RADIATION CARCINOGENESIS AND DELAYED LETHAL DAMAGE IN A HUMAN THYROID EPITHELIAL CELL LINE

John Mercer

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Radiation Carcinogenesis and Delayed Lethal Damage in a Human Thyroid Epithelial Cell Line

John Mercer

TL D466

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Dedication

I would like to dedicate my thesis to my Mother and Father who have been sources of unending encouragement and love throughout my academic career.

My mother died on the 4th August 1998 after a valiant 4 year struggle against cancer she was only 56 years old. This work is a testament to her love and encouragement.

"There are three things that last, faith, hope and love and the greatest of these is love."

(I Cor. 13: 4-13)

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GLOSSARY

DLD:	Delayed Lethal Damage
DNA:	Deoxyribonucleic acid.
dNTPs:	deoxyribonucleoside triphosphate.
dsb:	double strand break.
DMEM:	Dulbeccos's Modified Eagles Medium.
DMSO:	Dimethyl Sulphoxide.
EDTA:	Ethylenediaminetetraacetic acid.
FCS:	Foetal Calf Serum (Bovine fetal serum).
F12:	Ham's F12 Medium.
Gy:	Gray.
HaeIII:	DNA Restriction enzyme (GGCC)
Hoechst 33258:	DNA stain.
HTori-3 :	SV40 Immortalised human thyroid cell line.
keV:	kilo electron Volts.
kVp:	kilo Volts peak.
LET:	Linear Energy Transfer.
MeV:	Mega electron Volts.
MVp:	Mega Volts Peak.
nM:	nanometers (10E-6)
pM:	picometers (10E-9)
PBS:	Phosphate Buffered Saline.
PLD:	Potential Lethal Damage.
RCF:	Relative Centrifugal Force
RBE:	Relative Biological Effectiveness.
RPE:	Relative Plating Efficiency.
RPM:	Revolutions Per Minute.
SDS-PAGE:	SodiumDodecylSulphate-PAGE
SLD:	Sub Lethal Damage.
SF:	Surviving Fraction.
SF2:	Surviving Fraction at 2Gy

.ssb:	single strand breaks
SV40:	Simian Virus 40.
Taq:	Thermus aquaticus
TBE:	Tris-borate and EDTA
TEMED:	N,N,N,N – tetramethylethylenediamine
Tris:	Tris (hydroxymethyl) methylamine

Abstract

The human thyroid epithelial cell HTori-3 has been transformed with doses of either chronic and acute x-rays or strontium beta particles. Models of the past have relied upon animal cell systems to mimic *in vitro* carcinogenesis. The HTori-3 system hoped to overcome the limitations associated with these types of models by using a human thyroid cell line immortalised with the SV40 virus.

HTori-3 human thyroid epithelial cells were irradiated *in vitro*, passaged and then transplanted into nude mice. Tumours that grew over a 2-6 month period were excised and re-established in culture. Samples were stored and all tumours were taken for histological examination. Chromosome spreads confirmed the human nature of all tumours.

Following exposure to acute x-rays in the range of 0.25-2.0 Gy 13 tumours were observed in 25 recipients. Following 0.25-2.0 Gy of chronic x-rays 10 tumours from 25 recipients were observed. From a single 2 Gy exposure of strontium beta particles 3 primary tumours from 5 recipients were observed. The largest of these was re-transplanted in nude mice resulting in 100% incidence. All tumours were classified as undifferentiated anaplastic carcinomas. A small number of tumours were observed in the control cell lines, these may be the result of a general instability found with the partial transformed parental cell line.

All 2Gy tumours and those previously established from this laboratory after alpha or gamma radiation were used to test for the presence of the delayed lethal death phenotype. A number of cell and molecular endpoints were used. These included plating efficiency, cell adherence, micronucleus formation and p53 status. In all incidences, the reproductive viability of irradiated cells was below that of non-irradiated cells at up to 4 weeks post-irradiation.

The HTori-3 cell line and the techniques used to study the delayed effects of radiation may be applicable to other cell systems and may be a useful model to study the long-term effects of radiation induced genomic instability.

Chapter 1

INTRODUCTION

1.1 General Introduction

Ionising radiation is something to which we are all exposed, those who work with or are exposed directly to radiation have an increased risk of carcinogenesis. Although all the steps in the progression from an irradiated cell to a fully malignant cancer are not fully understood, research into this area will help formulate our understanding and direct solutions. A key problem within this field is the delayed effects of exposure to radiation. It is the subtly changes induced by low and chronic doses that may go unnoticed and yet may be the cause of the greatest concern.

One way of understanding the complexities involved in the transformation of human cells is to develop *in vitro* models that mimic the events that are occurring *in vivo*. The use of cultured animal cell systems has yielded important answers to many questions. However, there are intrinsic problems with these models, the use of human cells to solve a human problem are likely to result in a clearer picture of the processes involved.

This work attempts to use a human thyroid epithelial cell line designated HTori-3 as a model of *in vitro* transformation. A variety of ionising radiation sources will be used to transform these cells. Tumours produced can then be studied at the cellular and molecular level. A number of *in vitro* assays will be used to test the changes that have occurred during transformation, the model permits the comparison of

transformed cell lines to the non-transformed parental cell line from which all tumours are originally derived.

Genomic instability has been defined as the increased rate in the accumulation of new DNA mutations over time and is a common characteristic of both tumour and non-tumour lines. Genomic instability is expressed *in vitro* through a variety of phenotypic changes, these include decreased plating efficiency, elevated micronuclei formation and increased cell cycle time. Data from these and other variables will be collected from both tumour and non-tumours. In an attempt to study the delayed characteristic of *in vitro* irradiation on human cells data will be collected immediately following radiation (T=0 weeks) and at intervals post irradiation (e.g. T=2 weeks, T=4 weeks, etc). Tumours produced from this model will also be analysed for genetic alterations characteristic of radiation induced genomic instability using appropriate molecular techniques.

1.2 Initiation, Promotion and Progression

To understand how radiation transforms human cells from normal to malignant it is first important to understand how radiation induces damage in cells. DNA is the critical target for radiation induced damage which can form a complex array of changes to its structure, from minute point mutation to large chromosomal rearrangements (Watson *et al,* 1994).

