XVI: Classic Radiobiology Methods

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Basic Methods in Radiation Biology:

Both students and established scientists transitioning to the field of radiation biology have at their disposal both classical and new sophisticated methodologies for studying the effects of ionizing irradiation on cells, tissues, organs, and organ systems, as well as total body effects. These range from classical clonogenic radiation survival curves (which are applicable to the study of freshly explanted committed tissue stem cells, and their differentiated progeny) to a spectrum of molecular biologic techniques for quantitation of genetic and epigenetic changes caused by radiation either directly or indirectly mediated by cytokines or cell contact between cell populations. The present chapter deals with the specifics and applications of each of a variety of available assays, and how to use each to answer specific questions.

The Clonogenic Radiation Survival Curve:

The classic publications of Puck (1) and Elkind (2) provided the basis for the study of ionizing irradiation effects on cells in tissue culture. These assays remain the foundation of radiobiology. An understanding of the importance, limitations, and the interpretation of data and appropriate uses of this technique remains essential to the field.

What is the Clonogenic Radiation Survival Curve?

Ionizing irradiation effects on living organisms start with the radiation chemistry of water. Hydrolysis of water leads to formation of free radicals, which interact with nuclear, cytoplasmic, and membranous structures in cells. The presence of oxygen in cells provides another target for radiochemical generation of free radicals. The clonogenic survival curve is an assay for direct, as well as delayed effects of irradiation on cells in culture (1-2). The assay requires an understanding of the principles of the radiation dose response curve, concept of delay after irradiation to detection of induced cell death, and the role of cell cycle in the measurable effects.

***In Vitro* Studies:**

Cells including established cell lines, or those from freshly removed tissues which adhere to a plastic or glass surface, are irradiated in either suspension culture before transfer to flat surfaces, or after they are attached. Cells are irradiated to doses ranging from 0 Gy (baseline control) up to and exceeding a dose, which will kill all cells in the assay. For mammalian cells in culture, this dose is usually between 1 and 8 Gy (8 Gy = 800 cGy = 800 RAD). [Other sections in this web-based textbook describe the importance of radiation dosimetry in calibrating the irradiator used to carry out the dose-response curve experiment, and the importance of dose rate (cGy/minute) on the delivery of each of the doses in the dose response curve.] It is essential to report, the dose-rate information in the methods sections of manuscripts when publishing data on clonogenic survival curves because of the inherent fluctuations in results based on this principle.

Cells, which can form colonies on a flat surface, are usually irradiated in suspension in tissue culture tubes to each of several doses, and then are transferred (plated) to a flat surface. There has been relatively little attention directed to the types of tissue culture plates (plastic or glass) used for radiation survival curves. Original studies were done with glass tissue culture plates; however, multiple plastic culture plates and vessels have been used each with different inherent and potentially volatile components of the plastic product. These chemicals can influence radiation killing of cells in culture.

The critical importance of control (0 Gy) data points to establish a baseline (cloning efficiency) must be emphasized. When comparing two cell lines, the cloning efficiency in the absence of irradiation must be similar and must be standardized. For example, depending on the size of the surface area to be measured, plating cells at a density of 1000 cells (per 10 ml volume of medium in a 40 cm^2 surface area) may yield an average of 50 colonies 7 days later, each of which has greater than 50 cells per colony. After 7 days incubation at 37^0 C, there should be 7 cell divisions for most permanent established cell lines. Such data cannot be reliably compared to that using another cell line, which when plated at the same density in the same vessel produces less than 1 colony per dish. The latter cell line would require a plating density of 100 to 1000-fold greater number of cells to get the same 50 colonies. This means the latter line has a reduced plating efficiency. In order for robust data to be derived, it is important to establish that plating efficiencies (PEs) between cell lines, cell populations, and between experiments are comparable. Inferior health of a cell line (mycoplasma contamination) or inherent differences in growth in a particular culture medium using fetal bovine serum and/or antibiotics, as well as antifungal additives may explain a reduced PE.

The number of colonies per 1,000 or per 10,000 cells plated in the absence of irradiation is known as the plating efficiency. Cell lines or tissue samples of comparable plating efficiency can be realistically compared in our clonogenic radiation survival curve. It is well known that the establishment of cell lines in tissue culture and derivation of sub-clonal lines (cell lines derived from a single cell) have a higher plating efficiency than freshly removed tissues. This fact is based upon the conditions of tissue culture. Most cell cultures are carried out in fetal bovine serum, and in some cases, human serum or human plasma. Not all cell populations can grow under these conditions. The use of antibiotics in the culture medium to prevent contamination during the 7 – 14 days in the incubator is often a confounding factor and can reduce plating efficiency of some freshly removed mammalian cells and their dispersal in single cell suspensions. Explanted tissues contain cells, which were in contact in vivo.

Why is plating efficiency so important?

There are confounding variables that may decrease the health (plating efficiency) of one cell population compared to another. Cell lines in tissue culture, as well as fresh tissues, should be screened for contaminants such as mycoplasma, bacteria, or viruses that can decrease the plating efficiency by decreasing the health of one cell line or cell population in culture.

The clonogenic survival curve must allow time for irradiated cells to go through 7 cell divisions to reach a colony of 50 cells or greater per colony. To carry out these studies, there is need for

basic technical expertise to distribute cells evenly across a flat surface of a culture plate. Clumping of cells in the middle of a plate may result in close proximity of single cells, which can form colonies that can touch each other by day 7 and will make scoring impossible.

Colony formation is scored when cells from unirradiated control culture plates have undergone seven cell doublings, which is the number of doublings required to give a 50 cell colony. Genetic factors can influence PE. For example, cells from a mouse deficient in TGF-β (transforming growth factor-β homologous recombinant negative, TGF-β-/-) have a reduced PE (Fig. 1) compared to cells from a heterozygote TGF-β+/- or wild type control C57BL/6 mouse TGF-β+/+ (Fig. 1).

