Micronuclei Induction in AG01522 Cells is Independent of Temperature and Linear Energy Transfer

By

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Abstract

The bystander effect describes radiation-induced biological effects in non-irradiated cells that have received signals from irradiated cells. In a co-culture experiment, the bystander signaling is proposed to occur via the medium. Using a co-culture setup, the work in this thesis investigates the effects of temperature as an experimental parameter and linear energy transfer (LET) dependence on the bystander effect. Using the micronucleus assay and primary human AG01522 fibroblast cells co-cultured as both the target and bystander cells, the incidence of micronuclei in both X-ray irradiated and alpha particle irradiated bystander experiments were ~2 fold over control averages. In the temperature experiment, there were no significant differences between bystander cells co-cultured with cold (4°C) target cells and those co-cultured with warm control target cells. These results have shown, for AG01522 fibroblasts, that the bystander effect is independent of temperature and LET.

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1. Introduction

In the past couple of decades growing evidence has shown that radiation induces biological effects in non-irradiated cells that are assumed to have received signals from irradiated cells. Current research investigating these effects has shown improvement in providing a scientific mechanism for this phenomenon, but there have not been conclusive results. In this thesis, the bystander effect has been investigated further by comparing dependencies of different LET radiations and observing the effects of a lower temperature during irradiation.

1.1 Background of Radiation Biology

The study of radiation dates back to more than a century ago. Roentgen discovered X-rays in 1895 [1]. Soon after, the first known medical use of radiation occurred in 1897 to treat tumors [2]. The first documented observation of the bystander effect came during the 1940s when researchers described the bystander effect as ionizing radiation (IR) induced damage in non-target cells [1]. At that time, researchers borrowed the term "bystander effect" from genetics terminology to infer that many cells were killed after only one targeted cell killing [3]. Yet in the 1940s, the bystander effect itself never became a focus of research: the researchers believed that IR induced damage was caused by unrepaired or misrepaired nuclear DNA, and thus continued to follow a DNA-centric paradigm in radiation damage. In the 1950s and 1960s, researchers developed tools to focus microbeam radiation on non-nuclear targets such as cytoplasm. As previously believed, cell killing was most prominent upon irradiation of the nucleus [4]; however, new research began to investigate non-nuclear targets. This was the first step towards a non DNA-centric paradigm in radiation damage. In 1992, Nagasawa and Little described the bystander effect and established a surge of interest in bystander research [1, 5].

At this point, the bystander effect has been established and current research continues to challenge the original DNA-centric paradigm [3, 6]. It is known that mutations and chromosome aberrations are partially responsible for

the bystander effect caused in non-target bystander cells (never before exposed to irradiation) by nearby irradiated cells [3, 7, 8, 9]. However, no definitive evidence has come forward to provide a clear understanding of the basic mechanisms of how damage is transmitted from the irradiated cell to the neighboring non-irradiated cell.

The research surrounding radiation oncology and specifically the bystander effect has been controversial, leaving many unknowns to be investigated. There is evidence of genetic predisposition to radiation damage in both direct and indirect cells [6]. There are also cells studied currently that do not seem to send or respond to signals assumed to be associated with the bystander effect [6]. These different results have been recorded due to different cell lines, culture systems, method of radiation, or in vivo subjects. These questions leave much to be discovered.

1.2 Bystander Signal

For clarification purposes, directly irradiated cells release a bystander signal, which is transferred to neighboring non-irradiated cells. Some researchers have used Poisson statistics and a broad beam irradiation to quantify how many cells were targeted and how many cells were damaged. The discrepancy in these numbers accounts for the bystander effect. More current research employs microbeam radiation, which can target a single cell in a population, and endpoints are recorded to show bystander effects.

The bystander effect typically shows a saturated damage curve as dose increases, which suggests signaling as opposed to a direct cytotoxic substance, since the latter would likely yield a linear dose response curve [10]. Medium from irradiated cells (often referred to as irradiated conditioned medium (ICM)) has been shown to recreate the bystander effect when added to normal cells [10, 11]. In some experiments, gap junction inhibitors, when added prior to irradiation, have also been able to significantly decrease the presence of bystander effect. Furthermore, Mothersill et al. proposed that the bystander signal has characteristics similar to proteins because the effects are present when the ICM

is cooled to -20°C but destroyed if ICM is heated to 70°C [10]. More discussion surrounding specific signaling mechanism is addressed along with proposed mechanisms.

One interesting parameter to be studied is the difference in signaling or bystander effects using high and low linear energy transfer (LET) radiation. LET relates to the stopping power of an incident charged particle, or in the case of non-charged particles the stopping power of secondary charged recoil particles produced by the incident neutral particle [12]. Radiation with high LET generally causes more biological damage per unit dose than radiation with a low LET value [12]. LET values are difficult to ascertain from particles but can be estimated using a tissue equivalent counter and a pulse-height spectrum measured in the radiation field [12].

For the purposes of evaluating the bystander effect, many endpoints at low doses can be analyzed by contrasting the high and low LET radiations. High LET radiations such as α-particles have short tracks. The track density is proportional to the dose and dose response. The damage response remains fairly linear with dose until high doses where it plateaus. For low LET radiation such as X-rays, the damage response follows a much more shallow slope which steepens at higher doses [12]. The effects of both types of radiation balance incident damage with repair mechanisms within the cell, which can partially explain dose response. While LET has been well established to change the response in directly irradiated tissue, current research has not made it clear as to whether LET plays a significant role in the bystander effect. No studies have supported the idea that the magnitude of the bystander effect depends on fluence or LET of radiation [6, 13, 14]. There appears to be a different mechanism. Do high LET particles (which instigate a higher level of direct damage) also incite a more prominent bystander effect? The results from other published experiments as well as from the current experiment will be discussed further in the literature review and the discussion of this thesis.

What is known about signaling was discovered through testing mechanisms. Currently, researchers have not been able to prove a singular

mechanism for the bystander effect. However, research alludes to greater than 10 proposed mechanisms that have been implicated in bystander signaling. Scientists are working at every possible angle to test pathway hypotheses.

1.2.1 Consequences of Radiation

Depending on dose, type of radiation, and endpoint measured, the biological effects of radiation vary. Some occur instantly while delayed effects can be seen years later. Within the cell, post-irradiation, certain biochemical processes are affected within one second while cell division can be affected within minutes [12]. Bystander cells with post-irradiation genomic instability may be more prone to become cancerous [7, 12, 15]. And genetic effects can be seen in future generations [12]. The more detailed consequences of bystander effect include genetic/epigenetic changes, alterations in gene expression, activation of signal transduction pathways, and delayed effects in progeny [1]. Along with other types of damage, radiation-induced bystander signaling does not induce any mutations that do not occur naturally, these genetic effects are added to an existing spontaneous pool of damage [12]. Radiation damage to direct cells tends to alter DNA by a loss or deletion of a portion of a chromosome. However, radiation damage to indirect cells (among other types of damage) typically alters the DNA by a change at a single locus called a point mutation [4]. The latter suggests reactive oxidative species are involved in the radiation-induced bystander signaling which has cause the mutation.

For example, Koturbash et al. studied the bystander effect in an in vivo model. Their research supported the epigenetic consequence of radiation. DNA methylation is primarily responsible for two things: to regulate gene expression, and safeguard genome stability [7]. Their animal subjects showed a bystander response in the form of DNA strand breaks as expected in un-irradiated tissue. However, only DNA methylation in irradiated tissue showed a significant decrease. This supports the argument that bystander tissue contains genomic instability and contains damage that alters gene expression [7].

1.2.2 Endpoints Due to These Consequences

Effects on non-irradiated cells are seen in the form of many endpoints. Cells often incur damage directly to their DNA called mutations. Directly irradiated cells generally exhibit deletions of base pairs while non-target bystander cells more often undergo point mutations [4]. Both can be very destructive to cell function and viability, but point mutations are commonly less lethal. Other specific endpoints include sister chromatid exchanges (SCEs) [1, 5, 11, 16, 17], chromosomal aberrations [1, 3, 7, 15, 16, 17, 18], micronuclei (MN) formation [1, 2, 3, 11, 16, 17], neoplastic transformation [1, 16, 17], cell death [1, 10, 11, 16, 17], proliferation [1, 14, 17], and differentiation [1, 15, 17].

1.2.3 Proposed Mechanisms

The bystander effect is relatively new to research. There have been several proposed mechanisms that cause damage to non-irradiated cells; however, current research is not conclusive. In one review, Hamada proposes that it is likely a combination of different effects of signal and radiation that cause the bystander effect [1]. To better understand how they might fit together for a larger mechanism, the individually proposed mechanisms that are most commonly investigated are highlighted below.

Genetoxicity is a term meant to include mutations in nuclear DNA, mitochondrial DNA, chromosomal aberrations and MN formations. These all refer to genetic mutations in DNA of bystander cells. Chromatid type aberrations specifically relate to DNA base damage causing single strand breaks (SSB) and double strand breaks (DSB) [1]. Since these types of damage are hard to repair or sometimes are not repaired at all, they often lead to MN formation.

Some scientists have researched the cell machinery to repair damage induced by radiation or toxic signals coming from irradiated cells. This research has primarily investigated homologous recombination (HR) and non-homologous end joining (NHEJ) repair mechanisms [1]. HR is typically a slower process that uses the complementary strand to repair missing or incorrect nucleotides. NHEJ is a fast error prone process of linking broken DNA together without reference to accurate base pairing [7]. Rad51 has been found to be unregulated in both

irradiated and non-irradiated cells indicating persistent up-regulation in cell repair [7]. Deficiencies in these proteins have been linked to an increased damage in bystander cells [1]. Thus, they are most likely responsible for repairing most bystander induced DSBs [1].

Another proposed mechanism involves p53 tumor suppressor protein, which is important for DNA damage response [1]. It can be likened to a DNA damage checkpoint in the cell. Azzam reported up-regulation of p53 damage-response pathway in monolayer cell cultures exposed to low fluences of α-particles. The p53 genes in bystander cells were phosphorylated on serine 15, which suggests DNA damage [4]. Later research provided data indicating additional up-regulation of $p21^{Waf1}$, which is a p53 downstream effector and highly sensitive to stress [4, 16, 19].