1.2.1 Initiation

Initiation is the first alteration to the DNA helix, which is usually a rapid and irreversible process. The agent of initiation can be chemical, biological or environmental. Each of these mutagens has its own particular method of inducing a

lesion in the target molecule e.g. ionising radiation often causes point mutations, while UV produces bulky adducts. Any variety of DNA alterations can be thought of as the initiating lesion. These include point mutations, inversions, substitutions and amplifications. Proto-oncogenes, oncogenes and tumour suppressor genes are all potential targets.

1.2.2 Promotion

Promotion can be defined as the phenotypic changes that result from the primary initiating lesion. The cellular changes can be seen *in vitro* as differences between individual cells or colonies. These changes may include increased cell cycle time, increased tolerance to DNA damage or increased cytoplasmic volume. *In vivo* these changes may be exemplified as the formation of nodules or in the case of HNPCC (Hereditary Non-polyposis Colon Cancer) the formation of colonic polyps. The time course for these changes may be years or decades.

1.2.3 Progression

Progression is the final stage whereby a mutated cell has accumulated enough damage that it is able to break free from the normal growth constraints. The tumour now has an infinite potential for growth and reproduction. Moreover, it has metastatic potential. It is able to enter the systemic circulation and produce new tumours at sites distant from the original. Certain genes have been identified which when mutated transfer the cell from a state of promotion to independent progression and eventually to full tumourigenesis. P53 is a known candidate in HNPCC.

1.3 Clonal nature of cancer

It is now proven that cancers can arise from single cells (King, 1996). Although many mutated cells may be present in a population, selection pressure ensures that usually only one of these will survive to dominant. Evidence for this initially came from studies investigating cellular products e.g. immunoglobulin and certain isoenzymes. Under normal conditions a variety of immunogloblins are produced from the heterogeneous population of B cells, but a B cell cancer will produce only a single immunoglobulin. (Ruddon, 1995) Using molecular techniques that involve genetic analysis it has now been proven that all the cells in a tumour can contain the same mutation e.g. Philadelphia chromosome. Research into colorectal cancers has provided proof of the classical multi-step process from normal epithelium to a full malignancy (see figure 1.1). At each step specific gene loci have been implicated in the phenotypic changes that occur during the development of the tumour.

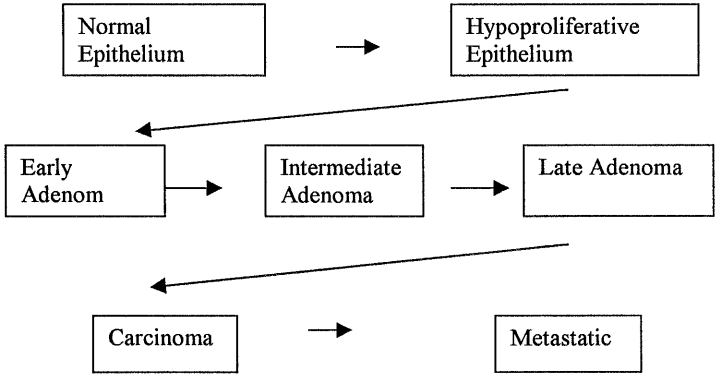

Figure 1.1 Multistage Carcinogenesis. Cancers like HNPCC are known to follow a stepwise progression from normal epithelium to full malignancy.

Investigations have shown that changes in the phenotype of the tissue can be directly correlated with losses at specific gene loci. It is not known whether all tissue have

to follow the same path to transformation or whether certain genes can be missed, skipped and gone back to or replaced with equivalents, though it is presumed likely.

1.4 DNA Damage

All cancers are characterised by mutations in their DNA. A long list of molecular rearrangements in human neoplasms has been created to which new members are continually added. Translocations such as the Philadelphia chromosome described by Nowell and Hungerford in patients with chronic myelocytic leukaemia (CML) spans lengthy sequences of coding DNA. Techniques to resolve these rearrangements, such as G-banding could only ever define areas on a chromosome of 5x10E6 base pairs. Deletions of 2 million base pairs or less would be difficult if not impossible to spot, bearing in mind that an average protein of MW 50,000 is encoded by 1200 base pairs. This means that over 1000 genes could be lost in the smallest area resolved by G-banding, (Ruddon, 1995). Mutations can come in many forms: deletions, substitutions, inversions and amplifications are just a few. Point mutations are one of the commonest types of mutations and are able to directly inactivate entire exons preventing gene transcription or inactivating protein products. It is thought that the accumulation of these mutations gives rise to the defective genotype termed aneuploidy also associated with oncogenic cells and cell lines. Aneuploidy refers to cells containing unusual numbers of chromosomes, either too few or too many. Where as polyploidy refers to the duplication of the entire genome. The increased frequency of mutations is thought to destabilise the genome and increase the rate of accumulation of mutations (Morgan, 1996).

All cells are subjected to a barrage of insults and genomic DNA is key target for most of these. It is DNA that holds the code for all molecular processes within the cell. Damage to key regulatory genes such as p53 by ionising radiation has been found to directly effect a cells reproductive capacity and its subsequent ability to transform. An important question is not only how ionising radiation damages DNA but what kinds of damage are induced so that by understanding the processes involved unwanted effects can be minimised.

1.4.1 Double Strand breaks

One of the most important types of DNA damage is the double strand break (dsb). A double strand break is defined as two complete breaks across the DNA molecular. Approximately, 40 double strand breaks are induced per Gy of radiation (Bryant and Blocher, 1980, Blocher, 1982). Work initially investigating DNA damage and repair using yeast mutants strongly supported the view that double strand breaks were the lethal event (Resnick 1978, Frankenberg *et al* 1984). Even a single double strand break could lead to cell death (Blocher and Pohlit 1982, Harbich *et al* 1984). More importantly, evidence suggests they are the lesions responsible for chromosomal aberrations, leading to gross rearrangements and possibly genome instability, this is detailed in later sections.