Why are seven cell doublings required for a clonogenic radiation survival curve?

The classic publications of Puck (1) and Elkind (2) used the seven cells doubling protocol to achieve a 50 cell colony, and this has become a standard for the field of basic radiobiology. The reason for this arbitrary colony size, which has been extrapolated to growth of hematopoietic cells in semi-solid medium (to be discussed below), is based on the fact that radiation killing may not be registered during or after the first cell doubling. DNA double strand break repair, which is a basic cause of initiation of the events producing apoptosis and cell death, may not register during the first cell doubling. In addition, growth arrest, which can be induced temporarily by irradiation (often called quiescence or if permanent, called senescence when applied to in vivo systems), may lead to a second or third cell doubling, but then a failure to achieve the subsequent cell 4 doublings necessary to produce a 50 cell colony.

Calculation of D_0 and ñ:

Some cell lines and freshly explanted single cell populations demonstrate little reduction in clonogenic survival experiments after modest radiation doses (1 – 4 Gy). Other cell lines and explanted cell populations show a significant reduction in colony formation with the same doses of irradiation. These early observations led to the concept of "sub-lethal irradiation damage". The dose of 1 – 4 Gy, which may produce significant reduction (over 90%) in clonogenic survival of lymphocyte colony forming cells, spermatogonia, or oogonia, may register no detectable reduction in colony forming efficiency of mesenchymal cells or fibroblasts. Plating triplicate or quadruplicate cultures after each irradiation dose allows quantitation of colonies at 7 – 14 days, and if dispersal of cells in all plates has been uniform, it should yield scoring of very tight data points and small standard deviations (and in multiple experiments, small standard errors). A sound laboratory technique allows for detection of difference between clonogenic survival reductions at low radiation doses compared to no significant reductions. Lack of significant reduction in cloning of cells after relatively low radiation doses (1-4 Gy) has been described as an experiment, which produces a "shoulder" on the radiation survival curve (See TGF-β+/- 2C9 cell line in right hand panel) (Fig. 1).

D_0 may be calculated by two methods. By definition, D_0 is the dose required to reduce the surviving fraction to 37% on the linear exponential portion of the survival curve. The data for the survival curve is plotted according to the Single-Hit, Multi-Target Model. Once the linear

portion of the survival curve has been determined, you determine the dose where 37% of the remaining colonies survived. For instance, if the linear portion of the curve begins at 3 Gy with a survival fraction of 0.1, then 37% survival will be a 0.037. If the dose resulting in survival of 0.037 was 4.5 Gy, then D_0 would be 4.5 – 3 or 1.5 Gy. A second way to determine the D_0 is to calculate the slope of the exponential portion of the survival curve. D_0 = 1/slope so if the slope is 0.66, then the D_0 is 1/0.66 or 1.51 Gy.

To calculate the extrapolation number (ñ), the survival curve data must be plotted according to the Single-Hit, Multi-Target Model (Fig. 1 left hand panel). Once the linear exponential portion of the curve is determined, a back extrapolation of this linear portion of the curve to the y axis or vertical axis is made. The point where the back extrapolation intersects the y axis, or y-intercept, is the ñ and indicates the width of the shoulder of the survival curve. The TGF-β+/- line has an ñ of 18.0 $\pm$ 3.6 and the widest shoulder (Fig. 1).

The computer software program utilized to calculate the shoulder on the survival curve is based on a log/linear plot of the data. The vertical axis (ordinate) is graphed on a log scale while the irradiation dose on the horizontal axis (abscissa) is plotted on a linear scale. All cell lines or cell populations, show a dose response dependent reduction in clonogenic survival at higher doses. A radiation dose, which reduces the survival fraction by 1/3 of a log, is described as the D_0 (Fig. 1). The linear portion of the survival curve is then extrapolated back to the vertical axis and back to 0 Gy irradiation dose on the horizontal axis (thus going back past the shoulder on the curve). The point on the vertical axis at which this line intersects the vertical axis is known as the extrapolation number or ñ. Cell lines or cell populations that have a very broad shoulder will have a large extrapolation number.

Cell populations such as clonogenic lymphocyte progenitor cells and derived cell lines may have little or no shoulder and will have a low extrapolation number (ñ). Original interpretations of the difference between two cell populations is still followed in radiobiology. The D_0 and ñ indicate that the "radiosensitivity" of the cell line is based solely upon the D_0. A cell line with a large shoulder on the survival curve may or may not have a large D_0. A relatively high D_0, which is a dose required to reduce survival by 1/3 of a log, may be the same between two cell lines, one with a low and one with a high shoulder. Therefore, the shoulder can be a separate measure of radiation effect if interpreted as the ability of cells to accumulate sublethal damage. In other words, the dose on the shoulder was not lethal (i.e. the cells withstood the killing effect at those lower doses). A broad shoulder may reflect better DNA repair, better cell cycle checkpoint control, higher antioxidant stores, and/or other mechanisms.

The scoring of colonies after 7 cell division and calculation of D_0 and ñ follow several specific principles of radiobiology.

How Does One Score a Clonogenic Survival Curve Assay?