Briefly, other responses to ionizing radiation include Histone H2AX phosphorylation [1, 19], activation of mitogen-activated protein kinase (MAPK) pathway [1, 11, 16], and lipid rafts [1]. There is evidence that serine 139-phosphorylated H2AX (γ-H2AX) forms foci at every site of DSBs [1]. Therefore, it is a response to radiation in irradiated and un-irradiated cells. Activation of the MAPK pathway has been shown to relate to signals coming from the plasma membrane and up-regulate after irradiation [16]. The bystander-induced activation targets nuclear factors and their downstream effectors' activator protein while also activating transcription factors [1]. Specifically, MAPK has been implicated in the following cellular events: proliferation, senescence, differentiation, and apoptosis [11]. Lastly, some evidence suggests that lipid rafts composed of cholesterol and sphingolipids are important in cellular processes such as signal transduction from cell surface receptors [1]. NAD(P)H oxidase and connexin (Cx) are proteins located in lipid rafts.

Evidence of signaling via gap junctions spans a large portion of the radiation-induced bystander research. Gap junctional intercellular communication (often referred to as GJIC) channels are composed of two Cx hexamers provided by adjacent cells. The result is a connecting route allowing passive transfer of ions small molecules (<1 kDa) between cells [1, 4]. Other

studies have suggested gap junctions are one of two pathways for bystander signaling (the other involves reactive oxygen species (ROS) and is described next). Some researchers believe GJIC effects are stronger than the ROS effects. Hall et al. used a densely plated cell culture (50%-60% cell contact) and found the mutation yield as high as four-fold of background mutation levels. When cells were transfected with dominant negative connexin 43-vector (DN6), GJIC were no longer available and the bystander effect was greatly reduced [3]. Interestingly, Little suggested that this mechanism might not be available in all cancer cells since intercellular communication is diminished in tumor cells [4]. Furthermore, studies using medium-mediated experiments proved that GJIC are not necessary to induce the bystander effect in non-irradiated cells, however high density of cells per volume of medium was essential [6, 5, 14]. Shao utilized DMSO and PMA, which can be used to reduce ROS and GJIC respectively. Results of using PMA-only in the cellular medium indicated that gap junctions were upstream from ROS activity [14]. At this point, GJIC is accepted as an adjunct mechanism but not as an essential one.

Medium-mediated effects are the second essential pathway for radiation-induced bystander effects. Reactive oxygen species (ROS) have been implicated in numerous studies as a possible mechanism to introduce damage in the non-irradiated bystander cells [1, 3, 4, 5, 16, 19, 20]. Nagasawa and Little, among the first to focus primarily on the radiation-induced bystander effect, suggested the irradiated cells secreted cytokines or other factors, which led to up-regulation of oxidative metabolism in bystander cells [5]. Azzam et al. supported ROS as a possible mechanism. Their research involved flavin-containing NAD(P)H known to produce ROS, which possessed the capability to affect signaling pathways. Protein accumulations of $p21^{Waf1}$ and p53 were products of these affected pathways and accumulation increased after irradiation [16, 19]. Azzam et al. also found after adding a flavoprotein oxidase inhibitor, there was a decrease in micronuclei formation after exposure to α-particles. These results implicated NAD(P)H in ROS formation and suggest that superoxides produced metabolically by target cells are important intermediates in

biological damage found in radiation-induced bystander cells. Hamada implicated TGF-β1, which is secreted from irradiated cells and could contribute to bystander cell increases in NAD(P)H oxidase activation [1]. This effect in turn leads to persistent ROS production and can invade the nuclei and other sites of DNA and produce mutations/damage [1]. Similarly, other research has suggested ROS generation may lead to formation of bystander factors that further the production of ROS and become a self-sustaining cycle with long lasting effects. Little discussed the similarities in the up-regulation of oxidative stress in bystander cells as compared to the up-regulation in progeny of radiation-induced genomic instability. Both contained DNA point mutations (in bystander cells 90% mutation are point mutations) [4]. Little also discussed oxidative stress as an important factor in point mutation formation. Furthermore, addition of radical scavengers has been shown to reduce the radiation-induced bystander effect in non-irradiated cells [14, 20].

Alternatively, Hall evaluated the initial effects of ICM on normal non-irradiated cells. He recorded the first change as a rapid calcium ion pulse followed 30-120 minutes later by changes in mitochondrial permeability and induction of ROS [3]. Similarly, Lyng et al. reported calcium fluxes, induction of ROS, and loss of mitochondrial membrane potential in bystander cells to be imperative in the bystander signaling process [11]. Specifically, when using apoptosis as an endpoint, the calcium ion flux played a pivotal role in the bystander effect. Voltage dependent calcium ion channel blockers inhibited calcium ion flux and showed control levels of apoptosis, which implicated these channels as important steps in the radiation-induced bystander effect. It is likely that ICM induced bystander effects causes intracellular elevation of calcium and specifically overloads the mitochondria. This led to transient loss of membrane potential and production of ROS. The ROS was implicated to activate the MAPK pathway and apoptosis [11]. Thus, calcium signaling has also been implicated as an important part of the signaling mechanism.

Though the evidence provided for all mechanisms is valid, more likely it is a cascade of events and multiple effects due to type of radiation, dose, and cell

type. Hamada suggests a cascade of events initiated by signals sent from the directly irradiated cell. He proposes that signals (cytokines, growth factors, membrane-permeable reactive species, and other unidentified soluble factors) are released from the irradiated cell and received by the bystander cell via plasma membrane. Other small molecules are directly transferred via GJIC where the two cells are in contact. He postulates that calcium channels are active and taking part in signaling. Receptors on the plasma membrane activate various pathways including MAPK and NAD(P)H oxidase which lead to more ROS production. DNA damage caused by ROS initiates cellular repair machinery and damage checkpoints [1]. In this theory, the amount of damage incurred depends on reparability and type of bystander cell receiving damage.

1.3 Experimental Approaches in Testing Bystander Effect

Setting up a bystander experiment can be done in several ways. The first bystander experiments used broad beam irradiation systems with mono-layered cell cultures. Direct and indirect cells were adjacent and gap-junction hypothesis was readily tested [1]. Typically irradiations only affected a small percentage of cells and researchers used Poisson distributions to determine the number of cells traversed by radiation (termed direct cells) for comparison with the amount of damaged cells [13, 16]. The first experimenters to use said statistical analysis was Nagasawa and Little [5]. The next evolution of the monolayered culture system involved using a medium-mediated culture system. In this system, cell contact between direct and indirect cells is not available and is used primarily to test for signaling via soluble factors transferred to un-irradiated cells and show damage [1, 3, 10]. Often, if the medium required transfer, the medium was strained in order to prevent transfer of any floating irradiated cells [11]. Using this method, the bystander effects have been shown to be dependent on target cell density. The effects are not seen if radiation is less than 0.25 mGy and they plateau before 10 Gy [3]. The bystander effect was still recorded even if the medium had been added 30 minutes post-irradiation. The last major

advancement in cell culture technique started 6-7 years ago [6] was the invention of microbeam irradiation.

This more modern procedure allows for a combination of the two methods described above [13]. Microbeams have been useful to understand microdosimetric aspects of bystander effect, aimed at determining dose-response relationships [6]. The precision of the microbeam can target sub-cellular structures and deliver exact quantities of ionizing radiation [4]. Therefore, two cells can be in contact and only one cell receive dose. Researchers have used fluorescence to mark cells and irradiated specific fluorescence markers, while the identifiable bystanders were not traversed by radiation [13]. This was the first study to visualize the bystander effect rather than infer via statistics. This is also one step closer to an in vivo model where cell contact and communication is imperative. This is important because studies done in ex vivo show that in vitro studies do not exactly mimic tissue in vivo. Cell communication via gap junctions intercellularly or via utocrine and paracrine factors are likely tissue specific [2].

1.4 Previous Research in Bystander Field

An introduction of relevant previous experimental results, which have led to current theoretical models and mechanisms, will be discussed here. Previous research has primarily tested LET dependence and developed mechanism theories and how they could be linked. Some of the first researchers to investigate the bystander effect were Nagasawa and Little in 1992. They provided a focus to the bystander effect by studying irradiation on a monolayer cell culture system (Chinese hamster ovary cells) by low fluence α-particles [5]. They showed a 30% frequency of sister chromatid exchanges (SCE) when less than 1% were traversed with a α-particle [5]. However, higher doses of X-rays were needed to produce the same effect, which also instigated the first discussion surrounding bystander effect and LET dependence [5]. Mothersill et al. found that cell-cell contact had no effect on the medium from irradiated epithelial cell cultures, which was used to reduce clonogenic survival of

bystander cells. The only factor found to effect bystander cell survival was irradiated cell density [10]. Another study irradiated with low fluence α-particles and stained cells with two different dyes. Then advanced microbeam technology allowed them to identify and irradiate only one color of dye. The bystander cells were then easily chosen to perform damage experiments. Again, particle fluence through the irradiated cell did not show significant changes in the amount of damage or magnitude of endpoints measured in the bystander cells. However, there was a particle fluence relationship evident in the damage of directly irradiated cells. Researchers observed proportionately more cell cycle delay in progeny of directly irradiated cells depending on particle fluence. However, when measuring the number of progeny able to enter S phase, only 28-42% of bystander and irradiated cells were able to enter S phase as compared to 95% of cells which were in G0/G1 phase during the irradiation [13].

Bystander effects have been more recently been studied in ex vivo tissue, which mimics a complex organism more effectively [17]. The results have shown a significant increase in MN as well as apoptosis as compared to background levels in bystander cells as far as 0.6 mm and 1 mm (respectively) from irradiated cells [17]. The study also highlighted differences in bystander results when comparing two different types of tissue, for example irradiating dermal tissue did not produce a bystander effect [17]. Another experiment directed at in vivo modeling found radiation exposure to half of an animal body led to DNA strand breaks, alteration of key protein levels relevant to methylation and silencing. These effects were seen in bystander tissue (not irradiated) at least 0.7 cm from the irradiated tissue [7]. Sedelnikova et al. suggested there are differences in research between in vitro models (which is most of the research in the bystander field currently) as compared to research in ex vivo models. Their ex vivo studies produced a 4-6-fold increase of DSBs in bystander tissue (using γ-H2AX foci formation analysis) over control tissue using α-particle microbeam, which lasted 6-7 days post-irradiation [15].