1.4.2 Single Strand Breaks

DNA damage that results in the loss of at least a single nucleotide, usually at the phosphodiester linkage, has been defined as a single strand break. Single strand breaks are more readily repaired due to the close proximity of the two ends of the lesion (Hutchinson, 1985). Single strand breaks are often caused by hydroxyl free

radicals but can be induced by both the direct and indirect mechanism of DNA damage (Hutchinson, 1985). Approximately 3000 single strand breaks (ssb) are induced per Gy of ionising radiation.

1.4.3 Other DNA damage

DNA protein crosslinks, sugar damage and base damage are other methods which have been shown to disrupt either the histone packaging of the DNA (Oleinick *et al,*, 1986), or the component parts of the helix structure. Both the direct and indirect action of radiation have been shown to produce these effects. (Hutchinson, 1985)

1.4.4 Chromosomal damage

Chromosome damage has been classically defined by Sax (1940) as the result of single interactions of ionising radiation with chromosomes. The one hit model suggests that any chromatid lesion induced is either repaired or remains unrepaired and appears as a break at the next metaphase when the chromosomes are re-condensed. Sax discovered that there was a linear relationship between dose and the induction of isochromatid breaks. However, Sax proposed that the initial lesion causes the loss observed. This is unlikely, as interstitial lesions can be of the order of 40MBp, the human haploid genome contain only 3000Mbp (Bryant , 1998). To induce such large losses of DNA exonuclease activity would have to occur on a massive scale initiating from the site of the double strand break. However, if two doublestrand breaks occurred on the same strand than the internal segment could feasibly be lost, though the chance of two dsb one a single strand is remote.

Revells model (1955, 1959) suggested that breaks occurred sometime after radiation by the failure of recombinational events at the damaged site. Although still requiring two double strand breaks (dsb) the model does help explain the number of exchanges that are observed in the sister chromatid assay. Differential harlequin staining by the incorporation of bromodeoxyuridine and giemsa staining readily shows switches of large chromosome segments. Bryant has recently extended this model by suggesting that single dsb breaks can be converted into chromatid breaks by a signalling mechanism that triggers the cell to initiate a recombinational exchange which if incomplete leads to a chromatid break (Bryant, 1998).

1.5 DNA Repair

Maintaining genetic stability is essential for life. DNA is a highly reactive molecular species and as such is in a continuous dynamic state of chemical flux. Enzymatic processes are involved in sustaining the integrity of the DNA molecule and are collectively referred to as DNA repair (Alberts, 1994). The rate at which stable changes occur in DNA is called the mutation rate (Alberts, 1994). Most mutations are deleterious, but a low frequency is required for evolutionary development. Therefore a balance between those mutations that can be tolerated and those which are lethal must be made. Spontaneous reactions such as those induced by background radiation for example or other chemical reactions producing free radicals alter 5000 purine bases in every genome in every cell every day (Alberts, 1994). Thermal collisions, solvent molecules and reactive species all contribute to the thousands of base changes occurring daily. On average one in every thousand

mutations is stabilised and incorporated into the genome. Therefore, approximately 50 mutations are accumulated in an average cells lifetime.

Over 50 genes have been discovered in yeast that are involved in different repair pathways. A number of known mechanisms exist in mammalian cells, these include: nucleotide excision repair mismatch repair, abnormal precursor repair, base excision repair, and use of O^6 –alkylguanine-DNA alkyltransferases. All are well documented, (King, 1996, Alberts, 1994, Ruddon, 1995).

Repair enzymes have been found to work in concert with some transcription factors and it has been found that actively transcribed genes are repaired more efficiently than those that are not (Alberts, 1994). Fast and slow repair are thought to be the two main mechanisms involved.

1.5.1 Mismatch Repair

All cells contain repair pathways that assist with the repair of damage sustained to the genome. Mismatch repair is currently one of the most important types repair because it is thought that damage to these genes prevent any further repair to single point and mismatch damage events. This is thought to create a positive feedback loop, whereby further damage is either mis-repaired or not repaired at all. The first mismatch repair genes hMSH3, hMSH6 and hPMS2 were found to form unique dimers: hMSH2, hMLH1 (Fishel and Kolodner, 1993). Subsequently other dimers were found to be involved in this pathway. Human MSH genes are Mut S homologues of the mismatch repair genes discovered in bacteria. (Fishel *et al*, 1993)

While human MLH1 another repair gene is a homologue of the Mut L gene in bacteria. (Modrich, 1994).

hMSH-2 and hMSH-3 hMSH-2 and hMSH-6 hMLH-1 and hPMS2

Another protein, GTBP was also found independently by groups in Rome and Boston and is thought to act as a proof-reader and possibly instigator of the mismatch repair cascade (Holzman, 1996). This gene has since been found as a germline mutation in hereditary non-polyposis colon cancer (HNPCC) which accounts for 15% of all colon cancers. Multiple roles have been discovered for mismatch repair genes (Davis *et al,* 1998). In addition to correcting the genetic anomalies that occur during normal cell replication, these genes fix environmentally induced DNA damage and have also been implicated in regulating the cell cycle.

Mismatch repair proteins are known to repair a variety of environmentally induced lesions, including those from ultraviolet radiation, ionising radiation and oxidation. Defective DNA results in defective transcripts and an altered protein function. Malformed proteins are likely to have deranged functions and an inability to identify mismatches. (Burke, 1990, Bignami, 1994). Double msh2 knockout mice have been developed to further study the role of this gene in tumourigenesis (Wind *et al,* 1995). Loss of this gene has been linked to the mutator phenotype and has been frequently observed in HNPCC (Kim *et al,* 1998, Mac Phee, 1995, Fishel *et al,* 1993).

Recent work by Reznikoff and Fishel has used the mismatch repair deficient colorectal cell line HCT116 to show the importance of these genes to repair DNA damage following ionising radiation (IR). They have shown that these cells, deficient in MLH-1 protein, are able to cross the G2/M boundary following ionising radiation leading to a tolerance of DNA damage (Davis *et al*, 1998).

1.5.2 Other Repair

Over 50 genes have been discovered in yeast that are involved in different repair pathways. A number of known mechanisms exist in mammalian cells, these include: nucleotide excision repair which is capable of removing bulky adducts, such as pyrimidine dimers caused by UV, and covalent reactions induced by such carcinogens as benzopyrene;

non-mismatch repair,

abnormal precursor repair,

base excision repair, and use of O^6 –alkylguanine-DNA alkyltransferases.