Most important is the individual observer's established definition of a 50 cell colony. As shown in Figs. 2A-C, which amplify the data shown in Fig. 1 for 3 cell lines, adherent cell colonies scored at day 7 demonstrate the difference between a colony that is at least 50 cells (solid arrow)

compared to a cluster of cells that is in this case 10 – 12 cells (open arrow). If cells are dispersed appropriately in the plate and allowed to settle evenly over the entire surface area, then the scoring of triplicate or quadruplicate plates at each time point should be very "tight" between replicates. The cluster of cells (white arrow, Fig. 2B), which did not reach the threshold of 50 cells or greater for scoring should not be discounted when understanding radiobiology, as it is not known whether this cluster would have grown to a size of greater than 50 cells if allowed to stay in the incubator for additional days. In this case, the time point of day 7 for scoring was chosen. If one waited for all clusters in the plate to reach the size of a colony, larger colonies already at 50 cells or greater at day 7 would have grown together and fused, forming a monolayer, thus making it impossible to score total number of colonies, which in this case was per 10^4 cells plated. For this reason, it is important to designate both comparable plating density and baseline plating efficiency to allow for scoring on a given day.

Plating density is the number of cells added per culture well. If cell lines are clonal (derived from a single cell) and have been passaged in culture for many weeks or months, the robustness of growth may allow plating of as few as 500 cells per dish. In contrast, if these are freshly removed cells from the bone marrow, intestine, or other organs, and sorted by flow cytometry, the plating efficiency may be very low due to the absence of time allowed for cells to adapt to growth in culture medium in fetal bovine serum, horse serum, and/or other combinations of growth factors. For this reason, said fresh tissues are sorted; populations of fresh tissues may require a plating density of much higher in order to get the expected number of colonies per plate of between 10 and 100. The number of 10 to 100 per plate has been designated as appropriate for scoring discrete colonies (providing cells are dispersed adequately) at day 7. Plating efficiency is a different term, and describes the "health" of cells or cell lines when two populations are compared. Plating efficiencies must be comparable between cell populations if accurate radiobiological characterization is desired. For example, a clonal cell line, which has a plating efficiency of around .01% cannot be accurately compared to a cell line with plating efficiency of 10%. The former cell line should be screened for contamination with mycoplasma, a lytic virus, or other contaminant that may have lowered the plating efficiency.

In some cases, there may be a genuine need to increase the plating efficiency of a cell line by removal of a specific growth inhibitory factor from the culture conditions. In these cases, a separate research question arises. To solve this problem, cell lines with a low PE should be cloned in single cell wells in the absence of an inhibitory factor (for example, penicillin/streptomycin antibiotic, which is usually added to culture conditions.). If they all show the difference independent of a toxic growth condition, then the data represents a new research question. Why is the dose more radiation toxic to one cell line?

Once data is collected, data points scored, and repeat experiments carried out to ensure the reproducibility of a finding, there is need to calculate D_0 and ñ, the relevant parameters for determining radiosensitivity of a particular cell line or population, as described above. A software program, which takes into account the single hit and multi-hit compared to the linear aggression model, is often used. Some scientific journals require a presentation of the data in both formats, while other publications may require the use of one format consistently. It is

crucial to understand the difference between the mathematical modeling behind each of these two methods, there are numerous publications which describe the logic behind these parameters.

Other Cell Populations: Hematopoietic Progenitor Cells:

Cell populations, which grow in semi-solid medium, or in suspension culture, but do not form adherent colonies on a plastic or glass surface, present a separate challenge.

Lympho-hematopoietic colony forming cells from bone marrow, spleen, or lymph nodes, and some stem cell populations (7) from epithelial organs will form nonadherent colonies in 0.8% methylcellulose containing medium or 0.3% agar containing (semisolid) culture. Scoring is carried out the same way as looking at colonies of greater than or equal to 50 cells after 7 cell doublings (usually at day 7); however, longer intervals may be required (6). The 3-dimensional nature of the culture requires using the inverted microscope but changing the focal plane for each microscopic field to ensure visualization and scoring of all colonies. It is important to note the viscosity of the semi-solid medium in the methodology for this culture system, as this is critical to prevent cells from floating to the bottom of the dish, and forming adherent cell colonies. This adherence phenomenon occurs in the case of some cell types (macrophages from bone marrow) or can occur if there is inability of cells to grow in the semisolid medium. For some cell phenotypes, as in the case of lymphoid colony forming cells, these cells may not grow at all on the flat surface area, and require the semisolid medium (6).

How Can Radiosensitivity be Calculated in Cells that Do Not Form Colonies?

Cells, which do not form colonies but do grow in suspension culture cannot easily be tested in a clonogenic survival curve assay; however, single cell plating using 96 well (500 microliter) tissue culture wells can facilitate generation of radiation survival curves. It is imperative to ensure that only one cell is deposited per well at the time of plating via visual inspection. Once this has been accomplished, scoring numbers of single cells that have grown into a colony can be performed 7 days later. This procedure can still generate a radiation survival curve as is the case with some hematopoietic cell populations grown in liquid medium, provided the medium is “supplemented” with specific growth factors.

Assays for Rapid Ionizing Irradiation Effects on Cells in Culture:

While the clonogenic radiation survival curve is the “gold standard” for reporting the effects of radiation on cell lines in culture, it does require incubation through seven cell doublings. Since its original description of irradiated effects on cells in 1956 (1), much work has been devoted to understanding the events, which occur during the colony during the 7 cell doublings.

While classical radiobiology suggests that irradiation killing may not be registered after the first cell doubling, the molecular mechanism of this phenomenon has yet to be fully explained. One hypothesis is that irradiated cells, which go through a first cell doubling, have induction of genes that produce inhibitory growth factors such as TGF-β, IL-1, TNF-α, and others (4). These growth factors can inhibit further cell divisions that might otherwise have occurred in the

absence of these inhibiting factors. Studies showing different radiation survival curves for similar cell populations comparing TGF-β homologous recombinant deletion (knockout) mouse bone marrow with wild type marrow support this hypothesis (Fig. 1). The colonies of TGF-β-/- (Fig. 2C) cells are smaller than those seen in TGF-β+/+ or TGF-β+/- cultures (Figs. 2A-B), but still scored as greater than 50 cells (black arrow) compared to smaller clusters (white arrow). The D_0 and ñ can still be calculated, but the difference is apparent from visual inspection.