Research supports oxidative metabolism as a key player in the signaling mechanism. Azzam et al. used low fluence α-particles to test oxidative

metabolism [16]. Their study concluded that reactive oxygen species produced by NAD(P)H mediated several stress-inducing pathways (p53 and $p21^{Waf1}$) as well as micronuclei formation in human fibroblast cells. Hall has suggested that bystander cells have a rapid calcium ion pulse after irradiation to neighboring target cells, which changes the mitochondrial membrane permeability. This change in the mitochondria produces more reactive oxygen species [3], and is implicated in bystander damage. Shao et al. used high LET particle microbeam to selectively irradiate human fibroblast cells [14]. When DMSO and PMA were used to reduce the reactive oxygen species and inhibit gap junctional intercellular communication respectively, the bystander effect was almost fully diminished [14].

The presence of extracellular factors in the bystander effect has also received attention in terms of linear energy transfer radiations in both normal and tumor cell lines. Mothersill and Seymour discovered that medium from γ-irradiated epithelial cells reduced clonogenic survival in un-irradiated tissue[10]. The same effects have been found after irradiation with X-rays of primary human fibroblast [19]. These same effects have been duplicated using α-particles in fibroblast tissue [13, 15, 20]. Another study compared high-LET ^{40}Ar or ^{20}Ne using a high-LET heavy particle microbeam on normal human skin fibroblast [14]. When targeting >1 cell in the culture system, they recorded a ~2-fold increase over the control levels of MN in bystander tissue independent of number and LET of particles.

1.4.1 Temperature Research

Scientists are thinking creatively to try and study every angle of the bystander effect. Although not clearly related to bystander effect at the time of study, there have been correlations between temperature and radiation protection as early as 1952 by Storer et al. Storer et al. saw reduced morbidity after chilling mice body temperatures to 0°C. They also noted that the animals were not breathing at this temperature, and thus tissue oxygenation could be an important factor in radiation desensitization at this lower temperature [21]. In

1956, Hornsey found that newborn rats and mice had increased survival rates by decreasing the body temperature to 0-1°C before irradiating with X-rays [22]. The dose required to give LD50 at 30 days was 2-4 times more than the control animals at normal body temperature [22]. Similarly, another temperature article published in 1959 detailed an experiment relating temperature to hypoxia. Profound hypothermia led to anoxia and anoxia reduced the body's sensitivity to X-ray irradiation [23]. Just a few years later, another study tested temperature dependence of irradiated mammalian cells (HeLa). Sensitivity to radiation below -145°C was temperature independent; however, irradiations performed from -145°C to 35°C demonstrated a correlation of sensitivity that increased with temperature. Overall there was a 1.59 factor sensitivity increase in HeLa cells over a range of 5-40°C. The authors concluded temperature protects by slowed rate processes and recombination of molecules in the metionic[1] state [24] before deleterious reactions could happen [25]. Many years later, Elmroth et al. argued that enzyme reactions are dependent on two factors for maximum efficiency, thermal energy and conformational stability of proteins [26]. They stated the effect of temperature on the bystander effect was most likely different for normal and tumor tissue because the intermediate metabolism is different. Testing the influence of temperature DNA supercoiling after irradiation, they found 2°C temperatures during irradiation appeared to protect the MCF-7 cells from radiation-induced inhibition of nucleoid rewinding. Thus, supporting the argument that decrease temperatures during irradiation provide protection to DNA-matrix damage induced by radiation [26]. Other researchers studied if X-

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[1] **"Metionic reaction describes the one that immediately follows the passage of the ionizing particle. Depending on the conditions, there may be many types, and the reaction may concern a single molecule or a whole structure. The metionic reaction may be recapture of an electron, repair of a broken bond, reaction with a neighboring non-ionized molecule, reaction between two or more ionized target molecules, and so on; reaction with radiation-formed radicals and peroxides would also be possible types. Clearly some metionic reactions will be such that restoration of the target to a functioning condition will be possible, whereas others will lead to a product that the metabolic processes of the cell cannot handle. The chemical environment of the target at the time of irradiation could be expected to influence a wide range of metionic reactions, and it is at this stage that modifying agents probably exert their effect. Apparently, if oxygen enters the metionic reaction, it can form a product with the target which makes it incapable of restoration." [24]**

ray irradiations during different cell cycles affected micronuclei formation, because stage of cell cycle has been strongly implicated to influence the radiosensitivity of irradiated cells [27]. This was also tested using temperature as a parameter [27]. For MCF-7 cells, the most sensitive cell cycle phase was G1 at 37°C while there was a strong protection for the same G1 phase cells at 2°C. The temperature protection was not seen in other cell cycles [27].

This research provided a basis to investigate if there are similar changes in sensitivity in the bystander effect when temperature is a parameter. Little reviewed one example of temperature effects on the bystander effect. When performing a medium-mediated transfer experiment, if the ICM is frozen or heated the bystander effects are lost [4]. This research reports, along with the knowledge that lower temperatures slow down cellular function [25, 26] give reason to believe that perhaps lower temperatures during irradiation could later the signaling response of the directly irradiated cell and provide protection for bystander cells. Further research must be done in order to detail exactly how temperature affects bystander damage and if it is an important consideration in radiation therapy or health radiation safety.

1.5 Goals of This Research

The work in this thesis describes LET dependence and investigates the effects of temperature on the bystander effect. The objective of this research was to add information to the current discussion of LET dependence and observe the effects of temperature as an experimental parameter. In order to uncover the underlying mechanisms and identification of signaling molecules, clarification of LET dependence and in vivo relevance requires further attention [1].

The bystander pathway is fragile and sensitive to low levels of irradiation, which makes it a high priority in research. It could prove to be an important factor in dose treatment margins and health/safety calculations [17]. This is particularly true since the bystander effect is most relevant to low dose irradiations, as higher dose irradiations generally cause too much cellular damage to continue cellular function [2]. Likewise, it will be important in dose

calculations when there is dose heterogeneity (radionuclide treatments), which might have high dose components, but will contribute to bystander effects in areas where less radionuclide is absorbed [2]. More specifically, knowledge of the bystander effect can enhance tumor control by changing dose modeling [6] to kill more tumor bystander cells and help delineate abscopal effects [1, 2]. With the knowledge of the bystander effect, doctors and researchers could focus on tumor cell inactivation instead of targeting maximum number of cells [1], potentially exploit GJIC between tumor cells to increase effects of lower doses to a smaller volume [1], and study the impact bystander effect on secondary cancers [2].

2. Methods and Materials

The first goals of this thesis are to reproduce the bystander effect in AGO fibroblast cells using two different modes of radiation in order to test LET dependence and investigate if temperature plays an important role in the bystander effect.

2.1 Cell lines

Primary human AG01552 fibroblasts were used during this experiment to perform the cell cultures. Cells were originally obtained from the Genetic Cell Respository at the Coriell Institute for Medical Research (Camden, NJ, USA). Cells were grown in 37°C with atmospheric concentrations of 95% air and 5% CO_2. The cells thrived in α-modified Minimum Essential Medium (MEM, Sigma) supplemented with non-essential amino acids (MEM/MEAA, Hyclone), Glutamine (L-glut, Cellgro), 20% Fetal Bovine Serum (FBS, Hyclone), and penicillin-streptomycin solution (Cellgro). AG01552 cells were grown to confluency for each experiment, but were not used beyond passage ten. Cells were harvested by trypsinization, and re-plated at appropriate densities for the MN assay.

2.2 Radiation Setup/Equipment

2.2.1 Co-Culture Systems

For X-ray irradiations, the cells were plated at appropriated densities in standard six well plates (Falcon) 24 hours before the irradiation. The normal AG01522 fibroblast bystander cells were added to the medium post-irradiation within 5 minutes.

For α-particle irradiations, machined cell culture dishes were used to allow a co-culture system during the incubations. The stainless steel cylinders allowed replaceable 1.4-μm-thick Mylar to be stretched across the bottom of the dish. This created a 3.81 cm diameter surface for cell plating/growth. A secondary outer stainless steel ring fitted with Vinton rubber o-rings created the tight fit between the cylindrical dish and Mylar layer. When using alpha particles, the non-irradiated cells were added to the medium within 2 minutes following irradiations. In Figure 1, the cells attached to the bottom Mylar layer represent the directly irradiated normal AG01522 fibroblast cells. Also in Figure 1, the normal AG01522 fibroblast cells denoted "bystander cells" are not irradiated and plated on micro-cover slips. The fibroblast bystander cells were added after irradiations.

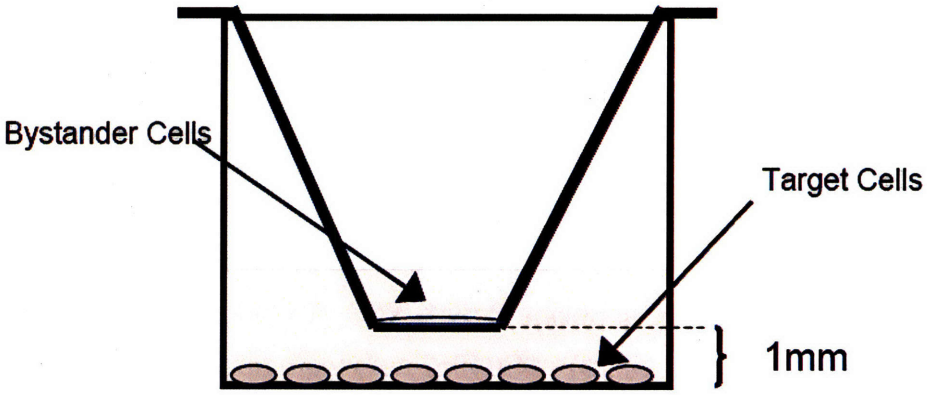

Figure 1: A co-cultured system. The cells on the bottom layer are irradiated. The non-irradiated cells on the insert (the bystanders) are assayed for damage.

Each dish, once assembled with all of the components necessary for the experiment, ran through an autoclave. During use and transport, the Mylar dishes were covered with 60 mm diameter plastic Petri dish covers. Before plating, the Mylar membrane was treated with FNC Coating Mix (BRFF AF-10, AthenaES, Baltimore, MD) to encourage cell adhesion.