(King, 1996, Alberts, 1994, Ruddon, 1995).

1.6 Genomic Instability

The consequence of exposure to ionising radiation is an array of damage, which includes gene mutations, chromosome aberrations, cellular transformation and cell death (Kadhim *et al*, 1995). It has been thought that these changes occur rapidly after exposure and that their fixation or repair occurs equally rapidly. However, growing evidence suggests that cells initially surviving irradiation and capable of proliferation may produce descendants with new chromosome aberrations (Watson, *et al*, 1997, Kadhim *et al*, 1992, 1995, 1996,). Genomic instability (GI) is a new

term to describe the multiplicity of nuclear aberrations found in cells and whose accumulation appear to increase spontaneously over time. Many, if not all cancers exhibit the phenomenon, moreover many normal cells are thought to acquire this phenotype after genotoxic insult. This increase in mutations is thought to hasten the transformation process. Small colony formation (Sinclair, 1964) and an increased cell cycle time (Thompson and Suit *et al*, 1969) were some of the first characteristics of the differences between irradiated and control cells to be noted. Delayed lethal damage was later termed to describe the induction of lethal mutations arising in the offspring of cells surviving irradiation (Seymour *et al*, 1986, Gorgojo and Little, 1989). A decreased cloning efficiency (Mendonca *et al*, 1989), decreased attachment ability (Little *et al*, 1991) and decreased ability to repair dsb (Little *et al*, 1992) were also found to be characteristic of the phenotype. It appears that a multiplicity of cellular and molecular changes occur following exposure to radiation the formation of giant cells and abortive colonies which is indicative of failed cytokinesis (Chang *et al*, 1992, while non-clonal chromosomal rearrangements indicate *de novo* mutations (Little *et al*, 1997). Suzuki has summarised some of these and the elevated formation of micronuclei and chromosome bridge formation using embryo stem cells as a source of primary tissue (Suzuki *et al*, 1998).

It is important to identify markers of instability so that those factors that may contribute to the phenomenon can be identified. Apart from the multiplicity of cellular alterations that occur in genomically unstable cells molecular rearrangements found in repetitive sequences of DNA have also been identified which may provide a rapid means of identify GI populations of irradiated cells.

1.6.1 Repetitive sequences

Ubiquitous repetitive nucleotide sequences include microsatellites, minisatellites and Alu repeats, all can be highly polymorphic. These sequences can be thousands of basepairs in length with up to a million copies. Although their exact function is a mystery, they are thought to contribute to the mutator phenotype (Loeb, 1991) and it has been suggested that they play a role in the evolution of new extronic sequences (King, 1996). Originally discovered in the 1960s by S.R.J. Britten and D.E. Kohne through a method known as reassociation kinetics, several types of repetitive sequences have since been found.

1.6.1.1 Microsatellites

Microsatellites are dinucleotide repeats. Often they contain either TG or CA nucleotides repeated 10-60 times. Over 100,000 copies of these unique repetitive sequences have been found interspersed throughout the human genome. Variations in the number of repeats at known loci have been found to change over time. This variation has been linked to genomic instability. It is thought that variations with the fidelity of the repair and duplication process involved during cell division are responsible for these changes. Changes in these sequences are indicative of changes else where in the genome.

1.6.1.2 Minisatelites or Tandem (VNTR) Repeats

Minisatelites or Tandem (VNTR) repeats are similar to microsatellites but are clustered towards the ends of chromosomes and telomeric regions. Loss of these CA sequences results in the telomeres becoming susceptible to exonuclease digestion (Jeffreys *et al*, 1985). Variations in these sequences are also thought to be

contributory to telomere rejoining which is often found in chromosomes that have been damaged after ionising radiation. There is little difference between species.

1.6.1.3 Alu and LINE repeats

These repetitive sequences were originally able to be cut by Alu1 endonuclease. On average these are 300bp repeats of which 10E6 copies are interspersed around the human genome, accounting for some 6-8% of all genomic DNA. Each 300bp may have two similar regions connected by an adenosine rich spacer. The length of sequence varies, but a consensus sequence has been found which aids PCR amplification. LINE sequences are 6000-7000 Bp containing several coding proteins followed by a poly A site. LINE sequences are thought to account for 5% of the genome. Both these inserts are variable within a population, and even during an individuals lifetime (King, 1996, Lengauer *et al*, 1992).

1.6.2 Oncogenes and Proto-Oncogenes

Ionising radiation is thought to induce cancers through activating oncogenes. Proto-oncogenes are found in all humans. These genes have a normal function until they are mutated and are then termed an oncogene, so as to distinguish them from there non mutated counter part. Oncogenes result from activating mutations that either raise the level of a protein in a cell or alter its function by mutation to the structural gene (Levine *et al*, 1994). Mutated forms of the gene result in a mutated protein structure and an abnormal function that may induce or encourage tumourigenesis

Originally, oncogenes were discovered through investigations in cellular transformation using RNA oncogenic viruses (Ruddon, 1995, King, 1996).

Oncogenes are involved in all aspects of cellular growth and reproduction. Over 60 oncogenes have been discovered, some of these will have an increased production of normal product while others still will have an altered gene product. The ras family of G-protein transducers is a group of oncogenes that include H, K and N-ras, all of which have been found mutated in a large number of neoplasms (King, 1996).

Ionising radiation is known to induce a host of genes. Genes induced to transcribe shortly after exposure to ionising radiation (IR) are termed early response genes (Mallya *et al*, 1998). It is thought that these genes transcribe proteins, which are involved in the cell's response to DNA damage and will include; DNA repair induction, cell cycle arrest and the induction of apoptosis. Mechanisms have evolved in the cell to prevent DNA damage from being passed onto progeny. The single most important gene that has been found to prevent this process is p53. A careful understanding how this gene works to prevent the passage of damage has become central to cancer research.