Other assays that cannot rely upon stability of cells in culture for seven days are available to compare ionizing irradiation effects.

Assays for Single Strand and DNA Double Strand Breaks and Repair:

More immediate effects of ionizing irradiation, which precede cell division can be scored using more molecular biologic tools and assays for the molecules, which arrive at the DNA double strand breaks within seconds after ionizing irradiation induction of the break. Ataxia Telangiectasia Mutated (ATM) phosphorylation occurs within seconds after ionizing irradiation, as the ATM molecules are one of the first to arrive at the DNA double strand break, followed by a series of 21 proteins in the Fanconi Anemia (FA) pathway. Scoring of these sites can also be carried out to quantitate immediate ionizing irradiation effects on cells in culture.

In situ microscopic staining for Fluorochrome labeled antibodies to the proteins that arrive at the DNA double strand break are available and the techniques available. These assays require histologic analysis of single cells in culture, which are examined by fluorescent microscopy. The techniques for these measurements require examination of a sufficient number of individual cells to provide for statistical analysis. Usually 100 microscopic fields with at least 10 cells per field are scored in triplicate per experiment.

The time course of appearance of DNA double strand breaks and their repair is also crucial to consider. For example, if breaks are repaired within 8 hours in one cell population, while delayed in a second population, scoring of the assay at 24 hours would give a false negative result with respect to the induction of DNA double strand breaks in the former cell line. Pilot experiments, also known as preliminary test experiments, should first be carried out by comparing two cell populations by carrying out these measurements at serial time points from as early as 5 minutes out to 24 or 48 hrs post-irradiation. The kinetics, or speed of repair, may differ between two cell lines. Therefore, these assays carried out at multiple time points with good statistical analysis of multiple examinations per experiment and multiple experiments may reveal differences in radiation repair between cell populations. Such analysis facilitated the discovery of novel radiation dose modifying proteins, microRNAs, and genes in cell populations from knockout, or transgenic mice, or during expression of late effects in cell populations removed from animals months after irradiation (4).

Analysis of DNA Single Strand Breaks, Induction of Mutations, and Epigenetic Changes:

As the complexity of cellular responses to ionizing irradiation has been elucidated over the past decades, more assays for different types of irradiation effects have been published. A definite

effect of ionizing irradiation has been shown to be the suppression of RNA transcription of some genes, and enhanced transcription of RNA for others (4). Changes in RNA splicing, expression of RNA transcript variants associated with irradiation, detection of microRNA species, and altered kinetics of degradation of RNA have all been published and associated with ionizing irradiation. Many of these assays are quite sophisticated and sensitive, but not specific to ionizing irradiation. Other forms of cell killing or delayed effects of ionizing irradiation in the presence of specific drugs may also induce these changes.

Epigenetic changes following exposure of cells and animal tissues to ionizing irradiation have recently been published. Histone acetylation and deacetylation at specific genetic sites have been shown to follow irradiation exposure. These assays have become increasingly relevant given the discovery of the importance of inherited, genetically stable changes in histone acetylation or histone methylation at specific genetic loci. Irradiation-induced changes can determine the activity of a specific gene, whether its messenger RNA will be transcribed, and whether the RNA will be activated to translate to produce a protein. Post-translational proteins can also be an effect of irradiation. These changes are relevant to radiation late effects including leukemogenesis and carcinogenesis as irradiation-induced changes in these areas may lead to evolution of abnormal clonal populations within a particular organ. Transfer of these changes during meiosis may lead to expression of these changes in progeny.

Ionizing irradiation induction of mutations can be quantitated by DNA sequencing and analysis of specific genes and the kinetics of repair or reversal of such mutations can be scored by assays at multiple time points after irradiation comparing sequencing information. Assay systems are appropriate for study of certain "fragile" sites in the genome such as in the X-chromosome and apply to the study of several syndromes or disease states, in which these mutation inductions have been associated with phenotypic changes in an organism.

Assays for Induction or Suppression of Specific RNA Transcripts:

Depending upon the question being asked, analysis of RNA transcription may be more important to an experimental model of radiation effects. For example, the difference in baseline levels of RNA transcripts, which are specific gene products, are detected in different tissues (4, 6, 8). Studies with both embryonic stem cells and inducible pluripotential stem cells show the cells can be "directed" in tissue culture to differentiate into specific lineages, and have shown cell phenotype specific differences in induction of specific RNA populations. Such changes can occur following ionizing irradiation, and independent of cell phenotypic differentiation (4, 8). The assay for real time polymerase chain reaction (rt-PCR) is a valuable assay system to quantitate such changes. Providing a standard curve for the rt-PCR technology is imperative, and variations in the sensitivity of the standard curve may change data from day to day (4, 6, 8). The investigators who use rt-PCR machines for quantitation of RNA rely upon the manufacturer's recommendations for specific equipment and primers purchased from specific vendors for RNA specificity. These assays can be very sensitive and valuable; however, they are also quite expensive.

With multiple specimens and need for reproducibility, the automated rt-PCR assays represent a significant step forward compared to Northern Blot assays for RNA levels, which would require large numbers of cells for each specimen to be studied, and significant technical resources compared to the rt-PCR "machine" (8).

Assay for Radiation Induction or Suppression of Specific Proteins:

Protein levels provide a valuable comparison between irradiated and non-irradiated cell populations, both in tissue culture and when removed from irradiated animals. A time course of induction or suppression of a protein may be relevant to the irradiation response. Finally, a pattern of protein induction comparing hundreds or thousands of proteins may be valuable to understanding radiation response and the effect of drugs, which may modify such a response.