2.2.2 *Irradiation Source*

The X-ray target cells were irradiated using a Phillips RT250 unit, with operating parameters of 250kVp and 15 mA. Irradiations were performed at room temperature via an X-ray beam 32 cm above six well plates. The irradiation procedures took a maximum of two minutes for each six well plate. The unit used a 0.4 mm tin plus 0.25 mm copper filtration system and a focus to target distance of 32 cm. The X-ray dose rate in the six well plates was 1.0 Gy/min.

The alpha particle source was a sealed planar ^{241}Am foil source custom manufactured by NRD, LLC, Grand Island, NY. and constructed and calibrated as described in Metting et al. [28] and as used in previous similar experiments [20]. The active layer was a mixture of americium dioxide and gold foil backed with silver and further coated on top with 1.5 μm of gold. Wang et al. reported that at cell irradiation position, the α-particle fluence was 998 counts/mm^{2}s^{-1}, average α-particle energy was 3.14 MeV, the average linear energy transfer was 128 keV/μm, and the average dose rate to the cells growing on the Mylar surface was 1.2 Gy/min. The source apparatus contained the ^{241}Am foil, a manual shutter, and a machined collar designed to fit the Mylar dishes. The gap between the surface of the source and the Mylar membrane was 5 mm and at 1mm above the Mylar membrane, the gamma ray dose rate component was negligible, on the order of 10^{-6} Gy/min [20].

2.3 Endpoints

The frequency of micronuclei formation was measured using a cytokinesis block technique [16, 19, 29, Anzenberg (not yet published)]. Micronuclei are formed when broken or detached chromosomes are separated from the spindle apparatus. After cells undergo mitosis, the fragments become trapped in the cell

cytoplasm and form micronuclei. The MN assay is frequently used for purposes of biological dosimetry, since the amount of radiation received is related to the number of MN formations in a sample. Binucleated cells are one stage in the process of cell division. The drug cytochalasin B blocks the cell cycle in anaphase by inhibiting cytokinesis.

In this study, after radiation treatments and appropriate co-culture incubation times, AG01522 fibroblast bystander cells were removed from co-culture system and put into 12 well plates (Falcon) with 2 ml fresh medium. Cytochalasin B (Sigma) was added to each well for a final concentration of 1.5μg/ml in AG01552. At this concentration, cytochalasin B was nontoxic in the cells. The cells incubated for 72 hours. After the three day incubation necessary for AG01522 fibroblasts, cells were fixed in methanol: acetic acid (3:1 v/v). The samples were given time to dry and then were stained using a nuclear stain 4',6'-diamidimo-2-phenylindole (DAPI) solution which had a final concentration of 10 μg/ml in water. Cells were rinsed with phosphate buffered saline (PBS) twice and mounted with FluoroGuard™ Antifade reagent (Bio-rad) to preserve the fluorescent stain. The micronuclei were scored if present with binucleated cells using a fluorescence microscope. At least 500 binucleated cells were examined from each cover slip, and only micronuclei associated with binucleated cells were considered for analysis. The reported frequency of MN formation in binucleated cell was calculated as #MN/#BN*100. The error associated with this frequency was one standard deviation calculated from the data samples. Data collected in the MN assay were expressed as % binucleated cells with MN.

After fixing and staining the cells, micronuclei appeared as fluorescent green round bodies apart from the nucleus. The minimum number of binucleated cells scored was 500 pair per cover slip. An example of a MN assay stain is shown below in Figure 2.

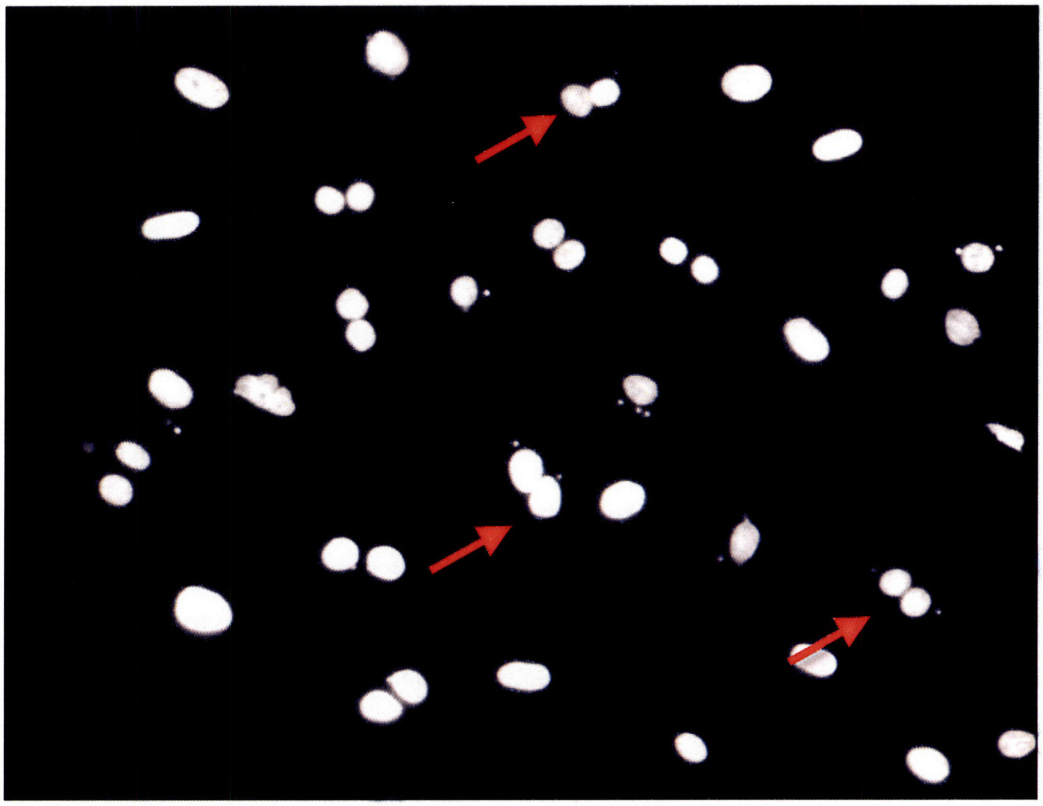

Figure 2: Binucleated cells (BN) with and without micronucleus (MN). The cells were stained with DAPI and recorded under fluorescent microscope. This view of MN assay shows MN near a BN cells. This particular image is taken from an AG01522 fibroblast, which have been directly irradiated at 2 Gy. The red arrows point to those binucleated cells that also have micronuclei.

2.4 Experimental Procedure

2.4.1 X-ray Bystander Experiment

Primary human AG01522 fibroblast cells were grown to confluency before harvesting for experimentation. For each experiment, the cells were plated 24 hours prior to irradiation to allow full attachment. To plate cells, the confluent cells were harvested using trypsin and incubated (for 25 minutes) to loosen the intracellular attachments. Medium was used to neutralize the trypsin and create a single cell suspension. For six well plates, a cell density of $1.0\text{-}1.3\text{x}10^5$ cells per 3 ml of medium was achieved by adding additional medium. Concurrently, the AG01522 bystander cells (non-irradiated cells) were plated on 18 mm glass micro-cover slips (VWR International) at a cell density of $8\text{x}10^4\text{-}1\ \text{x}10^5$ cells per 2

ml medium. Immediately prior to irradiation, the medium was changed in the six well plates as well as in the twelve well plates used for bystander cells.

The irradiation procedures took two minutes or less per six-well plate since the maximum dose delivered was 2 Gy and the X-ray machine had a 1 Gy/min dose rate. Irradiations were measured by time to achieve desired dose and the bystander cells were added to the medium within 5 minutes after the irradiation. The samples were incubated with the bystander inserts for approximately four hours.

2.4.2 *Alpha Bystander Experiment*

The alpha experiment protocol was very similar to the X-ray protocol. The primary human AG01522 fibroblasts cells were used and grown to confluency, and harvested in the same manner. However, since the Mylar membranes did not provide a good surface for cell attachment, FNC coating mix was used to treat the bottom of the Mylar dishes. Approximately 0.2 ml FNC coating mix was used for each Mylar dish and given several minutes to dry. Excessive amounts of the mix were removed by suction. Then, with a good single cell suspension, approximately 3-5x10^5 cells were added to each Mylar dish. Additional medium was added as needed to create a total volume of 4 ml. The bystander cover slips were prepared identically to the X-ray protocol.

On the day of irradiations, the medium was changed in both the Mylar dishes and bystander cover slips. The direct AG01522 cells were irradiated per alpha irradiation protocol at 1.2 Gy/min for long enough time to reach the desired dose. Immediately after irradiations, the bystander cover slips were added using plastic inserts machined to allow very little room between the Mylar surface and the bystander cover slip. The inserts allowed the cover slips to be submerged and thus co-incubated in the medium of the target cells. The direct+bystander Mylar dishes were then incubated for 4 hours at 37°C.

After the incubations, the bystander cover slips were removed and added to fresh medium in 12 well plates. The cytokinesis-block technique and MN assay were performed identically to the X-ray protocol.

2.5 Temperature Experiments

The temperature experiments were designed to mimic the bystander experiments with exception of one variable, temperature. Therefore, the harvesting and plating were performed in the same way with the same concentrations. However, the protocols changed on the day of irradiation. For cold temperature samples, instead of replacing the medium with warmed medium, cold medium was used. Also after the medium was changed, the target cells were incubated at 4°C for one hour prior to being irradiated. The temperature of the medium reached equilibrium with its surrounds in approximately 20 minutes, but to ensure that all cellular activity affected by temperature had stopped, the incubation time was one hour. The bystanders were not cooled; they were treated identically to the bystander experiments described above.

After the cold incubation, the target cells were irradiated as described in section 2.2.2. The X-ray cells were given approximately seven minutes to return to room temperature, while cells on the Mylar dishes were given 15 minutes post-irradiation to reach room temperature since the stainless steel conducted cooler temperatures for a longer period of time. After the samples had warmed up, the bystander cover slips were added and the 4-hour incubation proceeded just as in the bystander experiment. The warm controls in this experiment were also irradiated under the same time frame and therefore experienced a delay before adding the bystander cells.