1.6.3 Tumour Suppressors - p53 Guardian of the genome

The tumour suppressor p53 (figure 1.2) is crucial for controlling the cell cycle after genotoxic damage (Cox and Lane, 1995). When DNA is damaged by ionising radiation cells react rapidly by arresting cell division until the damage is repaired. If the p53 gene is damaged than cell cycle arrest is prevented and mutated cells are able to traverse the cell cycle leading to the accumulation of mutations and eventually carcinogenesis. P53 is the most commonly mutated gene in human cancers. Originally it was found to be a host protein through its binding to the large T-antigen of the SV40 virus (Lane and Crawford, 1979).

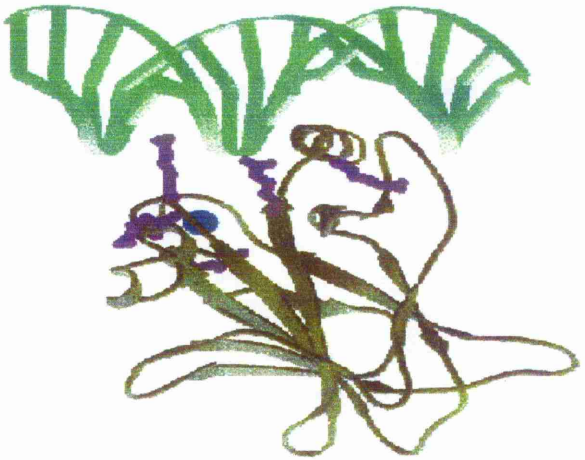

Figure 1.2 P53 interacting with DNA helix. (Modified from NCI Web site.) The purple regions of the diagram are those parts of the protein that have the most frequently mutated sites and in direct contact with DNA molecule. These regions are referred to as HOTSPOTS and are found between exons 5-8.

The p53 gene is found on the short arm of chromosome 17 at location 17p13.1 (Levine *et al*, 1993). It contains 11 exons, (see figure 2.5) encoding a 393 amino acid phosoprotein of 53kD (Yamaizumi and Sugano, 1994) (see figure 1.3). Initial studies suggested that p53 could act as an oncogene (Eliayahu *et al*, 1984, 1989) as cells transfected with the gene were neoplastically transformed. However, as mutant forms of the gene were used in these experiments later work redefined its role as a tumour suppressor, as the wild type gene arrested cell cycling.

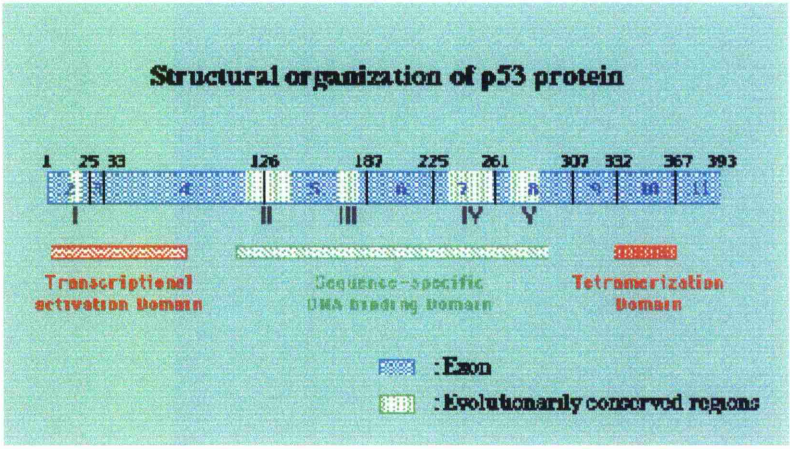

Figure 1.3 Diagrammatic Representation of p53 – modified from NCI Website. The 3D structure of the DNA binding domain has been carefully studied and has been found to consist of three loops. Many mutations that have been found in tumours which are in the DNA binding region of the molecule between codons 112-286.

Codons 110-307 ranging over exons 5-8 have been termed HOTSPOTS. (Hollstein *et al*, 1991). Wild-type p53 is an anti-transformation factor that has been used in some gene therapies. However, some mutant forms produce proteins with half-lives

much longer than that of the wild type normal protein (Levine and Momand, 1990). Using these and other properties a number of immuno-histochemical assays were designed for screening purposes (Rodrigues *et al*, 1990). The gene itself is a tetramer composed of three functional domains. These domains are involved in the regulation of transcription, the binding of specific DNA sequences and the formation of dimers. The binding of mdm2 to the transcriptional domain inactivates normal p53 function (Picksley and Lane, 1994). Several domains of the p53 protein have been isolated using X-ray crystallography, NMR and partial proteolysis (Picksley and Lane, 1994). The diagram over gives the current understanding of the functional domains of the p53 protein.

The frequency of mutations at a specific gene locus can be grouped together and frequently occurring mutations can be identified and compared in different tissues. This allows the identification of hotspots in mutation spectra. The work has identified the tissue specific mutations that lead to cancer. For studies investigating p53 the most frequent mutation is in codon 248, which codes for an arginine, this directly interacts with the DNA, whereas mutations of 249 have an indirect effect on binding. These studies have found for example that. Ovarian cancers show codon 273 as an especially susceptible, whereas codons, 157, 248 and 249 are hotspots found in lung cancer (Hollstein, *et al* 1991). Reasons for these variations have been attributed to potential differences in a tissue's response to DNA damage as well as differing metabolic and repair capacities of the different cell and issue types (Hollstein, *et al*, 1991).

P53 is known to mediate the G1/S boundary of the cell cycle (Kastan *et al,* 1991). After exposure to IR transcription of p53 increases dramatically.(Kastan *et al,* 1992). The high levels of stabilised p53 in turn induce GADD45 and WAF1/Cip1 (el-Deiry *et al* 1993) which mediates the G1/S boundary of the cell cycle and can mediate cell cycle arrest, both these genes have also been implicated in base excision repair (Mallya *et al,* 1998).

P53 has become of increasing interest in cancer research as 60% of all cancers can attribute loss of growth control to this gene (Levine *et al,* 1991, 1994). Over 90% of mutations in p53 are mis-sense and result in a malformed protein, which usually has an extended half-life. Of 252 mis-sense mutations found in one study 248 were found within this conserved region of the gene (Hollstein *et al,* 1991). These mutations can prevent the protein acting on down stream regulators of the cell cycle. Many mutations in p53 appear consistently in exons 5-8. This region of the gene has been conserved throughout evolution, with many divergent species having been found to have the same sequence. For example of the 200 mid-region amino acids, 169 are the same in human, monkey, rat and mouse.