Protein electrophoresis and antibody detection of "spots" on the blot of proteins can identify levels and modifications (e.g. phosphorylation, acetylation, deacetylation, etc.) of a specific protein (Western Blots) (4, 8). The Luminex assay (See Chapter on Fetal Irradiation), which facilitates this process on microscopic beads, can assay groups of proteins in specific categories including those associated with inflammation, DNA repair, and cell migration and interaction.

Chip Array Assays:

In the past decades, the availability of assay systems to screen hundreds or thousands of DNA fragments, RNA fragments, proteins, peptides, and small molecules has been available from a variety of commercial sources. These assay systems facilitate the collection of data and production of "heat maps," which provide a "big picture" visualization of the trends of change seen in multiple molecules of interest over time. Therefore, these assays allow detection of sweeping changes in the spectrum of induction or suppression of specific gene transcripts and products. Depending on the question being asked, these assays may be either useful or may complicate the experiment, as they provide a significant amount of noise in a system since primary events from irradiation, as well as secondary events (inflammatory changes) may cloud the data analysis.

In Vivo Studies:

Hematopoietic Stem Cell Assays: CFUs, competitive repopulation assay (3), and serial transfer assay. Other Tissue Stem Cells: Gut Colony assay for quantitation of intestinal stem cells are all sensitive and very reproducible (7).

Metabolomics:

The effects of ionizing irradiation on the functioning of organs and organ systems may change the spectrum of metabolic products found in plasma, urine, saliva, and other measurable fluids. These assays have become very important in biological dosimetry, and will be described in detail in other areas of this web-based textbook. However, assays are available for critical analysis of changes in each of these sampled fluids, which may follow ionizing irradiation. Critical to the

use of such assays is a clear understanding that multiple factors may change the pattern of detection and may not be specific to ionizing irradiation. However, in clinical radiation oncology, as well as in radiation counter-terrorism, biologic dosimeters are increasingly important to analysis of both the intensity of exposure to irradiation and the effect of radiation countermeasures. Several areas of this textbook will focus intensively on this topic.

Oxidative Lipidomics:

The language of cellular and tissue responses to irradiation has focused on DNA, RNA, protein, and metabolic products for the past decades. Recently, however, the observation of radiation-induced changes in lipids has been added as another diagnostic tool for radiation effects. Radiation-induced oxidative stress, which initiates from the hydrolysis of water, leads to generation of free radicals, which when combined with DNA, RNA, and proteins, induces measurable changes. Oxidative stress also induces changes in lipids. A major observation has been the discovery of ionizing irradiation specific changes in phosphatidylserine and cardiolipin (5). Cardiolipin is a critical lipid, which enters at the inner mitochondrial membrane to stabilize cytochrome-c in the mitochondria. Oxidation of cardiolipin is mediated by the peroxidase activity of cytochrome-c associated with induction of apoptosis. However, downstream peroxidation and peroxidation of multiple products of cardiolipin has been discovered. This discovery revealed an entire "language" of lipid signaling. However, the assays for oxidized lipids require the availability of specific and often quite expensive mass spectometry equipment for in vivo and in vitro assays of oxidized and peroxidized lipids. This topic will be covered in greater detail in a separate section of this web-based textbook.

Single Cell Microbeam Irradiation Assays:

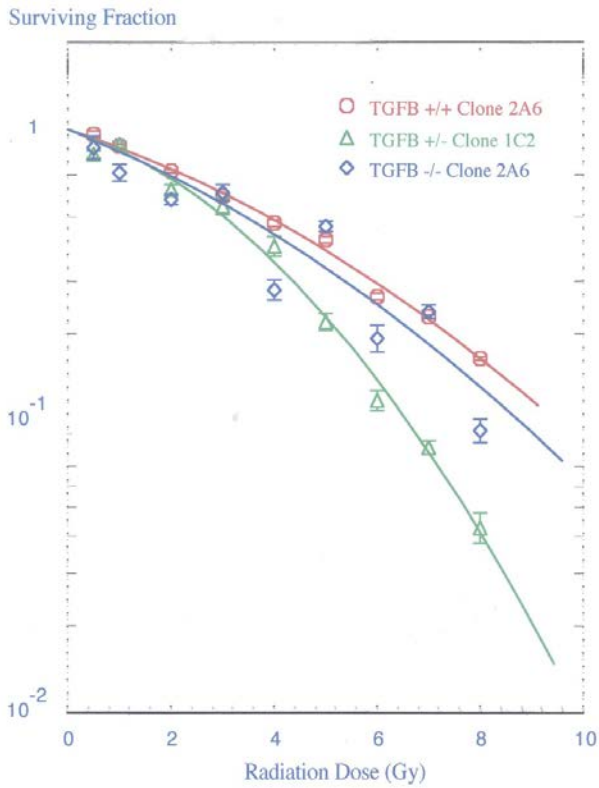
Single cell microbeam irradiation assays utilize microbeam irradiation devices with the capacity to deliver orthovoltage ionizing irradiation to insulated cell nuclei and cytoplasm. The purpose of these assays is to study individual cells compared to cells within a lawn of confluent monolayer cells in order to provide valuable information on cell contact in humoral mechanisms of propagation of radiation injury. These experimental systems are valuable to answer specific questions at the single cell level. The advantage of these kinds of systems is the ability to irradiate single cells.