2.6 Statistical Analysis

Data from at least three independent experiments, run in duplicates, were presented in graphical form as mean ± STDEV. Significance levels were assessed using a Student's *t*-test of SigmaPlot 2001 software; $p<0.05$ was considered to be statistically significant.

3. Results

The first goal of this experiment was to accurately reproduce the bystander effect in normal human fibroblast cells (AG01522) compared to directly irradiated cells using both X-rays and alpha particles. The goal was similar to previous studies (Anzenberg (not yet published), 19), which demonstrated medium-mediated bystander effects in normal AG01522 fibroblast cells produced by direct irradiation of human DU145 prostate tumor cells. It is hypothesized that the signal released from directly irradiated cells is moderated by components in the cellular medium. The moderation seems dependent on the magnitude of radiation delivered to/absorbed in target cells. This effect is seen as an increase in MN formation in the non-target cells as a function of higher doses of irradiation. The final goal was to test if cooling the temperature of the cells prior to irradiation could block the signal and reduce the MN formation, and thus reduce the bystander effect.

3.1 Direct Experiment

In order to reproduce published data regarding direct effects of high and low LET radiation (as compared to Yang et al. [19]), a test to quantify induction of micronuclei (MN) was used to measure damage in AG01522 human fibroblast cells. A comparison was made between direct MN inductions in AG01522 human fibroblast cells as compared to indirect MN damage in bystander cells. The direct assay was repeated twice to show a general trend compared to the bystander MN assay, which was repeated four to five times (See Figure 2). The directly irradiated fibroblasts showed a five-fold increase compared to a two-fold increase in percent MN formation in bystander fibroblast cells, which reached a plateau around 1 Gy. Figure 3 shows the incidence of MN in direct/indirect AG01522 human fibroblast cells.

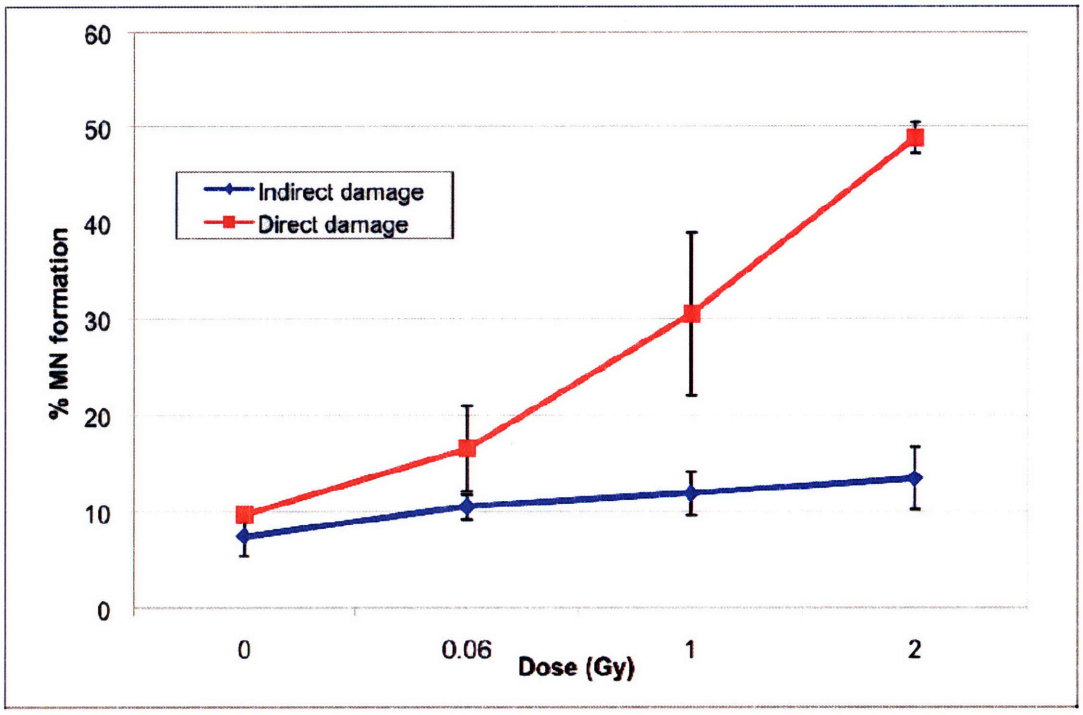

Figure 3: Micronuclei damage induced by X-ray irradiation. This figure shows the comparison of MN damage produced in directly irradiated cells (X-ray irradiations) versus bystander cells. The error bars represent +\- one standard deviation.

A similar comparison was made between directly irradiated AG01522 normal fibroblast cells and non-irradiated bystander AG01522 normal fibroblast cells using high LET α-particles (refer to Figure 4). The directly irradiated AG01522 cells were scored from the Mylar film and compared to the bystander fibroblasts, which were co-cultured post-irradiation. As shown in Figure 4, MN incidence in the directly irradiated AG01522 fibroblast cells increased until some point between 0.6 Gy and 1.2 Gy after which a decrease is observed. The sharp decrease in MN formation is not symbolic of radiation protection but is instead a decrease in the number of viable cells, which were able to reproduce and show MN damage. From the MN damage at these doses, the directly irradiated fibroblast cells showed a four-fold increase in MN, while the bystander fibroblasts show a two-fold increase over controls which reached a plateau at approximately 1.2 Gy.

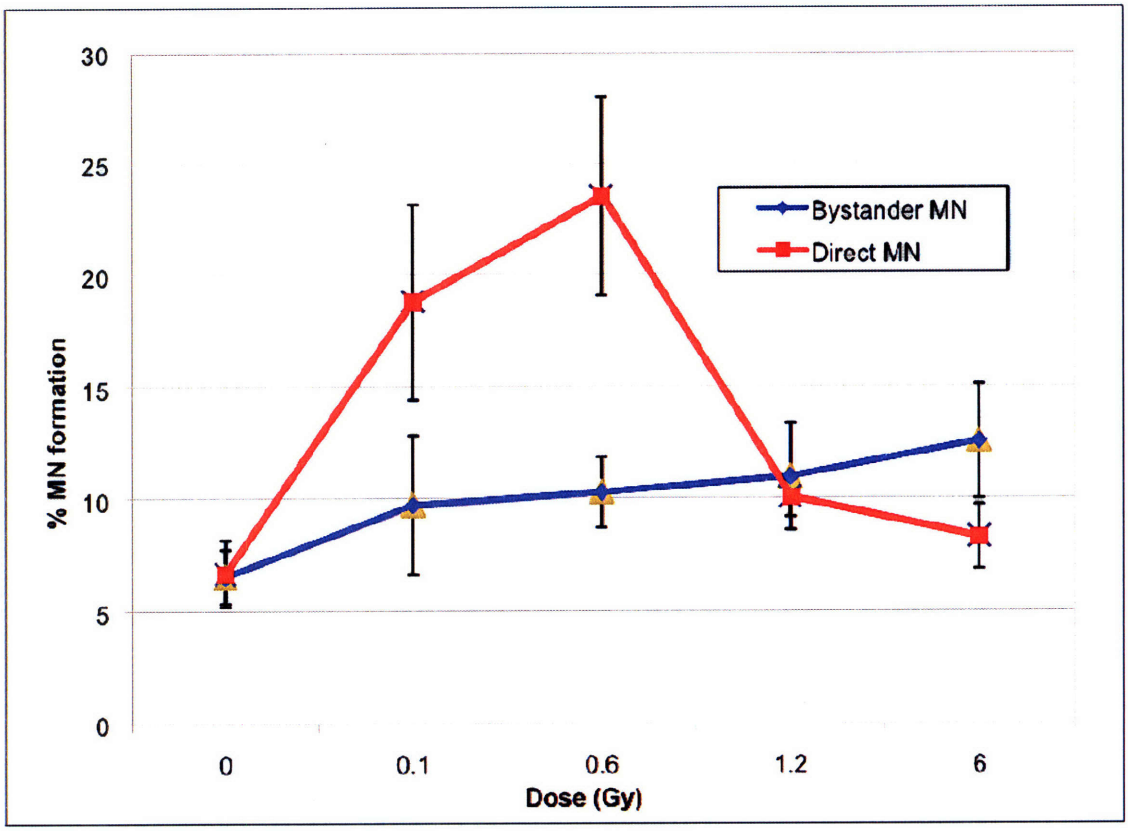

Figure 4: Micronuclei damage induced by alpha particle irradiation. This figure shows a comparison of MN damage produced in directly irradiated cells (using α-particles) versus bystander cells. The error bars represent +\- one standard deviation.

3.2 Bystander Experiment

To show the statistical relevance of the bystander effect in irradiated normal AG01522 cells co-cultured with un-irradiated normal AG01522 cells, the bystander experiment was repeated four times. In the X-ray bystander experiment, all three doses were statistically different from controls using a Student's *t-test* (refer to Figure 7). The percent of MN formed in the bystander cells compared to average control values showed a two-fold increase, which reached a plateau after 1 Gy. The following figures show example views of a MN fluorescent assay in the X-ray bystander experiment.

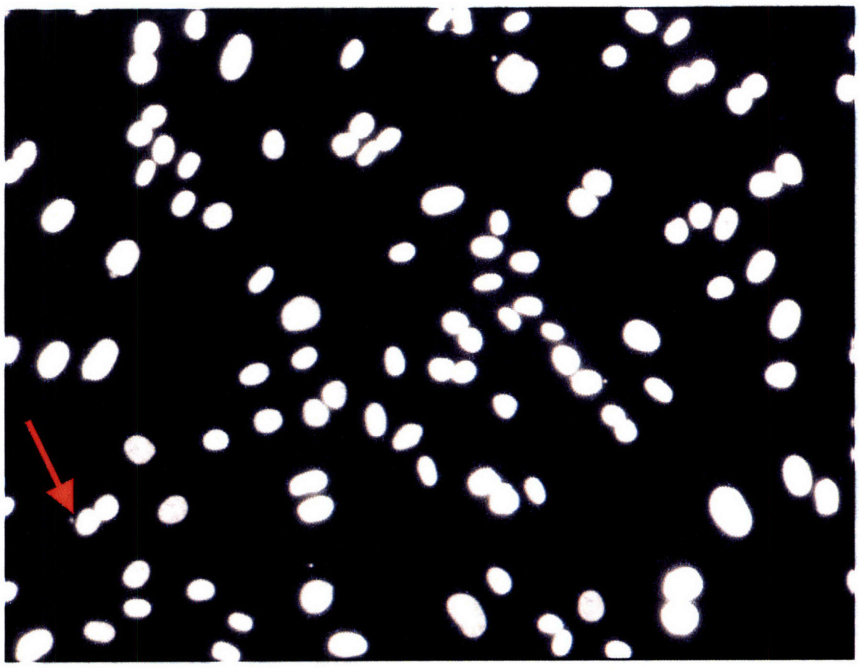

Figure 5: AG01522 fibroblast bystander control cells in X-ray bystander showing binucleated cells (BN) with and without micronuclei (MN). The cells were stained with DAPI and recorded under a fluorescent microscope. The red arrows indicate BN with MN.