P53 has been shown to be induced by double strand breaks (Lu and Lane, 1993) and has been found to bind to the ends of single strand breaks (Kastan, 1996). Though one of P53's main post induction functions is to bind to the 21kDa Cip1/Waf1 gene (p21) acting as a transcription factor for it (see figure 1.4). Cip1 in turn binds to cdk and cyclin proteins blocking their function and thus arresting mitosis. Once the cell has been prevented from traversing through the cell cycle, multiple repair enzymes can be up-regulated to scan the DNA molecule for damage. Once damaged DNA

has been satisfactorily repaired, the blocking mechanism is removed. Alternatively, if the damage sustained is too great than the newly emerged cysteine proteases related to ICE (Interleukin-1 beta converting enzyme) and CED (C.Elgans) proteins are transcribed for initiation of apoptosis (Nicholson et al, 1996).

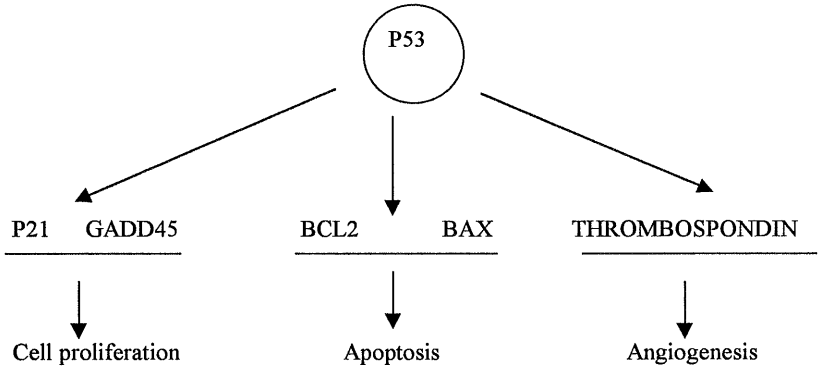

Figure 1.4 Functions of p53 – modified from King 1996. A large number of other proteins interact with p53, both upstream e.g. DNA PK, while others are downstream targets of the gene e.g. GADD45, mdm2 and the tumour suppressor p16. (Kastan et al, 1992, Oliner, 1993)

Although much work has been has been done on p53's role as a transcription factor in response to DNA damage, relatively little work has investigated the mechanism of its regulation. Recent work by Woo *et al* suggests DNA PK and therefore possibly the Ku proteins found in DNA repair scaffold are involved in the binding of p53 to DNA (Woo *et al*, 1998). This work also suggests the DNA PK could be a link between DNA damage and p53 activation. P53 is probably the best known protein whose deficit leads to the accumulation of DNA damage as checkpoints are omitted

from the cell cycle. However, a number of other genes are now implicated in DNA repair.

1.7 Radiation induced carcinogenesis.

The opportunities to study the effects of radiation in man are rare. Exposure to high doses of ionising radiation is an uncommon event and generally restricted to accidental situations. In contrast, animal experiments can be performed under standardised conditions and can provide information on the dose response relationships for radiation carcinogenesis. How radiation, especially low doses, cause cancer is a question that appears can only be elucidated by help of *in vitro* cellular models. However, these early models generally relied upon animal cell systems.

1.7.1 Animal Models of vitro transformation

Reznikoff (1973) showed how a cloned mouse embryo cell line from C3H mice, later designated C3H10T1/2, could be used to test the effects of ionising radiation in growing populations of cells. Embryo cells were sensitive to the post-confluence inhibition of cell division and thus any transformants would appear as piled foci on a confluent monolayer (Reznikoff *et al*, 1973). Kakunaga, (1973) used a similar assay with BALB/3T3 cells to test the properties of chemicals for their ability to transform cells *in vitro.*

With the advent of molecular biology and cloning techniques, a number of groups transfected known oncogenes into non-transformed cell lines, e.g. Shih used the mouse 3T3 line (1979). Transfected cells would be re-seeded into flasks and any transformed cells would pile-up forming foci, these could then be scored after 14-20

days (Shih *et al*, 1979; Cooper *et al*, 1980). Later experiments used different species of recipient and donor cells. It was found that this barrier was no obstacle for *in vitro* transformation. However, spontaneous foci were always present and had to be taken into account. Blair *et al*, extended this work in 1982 using nude mice to screen the foci produced from his transformation assay for the production of tumours. Molecular studies then allowed the confirmation that the human DNA transfected was present in the DNA of any of the transformants. The use of the G418 plasmid permitted antibiotic selection to confirm the transfection process and speed transformant selection which had previously been a time consuming and lengthy process (Wigler *et al*, 1979).

During the late 1970s to mid 1980s the 10T1/2 and 3T3 assays were used extensively to investigate *in vitro* transformation. The studies analysed the effects of radiation qualities, dose-rate, fractionation, the effects of cell cycling, and many others variables. Little (1979) found an increased dose resulted in high cell kill and increased transformation, while an inverse dose rate effect for neutrons i.e. decreased dose-rate of neutrons resulted in a higher transformation was a key discovery by Hill *et al*, (1982). Hei in 1988 showed that high LET radiation was more damaging than low LET. Some of this work was extended to show that there was an enhanced transformation by low dose fractionation and enhanced cell repair by high dose fractionation (Miller *et al*, 1988, 1989). However, it was not until 1992 that radiation induced neoplastic transformation was found to be cell cycle dependant (Miller *et al*, 1992). More recent work has shown that specific molecular lesions are responsible, at least in part, for transformation. C3H10T1/2 foci, type II

and III, formed after exposure to neutrons contain ras mutations which have been reported in about 25% of human tumours (Freyer et al, 1996).