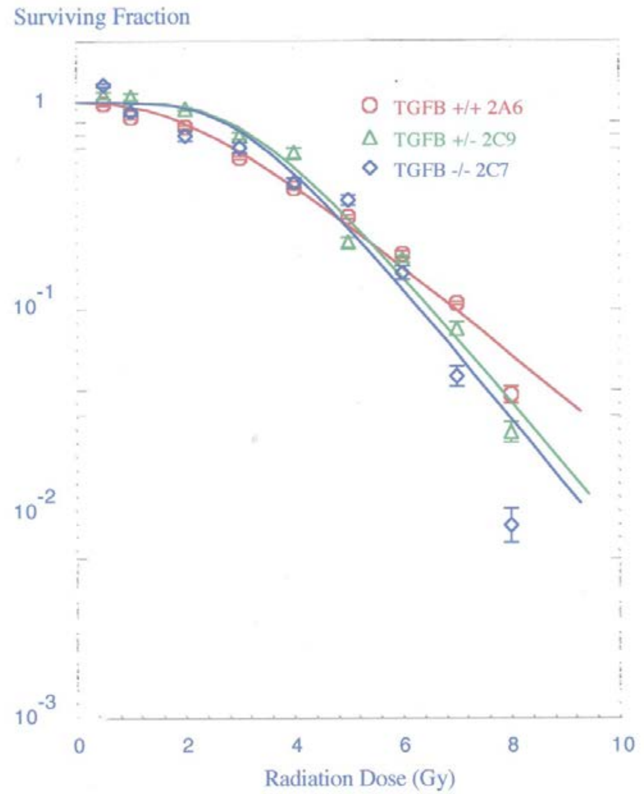
Other Co-Cultivation or Co-Incubation Assays:

A less expensive and alternative way to carry out the aforementioned studies includes irradiation of some cell populations and then admixture of these cells with other unirradiated populations. Biomarkers are available such as Luciferase+, Green Fluorescent Protein (GFP+), M-Cherry+, and fluorochrome expressing cells such that radiation of these biomarked cells could be studied in assays in the context of changes in adjacent non-biomarked cells. Another alternative approach that may also be potentially valuable includes irradiating non-marked cells and then transferring these to a lawn of unirradiated cells.

Figure 1: TGF-β-/- bone Marrow Stromal Cell Line Clonogenic Radiation Survival Curves

Single Hit/Multi Hit Model | **Linear Regression Analysis Model**





TGF-B Bone Marrow Stromal Survival Curves

Cell Lines	Plating Efficiency	Do (Gy)	ñ
TGF-B+/+	25.2 + 3.0	1.95 + 0.03	5.2 + 1.3
TGF-B+/-	18.1 + 5.5	1.33 + 0.03	18.0 + 3.6
TGF-B-/-	7.5 + 1.3	1.31 + 0.14	6.0 + 2.9

Figure 2A: TGF-β+/+ mouse marrow stromal cell line.

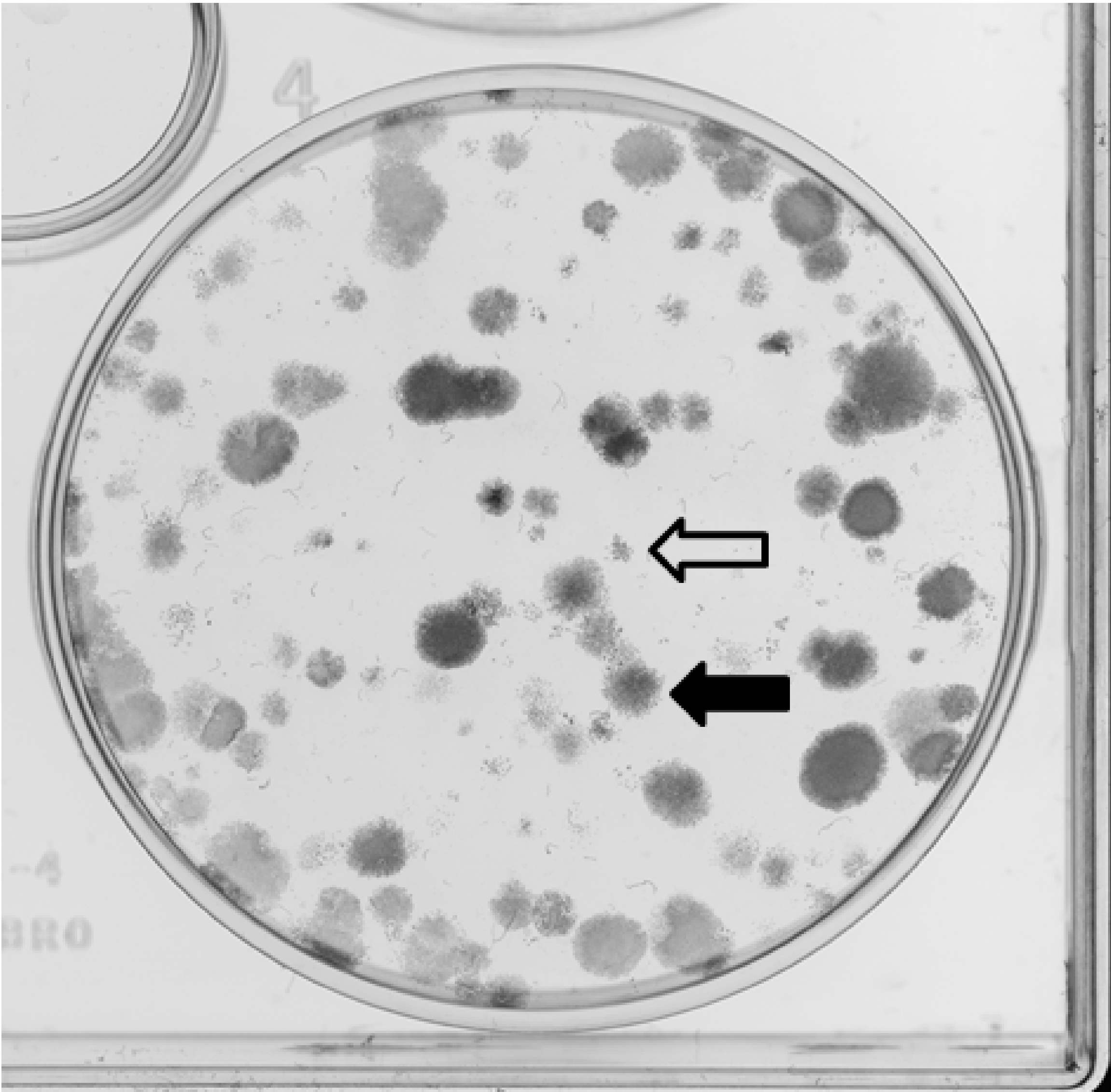


Figure 2B: TGF-β+/- mouse marrow stromal cell line.