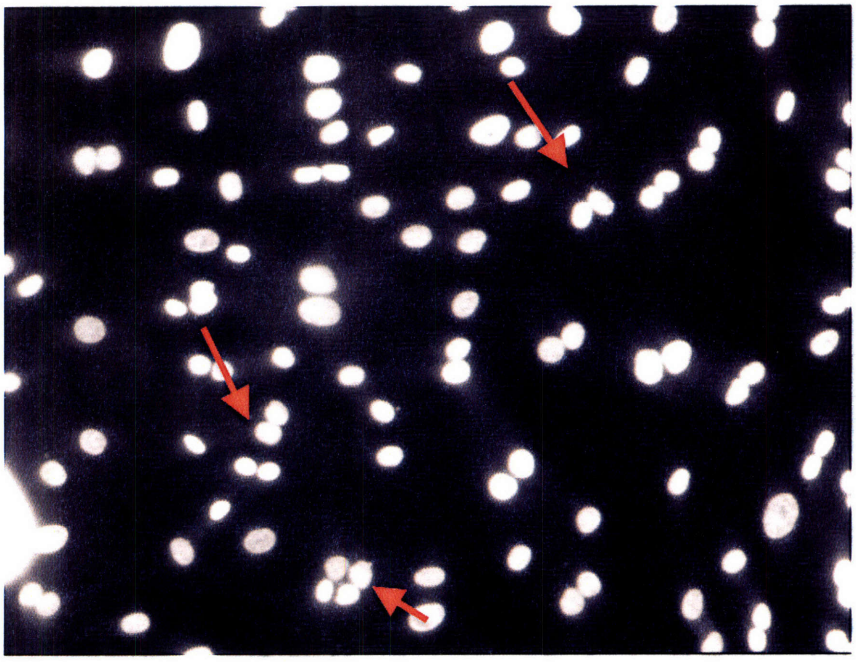

Figure 6: AG01522 fibroblast bystander cells which have been co-cultured with AG01522 fibroblast cells irradiated with 2 Gy in X-ray bystander experiment. Shows binucleated cells (BN) with and without micronuclei (MN). The cells were stained with DAPI and recorded under a fluorescent microscope. The red arrows indicate BN with MN.

The statistical results from all of the samples are shown in Figure 7.

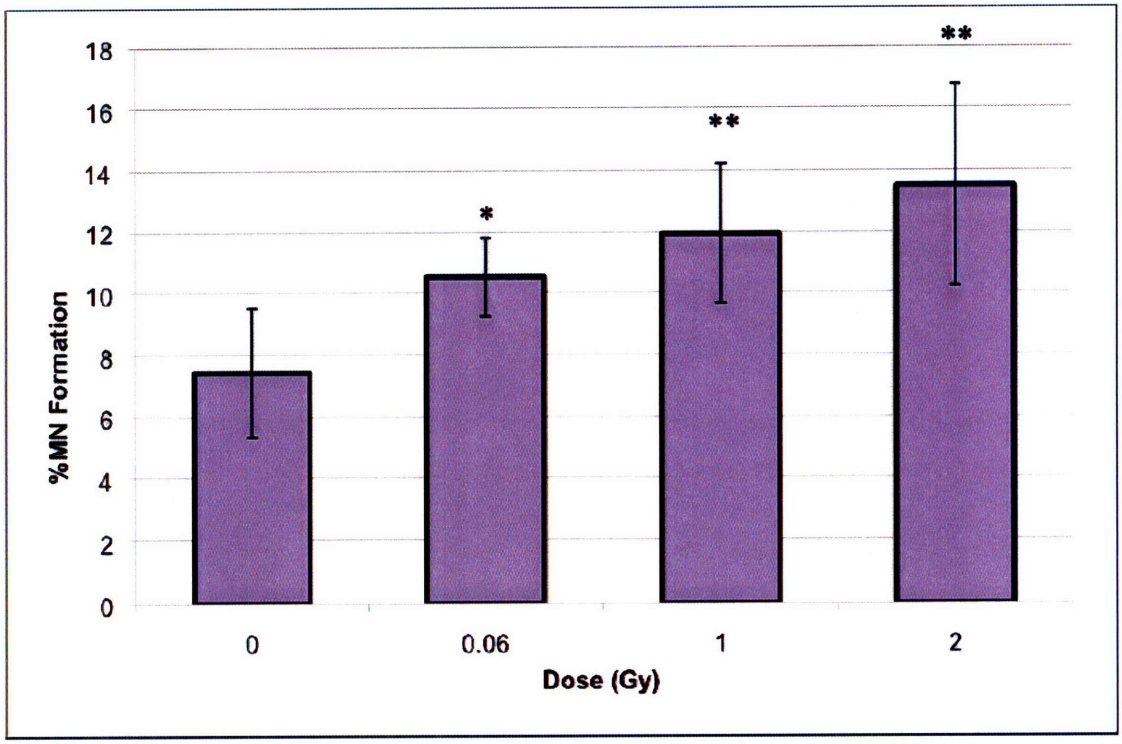

Figure 7: X-ray radiation induced bystander effect in non-irradiated AG01522 normal human fibroblast cells. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.

The next step was to reproduce the bystander effect in AG01522 normal fibroblast cells using high LET (α-particle) irradiations (reproduction of data in [19]). This experiment was repeated three times. As the data shows in Figure 8, the highest three doses were statistically different from controls using a Student's *t-test*. The percent of MN formed in the bystander cells compared to average control values showed a two-fold increase.

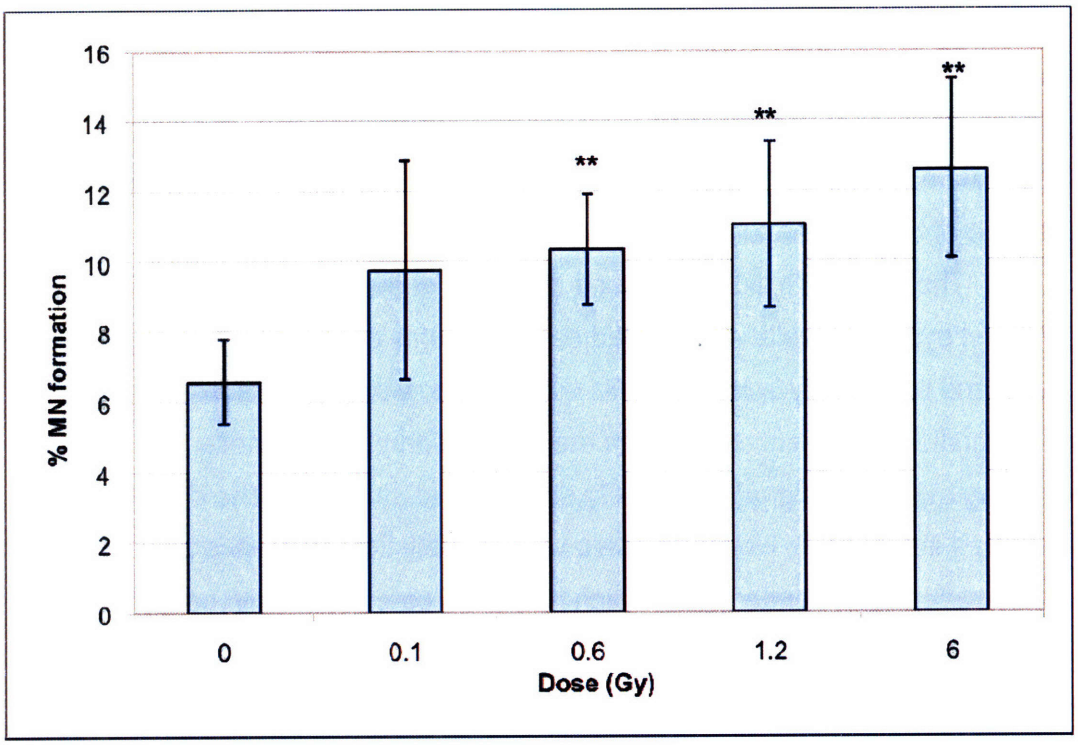

Figure 8: Alpha radiation induced bystander effect in non-irradiated AG01522 normal human fibroblast cells. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.

LET dependence is the important result from these two graphs (Figure 7 and Figure 8). Both show comparable MN induction at low dose irradiations as well as reaching a plateau of ~2-fold over controls at higher doses. This supports previous research that MN formation in bystander cells is LET independent [14].

3.3 Effects of Temperature on Bystander Effect

Because temperature has been shown to be a promising radiation-induced damage protector [21, 22, 23, 25, 26, 30], this is an interesting parameter to introduce to bystander research. Bystander experiments were performed similarly to experiments described previously but were cooled for one hour prior to irradiation and during irradiation. Other studies have shown that the most important temperature parameter is during the irradiation itself [25, 26]. The bystander cells were added to the irradiated cell culture after a delay post-irradiation that depended on the type of irradiation (see section 2.2.2). The

results from this experiment showed no statistically significant difference between warm controls with the post-irradiation delay as compared to warm controls that did not experience a delay in adding the AG01522 fibroblast bystander cover slips (data not shown). Therefore, the delay should not have affected the outcome of the temperature experiments.