A number of other investigators have attempted to mimic *in vivo* carcinogenesis *in vitro*. In 1988 Paraskeva used a non-tumourigenic epithelial cells designated PC/AA as a model for a multistaged colorectal cancer HNPCC (Hereditary Non-Polyposis Colorectal Cancer). The cell line used had been derived from a large pre-malignant colorectal adenoma from a patient with the disease. The cells were immortal in culture and retained colonic characteristics, such as mucin secretion, while also keeping a normal karyotype (Paraskeva *et al*, 1988). By using chemical mutagens such as Sodium butyrate and the potent carcinogen MNNG (N-methyl-N-nitro-nitrosoguanidine) it was hoped that the lines could be transformed into fully malignant cancers and any genetic changes dissected (Paraskeva *et al*, 1990). Specific abnormalities were noted in chromosomes 1, 6, 7, 13, 14, 17, 18, and 22. Later work showed that p53 mutations appeared as a late event in the transformation process and that Bcl-2 could be used as a possible sensor of nutritional stress that inhibits apoptosis and permits sub-lethal damaged cells to survive (William *et al*, 1997, Singh and Paraskeva, 1998).

The transformation of human cells is known to be complex multistage process and thus has proved difficult to induce using radiation. Some success using chemical carcinogens has been more fruitful (Chadwick *et al*, 1989 and Rhim *et al*, 1990). The use of hybrid or immortalised but non-transformed cell lines has been an approach to over come the difficulties of primary cell culture. (Redpath *et al*, 1987, 1990). While not ideal its application is a compromise, which can still yield useful

information regarding the stages involved in the multistep pathway to cancer. Early phenotypic changes can be observed at length. Changes in growth characteristics such as plating efficiencies and cloning ability can be investigated. Molecular alterations can be found through DNA analysis and associated with the different phenotypic changes observed.

1.7.2 Use of animals for screening purposes

The creation of syngeneic animals such as the nude or athymic mice has meant that large numbers of irradiated cells can be screened for tumourigenecity. Inbred nude mice lack hair and the thymus gland which is critical for the production of T lymphocytes involved in cell mediated immunity (Payne *et al*, 1976). Thus, these mice are unable to reject foreign tissue or explants. The ability of agents or treated cells to be screened in nude mice has greatly advanced our understanding of cancer and allowed the production of human cancers to be studied under carefully controlled circumstances.

1.7.3 Human transformation models

Attempts to achieve neoplastic transformation of human cells by radiation have generally proven unsuccessful. However, efforts to investigate the progression of events that cause human cells to become neoplastic in response to ionising radiation have been aided by the development of cell and tissue culture systems of epithelial cells. Early work showed that certain phenotypic and later genotypic alterations did occur. An increased cell lifespan in culture following gamma radiation was reported by Watanbe *et al,* 1992 after using human embryo cells. Anchorage independent growth, indicative of a primary neoplastic event, was reported using human foreskin

fibroblasts (Sheela and Kennedy, 1986). Molecular alterations like point mutations (Rossi, 1991) as well as gene amplifications and changes in gene expression became commonly documented features of human cells exposed to ionising radiation (Garte and Burns, 1991).

Human cancers are known to follow a stepwise progression. Human models that are able to mimic this would be invaluable in understanding the mechanisms involved. Though they would be equally useful in the screening of potentially transforming drugs e.g. Taxol (Hei and Hal, 1993). However, only with later work using human cells immortalised with viral constructs did it become possible to fully transform human cells so that they were able to form re-injectable tumours into nude mice (Su and Little, 1992). Cell lines produced from these tumours could then be studied in culture and later at the molecular level.

Non-tumourigenic primary human keratinocytes transfected with SV40 virus (RHEK-1) were some of the first used (Rhim *et al*, 1990). These models used ionising radiation or the retroviral addition of known oncogenes to complete the transformation. A non-tumourigenic human epidermal keratinocyte cell line immortalised by adenovirus type 12 and simian virus 40 was transformed by exposure to x-ray irradiation (Thraves *et al*, 1990). These cell lines showed classic morphological differences from non irradiated controls cells. This included the ability to form anchorage independent colonies and the formation of tumours in nude mice.

Later work by Pazzaglia *et al*, 1994 used a human uroepithelial cell immortalised with SV40 to produce an *in vitro* multistep model of transformation. Their data suggests that cells exposed to radiation then chemical carcinogens produced a variety of tumour types. Phenotypes ranging from very aggressive, invasive high-grade carcinomas to superficial low-grade indolent tumours were found. In addition, exposure of a low-grade indolent tumour, MC-T11, generated in this system to the same carcinogens, resulted in neoplastic progression as assessed by the production of high-grade aggressive cancers.

Radiation induced neoplastic transformation of human cells using the re-expression of the HeLa tumour-associated cell surface antigen, p75-IAP (intestinal alkaline phosphatase) has recently been developed. Loss of tumour suppressor function in neoplastically transformed HeLa x skin fibroblasts (CGL1) human cell hybrids was found after gamma radiation. This particular cell system was later developed into a quantitative *in vitro* model. (Mendonca *et al*, 1995). Hei (1997) has extensively used a papillomavirus-immortalized human bronchial epithelial cell line as a model for lung carcinogenesis. Malignant transformation of immortalised human bronchial epithelial cells by asbestos fibres has been a particular useful application (Hei *et al*, 1997).

In summary, some of the earliest *in vitro* assays used bacteria to investigate the harmful affects of chemicals. However, the development of specialised media, which permits the growth of long-term cultures, has allowed investigations into the causes of cancer in eukaryotic cells to be studied. The treatment of cells with cancer causing agents have proved an effective method of dissecting some of the steps in

the path to the transformed cell. However, the use of animal cell systems has limited its application. More recently, work using viral constructs which immortalise human cells have proved successful. Although more useful these systems have intrinsic limitations. Immortalised cell lines must be regarded as partially transformed which brings with the risk of spontaneous transformants. It is hoped that future cell lines such as those transfected with telomerase genes will overcome these problems.

1.8 Development of immortalised cell lines.

1.8.1 SV40 and Immortalisation

Isolated in 1960 SV40 is a member of the papova family, this group of viruses are small non-enveloped DNA virus of 5243 basepairs which will induce a tumour in infected cells (Tse-Hua Tan *et al,* 1986, Bargonetti *et al,* 1991).