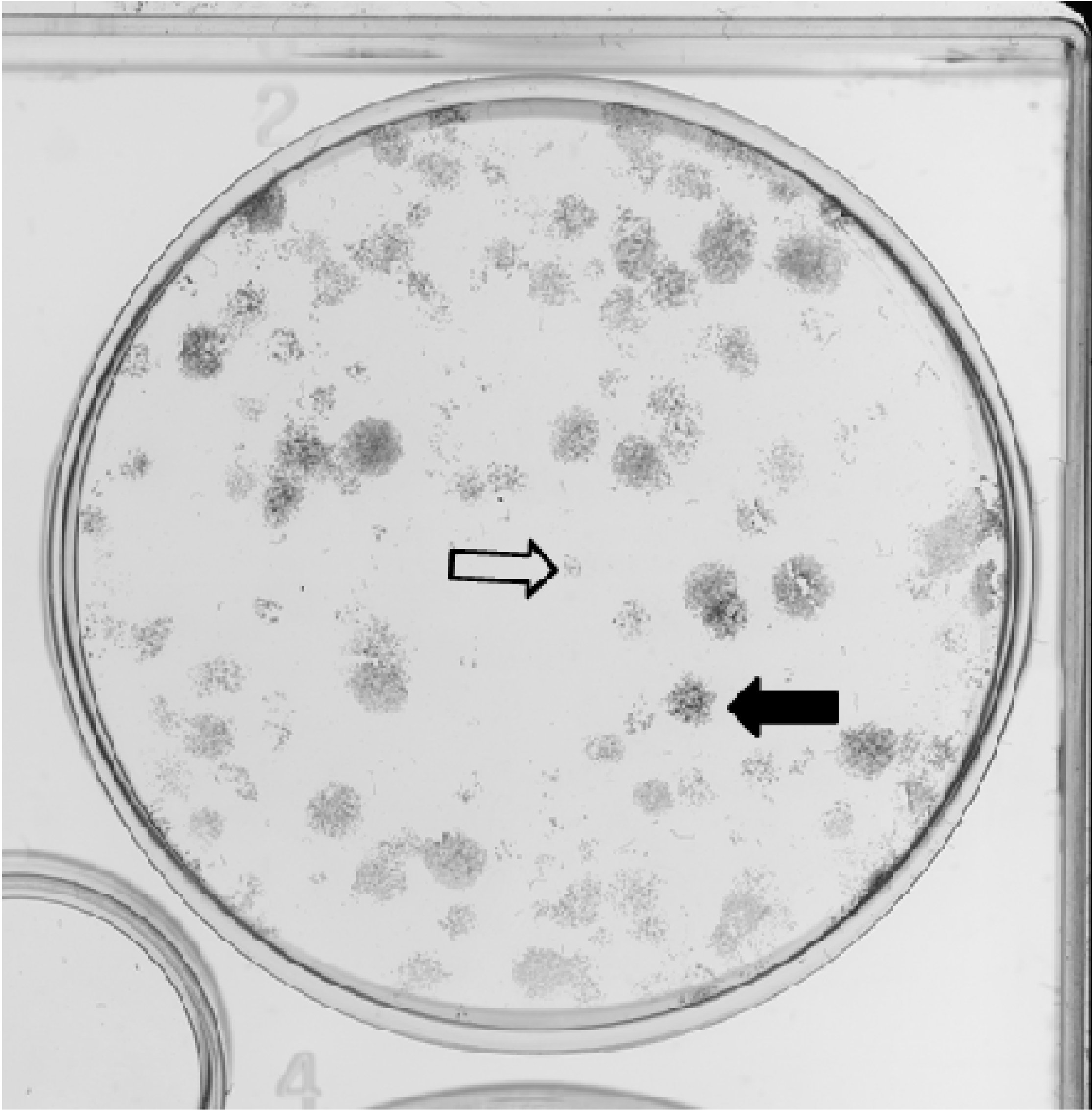
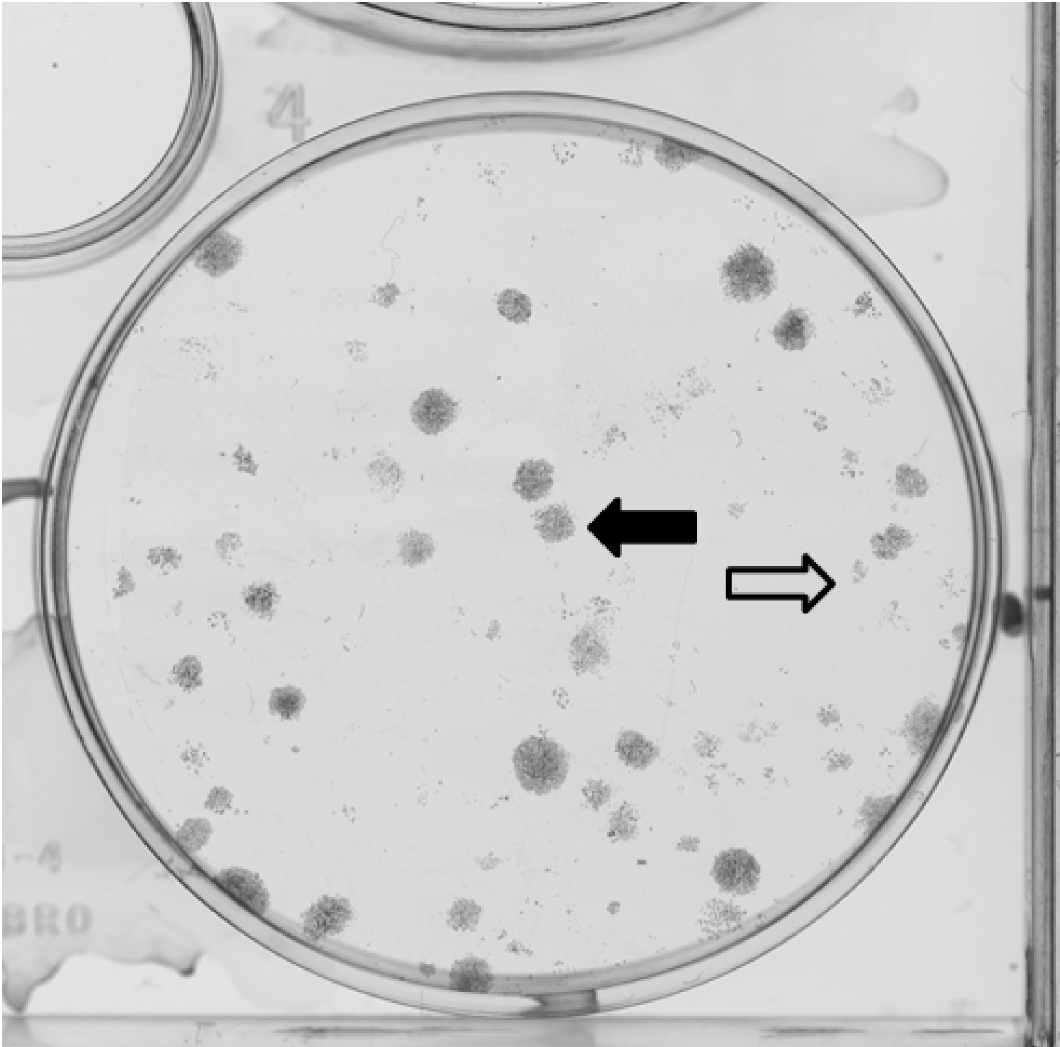