The low LET irradiations used X-rays to damage direct cells. AG01522 fibroblast bystander cells were added seven minutes post-irradiation, during which time the directly irradiated cells were able to warm up. As expected, there were significant differences between the control samples and those samples receiving bystander signals from 1 Gy directly irradiated cells. The data are presented in Figure 9. However, there was no statistical difference between the MN formation in bystander cells, which had been co-cultured with cells receiving the temperature treatment and those which were maintained at warmer temperatures. The cooler temperature did not appear to mediate or mitigate the signals sent from the directly irradiated AG01522 fibroblast cells. On the graph, cold-warm indicates the directly irradiated cells which were irradiated at the cooler temperature while the bystander cells remained at 37°C. Warm-warm refers to the fact that both irradiated and bystander cells were kept at warmer temperatures.

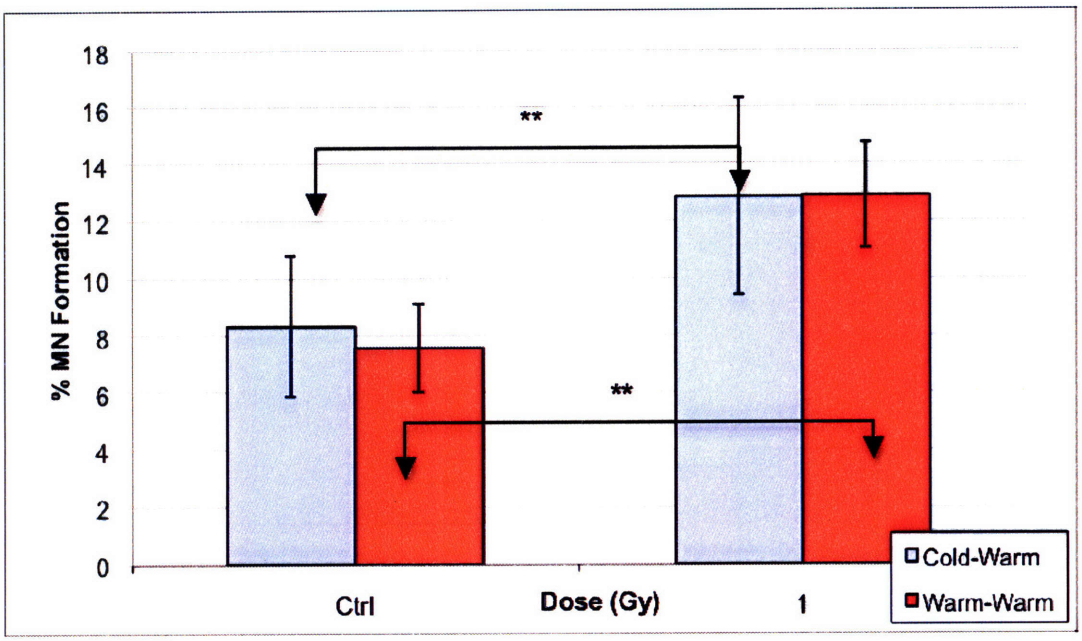

Figure 9: Temperature mediated bystander effects using X-ray radiation. There was no significant difference between warm and cold controls or between warm and cold radiations (1Gy). Cold-Warm denotes the direct irradiation occurring at low temperatures while the bystanders were kept warm. Warm-Warm denotes that both direct and bystander cells are kept warm. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.

The high LET irradiations used alpha particles to damage direct cells. AG01522 fibroblast cells were added 15 minutes post-irradiation, during which time the directly irradiated cells were able to warm up. As expected, there were significant differences between the control samples and those samples receiving bystander signals from 1.2 Gy directly irradiated cells. However, there was no statistical difference between the MN formation in bystander cells, which had been co-cultured with cells receiving the temperature treatment and those that were maintained at warmer temperatures. The cooler temperature did not appear to mediate or mitigate the signals sent from the directly irradiated AG01522 fibroblast cells. The data are presented in Figure 10. On the graph, cold-warm indicates the directly irradiated cells which were irradiated at the cooler temperature while the bystander cells remained at 37°C. Warm-warm refers to the fact that both irradiated and bystander cells were kept at warmer temperatures.

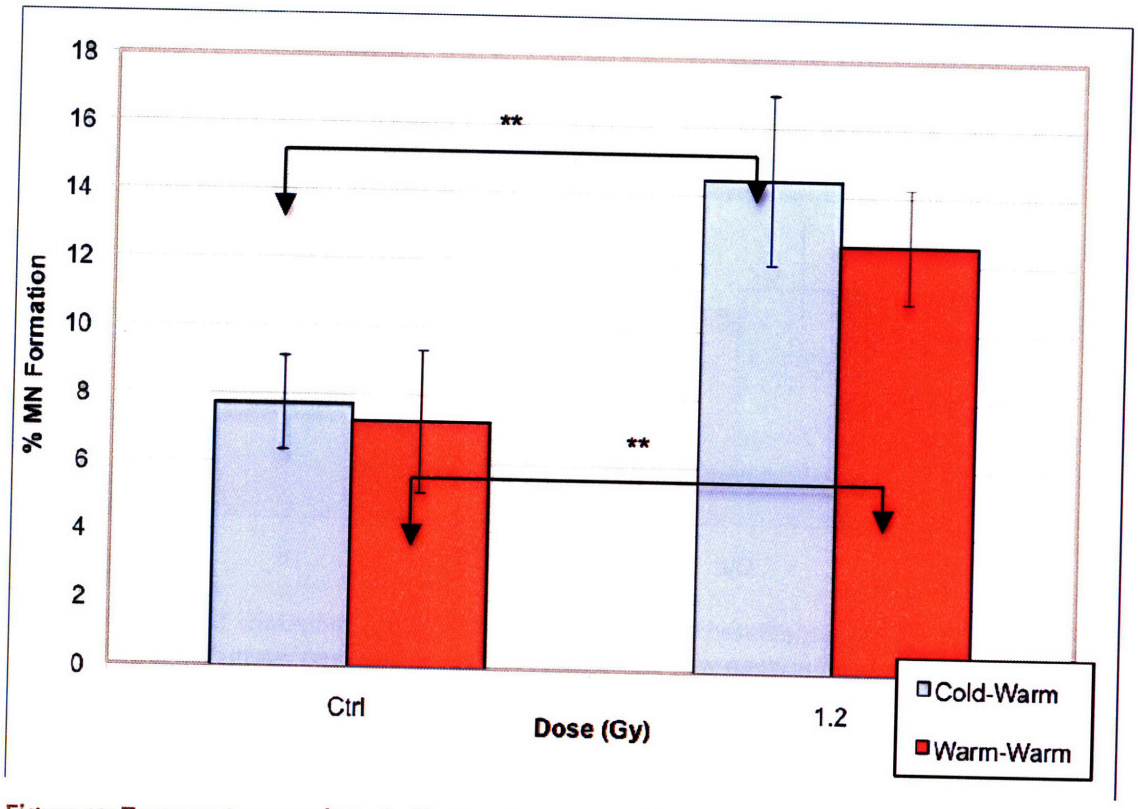

Figure 10: Temperature mediated effects using α-particle radiation. There was no significant difference between warm and cold controls or between warm and cold radiations (1Gy). Cold-Warm denotes the direct irradiation occurring at low temperatures while the bystanders were kept warm. Warm-Warm denotes that both direct and bystander cells are kept warm. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.

4. Discussion

The terminology "bystander" describes two different phenomena. The first is the signal itself that is released by the irradiated cell. The other is the effect of the signal on un-irradiated bystander cells. These appear to be modulated in some form by LET of radiation, dose, and cell types used in the experiment. The goal of this experiment was to compare the effects of two ionizing radiations with different LETs, replicate the dose curve, test bystander using a normal cell line (not tumorous), and observe the effects of temperature as an experimental parameter.

The LET dependent effects associated with the bystander effect have not been widely investigated. Much of the bystander literature has focused on low fluence α-particles most likely because that was the focus of the original research done by Nagasawa and Little [5]. It is also of interest because of the high LET dose the human population receives as a result of radon alpha particle daughters [31]. Lastly, new technology has evolved sending exact numbers of high LET particles at single cells, which has alleviated some uncertainty surrounding the prior broad beam methods that required Poisson statistics and encompassed a certain error [5, 15]. Therefore, this study compares LET effects to determine if there is a dependence of LET on damage in bystander cells.

4.1 Direct Experiment

The first task was to show a direct to indirect comparison in normal AG01522 fibroblasts. In both X-ray irradiations as well as α-particle irradiations, the bystander effect increased approximately 2-fold with respect to the control average. This finding gives evidence that the bystander effect using normal AG01522 fibroblasts cells is LET independent. However, the directly irradiated AG01522 fibroblasts appeared to be LET dependent as seen by the chaotic trend in α-particle irradiations as shown in Figure 4. In order to form MN, the cells must be viable and capable of reproducing. If the dose is too high and the incident damage is too great, the directly irradiated cells enter cell cycle arrest and are incapable of reproducing and incapable of showing the MN endpoint [19,16]. After a dose of 0.6 Gy the trend in Figure 4 becomes unpredictable because the cells irradiated with higher doses of α-particles lost cell function capabilities and ability to reproduce. The lower LET irradiation at high doses did not appear to inhibit cell reproduction (refer to Figure 3).

In contrast to the AG01522 directly irradiated cells, the normal AG01522 fibroblast bystander cells did not seem to be LET dependent. In this research, the AG01522 bystander cells showed a 2-fold increase over controls, which reached a plateau at approximately 1 Gy in both high and low LET radiations. However, others have found different cell lines to behave differently concerning

the bystander effect. Anzenberg et al. provided data to support LET dependence in the bystander effect when the cells used were DU145 tumor co-cultured cell experimental systems [Anzenberg (not yet published)]. This contrast supports the overall theme seen in research; the bystander effect is very much dependent on the cell culture system used in experimentation [6].

The results were as expected and there could be health benefits in radiation oncology if doctors knew at what dose of each LET impaired cellular function and specifically the ability to reproduce. There were no apparent changes in the results from MN formation even after directly irradiated cells were compromised; however, the cell killing is an important objective in radiation therapy. Therefore, further research should investigate samples directly irradiated at closer dose ranges in order to establish exact dose ranges where varying cell lines lose the ability to reproduce when hit with high LET radiation.

