+/+ and HCT116–/– cells exposed to photon signals emitted from ^{3}H-irradiated cell culture media and petri dish.

A.2 Cell Line Characteristics

Table A.2: Characteristics for the Cell Lines Used

Cell Line	HaCaT	HCT116 p53 +/+	HCT116 p53 -/-	SW48	HT29
Classification	*Homo sapiens*	*Homo sapiens*	*Homo sapiens*	*Homo sapiens*	*Homo sapiens*
Morphology	epithelial	epithelial	epithelial	epithelial	epithelial
Pathology	–	colorectal carcinoma	colorectal carcinoma	colorectal adenocarcinoma	colorectal adenocarcinoma
p53 Status	mutated	wild type	null	wild type	mutated
p53 Mutation(s)	3 pt mut, both alleles: His179Tyr, Asp281Gly Arg282Trp [2]	–	–	–	1 point mutation: Arg273His [1]
Doubling Time	21 h [3]	21 h [4]	21 h [5]	35 h [6]	26 h [7]
Source	Dr. Orla Howe, DIT	Dr. Robert Bristow, UHN, UofT	Dr. Robert Bristow, UHN, UofT	Mothersill/Seymour lab	Mothersill/Seymour lab

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Appendix B

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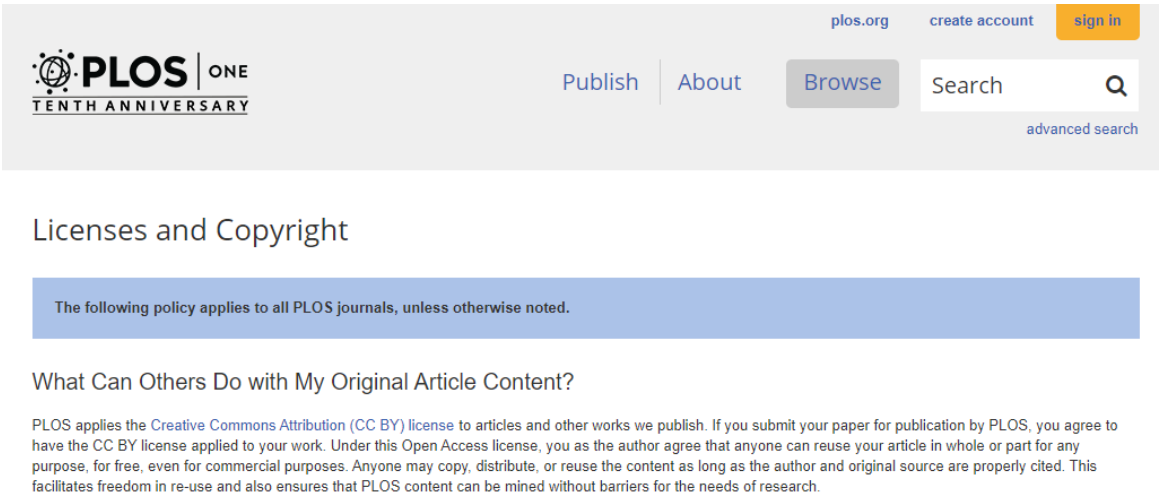

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Le, M., Mothersill, C.E., Seymour C.B., Rainbow, A.J., McNeill F.E. An observed effect of p53 status on the bystander response to radiation-induced cellular photon emission. (2017). Radiation Research,187: 169-185. DOI: 10.1667/RR14342.1

All the best, Judy

Judy E. Fye

Managing Editor

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