Figure 2C: TGF-β-/- mouse marrow stromal cell line.



References:

1. Puck TT, Markus PI. Action of x-rays on mammalian cells. J Exp Med 103: 653-666, 1956

2. Elkind MM, Sutton H. Radiation response of mammalian cells grown in culture: 1. Repair of x-ray damage in surviving Chinese hamster cells. Radiat Res 13: 556-593, 1960.

3. Wang L-J, Chen YM, George D, Smets F, Sokal EM, Bremer EG, Soriano HE. Engraftment assessment in human and mouse liver tissue after sex-mismatched liver cell transplantation by real-time quantitative PCR for Y chromosome sequences. Liver Transplantation, 8(9): 822-828, 2002.

4. Kalash Ronny, Epperly Michael W, Goff Julie, Dixon Tracy, Sprachman Melissa M, Zhang Xichen, Shields Donna, Cao Shaonan, Wipf Peter, Franicola Darcy, Berhane Hebist, and Greenberger Joel S. Amelioration of irradiation pulmonary fibrosis by a water-soluble bi-functional sulfoxide radiation mitigator (MMS350). Radiat Res., 180: 474-490, 2013.

5. Tyurina Yulia Y, Poloyac Samuel M, Tyurin Vladimir A, Kapralov Alexander A, Jiang Jianfei, Anthonymuthus Tamil Selvan, Kapralova Valentina I, Vikulina Anna S, Jung Mi-Jeon, Epperly Michael W, Mohammadyani Dariush, Klein-Seetharaman Judith, Jackson Travis C, Rochanek Patrick M, Pitt Bruce R, Greenberger Joel S, Vladimirov Yury A, Bayir Hulya, and Kagn Valerian E. A mitochondrial pathway for biosynthesis of lipid mediators. Nature Chemistry, On-Line: xx: 1-12, 2014.

6. Berhane Hebist, Epperly Michael W, Goff Julie, Kalash Ronny, Cao Shaonan, Franicola Darcy, Zhang Xichen, Shields Donna, Houghton Frank, Wang Hong, Sprachman Melissa, Wipf Peter, Li Song, Gao Xiang, Parmar Kalindi, and Greenberger Joel S. Radiobiologic differences between bone marrow stromal and hematopoietic progenitor cell lines from Fanconi Anemia (Fancd2-/-) mice. Radiation Research, 181: 76-89, 2014.

7. Wang Xinwei, Wei Liang, Cramer Julie M, Leibowitz Brian J, Judge Colleen, Epperly Michael, Greenberger Joel, Wang Fengchao, Li Linheng, Martin Martin G, Lagasse Eric, Zhang Lin, Yu Jian. Pharmacologically blocking p53-dependent apoptosis protects intestinal stem cells and mice from radiation. Scientific Reports, 5:8566, 2015.

8. Shinde Ashwin, Berhane, Hebist, Rhieu Byung Han, Kalash Ronny, Xu Karen, Goff Julie, Epperly Michael W, Franicola Darcy, Zhang Xichen, Dixon Tracy, Shields Donna, Wang Hong, Wipf Peter, Parmar Kalindi, Ferris Robert, Li Song, and Greenberger Joel S. Intraoral mitochondrial-targeted GS-Nitroxide, JP4-039, radioprotects normal tissue in tumor-bearing radiosensitive *Fancd2*$^{-/-}$ (C57Bl/6) mice. Radiat. Res., 185: 134-150, 2016.