4.2 Bystander Experiment

As mentioned before, the type of cell used in an experiment can greatly affect the results. Some cell lines have been known to not respond to signals released from directly irradiated cells [6, 17, 4]. Other studies have shown 2-fold increases in MN formation in DU145 tumor cell co-culture system irradiation by X-rays [19, Anzenberg (not yet published)] and AG01522 fibroblast cells in a medium-transfer experiment after heavy-ion irradiation [32]. Alternatively, using α-particle irradiations, Nagasawa repeated three separate research projects (CHO cell lines and mouse cell lines) and demonstrated a plateau at around 1.4-fold of background levels [5]. Koturbash et al. also showed an increase of 1.3-fold over background in vivo using a mouse model and measuring γH2AX [7]. The results from the experiment described in this paper do agree with the other experiments using the same cell lines. However, the results are remarkably different across different cell lines.

Also it should be mentioned that although most of the data points in the α-particle bystander experiment (results shown in Figure 8) were significant, 0.1 Gy dose in the α-particle irradiation was not. The data point remained in line with

the general trend, but likely was not significant because the experiment was repeated separately only three times. The results at this particular data point were not as precise and thus created a larger standard deviation.

4.3 Effects of Temperature on Bystander Effect

As early as the 1950s researchers were investigating temperature as a protector from radiation damage. Belli et al. concluded in 1963 that temperature protects tissue by slowed rate processes and recombination of molecules in the metionic state before deleterious reactions could happen [24, 25]. This result was seen in a number of other studies [22, 23, 25, 26]. However, it has not been questioned whether these protective effects can be carried out in bystander cells. Lower temperatures stabilize protein conformations while slowing other cellular processes [25, 26]. If temperature could provide radiation induced damage protection with regards to signal moderation, then we would have expected to see the effects of the signal mitigated between direct and indirect cells.

Independent of LET, the results from these experiments did not show a significant dependence or change when the irradiated AG01522 cells were incubated and irradiated at less than 4°C. However, Anzenberg et al. (not yet published) showed protection at this lower temperature with a DU145 co-culture system.

The comparison of results from AG01522 fibroblasts with DU145 tumor cells again supports the argument that different cell lines react very differently within a bystander experiment. However, the proposed medium-mediated signaling between the two is considered to be reactive oxygen species (ROS). Including only medium-mediated signaling, Anzenberg et al. found that the type of signaling (nitrogen oxide (NO) or general ROS) that occurred between direct irradiated DU145 cells and either DU145 or AG01522 fibroblast bystander cells was dependent upon the type of incident radiation on the irradiated cell. The study also suggested that signaling depended on the receiving cell type. There was no evidence in the study discussed here to support a LET dependence, but the comparison of results suggest that temperature protection may only be

available to certain cell types, which in turn could provide more definitive evidence that different types of signaling occur in different cell lines. This result is not too surprising, since the intrinsic communication between tumor cells is different than that between normal cells. It has already been postulated in other studies that the signals are inherently different [2]. However, whatever protection could be offered direct cells by lower temperatures does not seem to decrease the formation of MN in AG01552 fibroblast bystander tissue.

This theory is interesting because the lower oxygenation of tissue seems responsible in direct cells for this protection. The relationship to oxygen suggests a radical process is involved and less prevalent (less available oxygen) at lower temperatures [21, 22, 23, 30]. Likewise, the most widely accepted mechanisms in the bystander effect also include ROS mechanisms in some shape or form. However, at lower temperatures the protected cells are still able to release signals to damage bystander cells. Hamada et al. suggests that ROS play a larger role in bystander tumor cells, whereas NO plays a larger role in normal bystander cells. If true, this would support the conflicts seen above with temperature. Since tumor bystander cells rely more heavily on ROS signaling reception, the temperature can exhibit a larger effect. However, since temperature does not change NO signaling there would be no temperature dependent bystander effect. The results and information currently known in this area of bystander research are inconclusive. There are clear contradictions in research and signaling appears to rely on LET, cell lines (of bystander and direct cells), and dose. The results from the temperature experiment leave more questions unanswered. The next step might be to cool the bystander cells as well and see if the temperature protection could be provided at the reception of signals from directly irradiated cells.

5. Further Research

Concerning the results presented here, there are more questions to address. In the future, researchers should perform more direct α-particle experiments with doses closer in range to pinpoint a narrow distribution in which

the direct cells arrest in cell cycle. This could be repeated for various cell types. In addition, studies should include more in vivo modeling to determine if signaling is different when the communications systems are more complex [2]. In vivo models account for gap junctional intercellular communication (GJIC) as well as signaling that occurs via medium. But as Prise et al. have shown, different ex vivo tissue types respond differently to bystander signaling [2].

There is also a need for continued rigorous investigation at low dose irradiations (especially low LET) to support the current idea that radiation-induced genomic instability is most important in the 0.1 Gy range. This is arguably most important since the majority of the human population will not encounter any other radiation than low fluence background levels or rare medical tests. Similarly, more in vivo research to investigate radon involvement in low doses and human exposure in high radon areas would be necessary because radon exposure affects every human being as it accounts for half of our annual background dose [12].

Since the effects of temperature on bystander tissue clearly depend on cell type [Anzenberg (not yet published), 2], more studies should be performed to elucidate these parameters and use the knowledge to learn more about various signal processes. One idea would be to follow would be to test bystander tissue incubated at lower temperatures during co-culturation to learn if temperature protects the bystander cells at the step of signal recognition or reception.

All of these areas of future research should be kept within the common goal of improving radiation protection. By further defining and characterizing the bystander effect, there will be smaller margins of error in dose calculations and scientists will better be able to assess the damage of low levels or in some cases background levels of low fluence radiation.

6. Conclusion

There is a vast amount of research in a number of different cell culture systems that proves the bystander effect is real. However, the mechanisms and signaling factors provide a great challenge to radiation biology. It is still unclear

how direct intercellular communication through gap junctions or via signaling factors released into the cellular medium both contributes to damage in the bystander cells. But even assuming that question could be answered; there is still a wealth of knowledge to be uncovered concerning the signals themselves. There are many studies focusing on one part of the signaling proposed mechanisms, for example ROS damage or increased levels of p53, but no study has yet to demonstrate the order in which all of these pieces come together to model the bystander effect via gap junctional intercellular communication, via medium-mediated signaling, and in vivo which combines both signaling pathways.

While it seems to follow logic that these effects are relevant to current radiation health safety as well as medical uses of radiation, there are too many unknowns to put any plan into action. The effects of damage in bystander tissue adjacent to irradiated cells undoubtedly play a role in margins of error in dose calculations to tissue, but the variables which alter its effects are dose, LET, radiation quality, and cell lines. This phenomenon will likely become an issue in vivo as well because the lung tissue is very different than the tissue in a tumor or heart. Therefore, the dose calculations incorporating the bystander effect will have to be very thorough and complicated.

This study provides additional evidence to the field of bystander effects. There is a narrow range of dose with high LET radiation that arrests the cell cycle in directly irradiated AG01522 fibroblast cells and prohibits cellular reproduction. This finding could be investigated further to specify the narrow range and show further implications for its use. Secondly, the bystander effect in normal co-cultured AG01522 fibroblast cell lines did not appear to be dependent on LET. Both high LET and low LET radiation reached a plateau after 1 Gy, which doubled the background level of MN induction. Lastly, the expected temperature protective effects in bystander cells were not seen in high or low LET radiations of normal co-cultured AG01522 fibroblast cell lines. The most probable reasoning being that a) the cell lines might not respond to temperature effects in

signal moderation or b) the inherent signal released by AG01522 cells is not depending on oxygenation and thus temperature.

The results from this experiment both agreed and contradicted some expectations formed after review of other published articles. Most importantly, it provided a few answers and a lot more questions to the field of research. This research suggests there are LET dependent effects in directly irradiated cells, however the effects on normal AG01522 human fibroblast bystander cells appeared to be uniform regardless of radiation quality. Also in this line of cells, there was no obvious dependence on temperature to change the bystander effect outcome. This information along with the new questions it poses should be used in future research. As always the end goal is knowledge and improvement in the quality of healthcare and radiation protection.

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8. Appendix/List of Figures

Figure 1: A co-cultured system. The cells on the bottom layer are irradiated. The non-irradiated cells on the insert (the bystanders) are assayed for damage.

Figure 2: Binucleated cells (BN) with and without micronucleus (MN). The cells were stained with DAPI and recorded under fluorescent microscope. This view of MN assay shows MN near a BN cells. This particular image is taken from an AG01522 fibroblast, which have been directly irradiated at 2 Gy. The red arrows point to those binucleated cells that also have micronuclei.

Figure 3: Micronuclei damage induced by X-ray irradiation. This figure shows the comparison of MN damage produced in directly irradiated cells (X-ray irradiations) versus bystander cells. The error bars represent +\- one standard deviation.

Figure 4: Micronuclei damage induced by alpha particle irradiation. This figure shows a comparison of MN damage produced in directly irradiated cells (using α-particles) versus bystander cells. The error bars represent +\- one standard deviation.

Figure 5: AG01522 fibroblast bystander control cells in X-ray bystander showing binucleated cells (BN) with and without micronuclei (MN). The cells were stained with DAPI and recorded under a fluorescent microscope. The red arrows indicate BN with MN.

Figure 6: AG01522 fibroblast bystander cells which have been co-cultured with AG01522 fibroblast cells irradiated with 2 Gy in X-ray bystander experiment. Shows binucleated cells (BN) with and without micronuclei (MN). The cells were stained with DAPI and recorded under a fluorescent microscope. The red arrows indicate BN with MN.

Figure 7: X-ray radiation induced bystander effect in non-irradiated AG01522 normal human fibroblast cells. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.

Figure 8: Alpha radiation induced bystander effect in non-irradiated AG01522 normal human fibroblast cells. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.

Figure 9: Temperature mediated bystander effects using X-ray radiation. There was no significant difference between warm and cold controls or between warm and cold radiations (1Gy). Cold-Warm denotes the direct irradiation occurring at low temperatures while the bystanders were kept warm. Warm-Warm denotes

that both direct and bystander cells are kept warm. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.

Figure 10: Temperature mediated effects using α-particle radiation. There was no significant difference between warm and cold controls or between warm and cold radiations (1Gy). Cold-Warm denotes the direct irradiation occurring at low temperatures while the bystanders were kept warm. Warm-Warm denotes that both direct and bystander cells are kept warm. Errors show +\- one standard deviation. One asterisk describes $p<0.05$, two asterisks describe $p<0.01$. Statistical significance is based on comparison to control.