The large T-antigen of the simian virus-40 binds to p53 (Levine, 1986). This inactivates the cell cycle arrest properties of the cell (Vogelstein, 1991). The process of transfecting cells with simian virus 40 effectively immortalises the cell line, (Lemoine, 1989) but in so doing may reduce its ability to repair damage. Under normal circumstances any damage sustained through chemical or background radiation would induce an arrest and initiate cell cycle repair processes. (Kastan *et al,* 1991). However, if cells are irradiated, p53 is induced and protein transcription initiated. Levels of the large T-antigen will bind to p53 and inactivate this arrest. If repair mechanisms are still functionally intact than repair will be started but cell division may continue. Damage may be mis-repaired or not repaired at all. This may lead to an accumulation of damage which if let unchecked could lead to enhanced transformation *in vitro*. Conceivably, this process could occur with out the

need for radiation and may account for the relatively small instances of apparently spontaneous transformation *in vitro*.

1.8.2 HTori-3

HTori-3 is a non-tumourigenic thyroid epithelial cell line. The cell line was developed by Levine *et al*, (1986) from tissue derived from a 35 year old female lobectomy patient. Tissue was treated with a mixture of digestive collagenases, and then filtered through a nylon mesh. The cells were then washed in Hanks calcium and magnesium free balanced salt solution (HBSS). The follicular epithelium were then plated as a monolayer with 10% FCS and 0.1mU ml^{-1} of bTSH (bovine Thyroid Stimulating Hormone). Transfection of 1μg of SVori- plasmid (which comprises of the 5.3 kb SV40 genome with a six base pair deletion that eliminated the Bgl1 site at the origin of replication cloned in pMK16) was performed using the strontium phosphate co-precipitation method (*Brash et al*, 1987). Confirmation of expression of SV40 was determined by an indirect immunoperoxidase procedure using monoclonal antibodies. Iodine trapping and a number of other variables including anchorage dependant growth and screening in nude mice was also confirmed. The two best cell lines HTori-3 and Htori-5 were then made available for general use. Levine states that these cell lines may be "very suitable for neoplastic reconstruction experiments"

1.8.3 Tumour cell lines

The HTori-3 cell line has previously been used in our laboratory for investigations into the effects of single and fractionated exposure of gamma radiation (Riches *et al*, 1994) as well as single exposure to plutonium alpha particles *in vitro* (Riches *et al*,

1997). A number of alpha and gamma induced tumours were formed from these investigation which now been used in this present study. The human origin of the alpha and gamma tumours was confirmed by immuno-histochemistry, DNA fingerprinting and chromosome analysis. The experimental model used was thought to provide a suitable system to begin to study the molecular mechanisms involved in radiation carcinogenesis

1.9 Chernobyl and the Thyroid gland

The cells used throughout this investigation are HTori-3, thyroid epithelial cells. These cells are immortal in culture and are easy to maintain under standard conditions. It was anticipated that HTori-3 could be used as a model of in vitro transformation, not only thyroid tumourigenesis but epithelial carcinogenesis in general. In the wake of the Chernobyl disaster of 1986 no model of thyroid tumourigenesis existed. Thus it was anticipated that HTori-3 would provide an invaluable model that would aid research in the development and progression of the disease.

1.9.1 The Thyroid Gland

The human thyroid is a lobulated endocrine gland situated in the neck in front of the upper trachea. Embryologically it is derived from an epithelial down growth of the primitive oral cavity (Mansfield, 1949). The gland produces three hormones:

1. Tri-iodo- thyronine (T3): Regulates basal metabolism, role in growth and maturation of nervous tissue.

2. Thyroxine (T4), converted to T3 once in general circulation. The hormone A.D.H., released from the anterior pituitary gland controls release of T3 and T4

3. Calcitonin is a polypeptide hormone, which regulates blood calcium levels in conjunction with the para-thyroid hormones. The hormone lowers calcium levels by independently controlling the decalcification of bone by osteoclastic reabsorption.

The thyroid gland is unique in that it stores large amounts of hormone in an inactive form. Iodine is absorbed through the diet and is soluble in the blood stream where upon it is sequested by the thyroid for hormone production. (Werner *et al*, 1957)

Large quantities of radioactive iodine were released by the nuclear disaster at Chernobyl (Hillbrandt *et al*, 1996, Dubrova *et al*, 1996). Under these circumstances, the thyroid gland becomes a target for the accumulation of this radioactive isotope and a site for intracellular irradiation. Some of the youngest victims of the Chernobyl disaster were *in utero* but the thyroid gland is known to sequester iodine from 12-14 weeks gestation (Baverstock *et al*, 1992). Assays of the past have relied on murine or rodent cells. While useful, it is not always possible to extrapolate the results from these experiments to human or mammalian cells systems. The difficulty of culturing human cells has restricted their use in these types of experiments. A few isolated examples of *in vitro* transformation have been reported in the literature (Kakunaga, 1978, McCormick *et al*, 1988). More successful methods have been published using adenovirus or SV-40 (Lemoine *et al*, 1989). Thus, it was anticipated

that the use of HTori-3 cells would be a good model to study the *in vitro* effects of cellular irradiation and human tumourigenesis.

1.9.2 Accident at Chernobyl

Radiation induced tumours, especially of the thyroid gland, is a rare event and is generally restricted to accidental situations. However, when these accidents occur they provide an invaluable insight into the mechanisms involved in radiation carcinogenesis. In the Russian region of Belarus on the 26th April 1986 a breakdown in reactor number 4 leads to the release of a plume of radioactive material over Chernobyl. Gomel lies immediately north of the reactor and is known to have received a high level of radioactive fallout after the accident. (Baverstock *et al,* 1992). Large amounts of radioactive iodine 131 were released into the cloud during the accident, which drifted over northern Europe for a number of weeks

In 1990 a great increase in the frequency of thyroid cancer in children in Belarus is reported. A staggering 1500% increase on 1986 (Baverstock *et al*, 1986). Clinical investigations show that nearly all the thyroid tumours are of the papillary type, 128 out of 131. These tumours are aggressive often metastasising to the lung. Spontaneous thyroid carcinoma were a rare event in the area surrounding Chernobyl prior to the accident, between one and two cases per year (Baverstock *et a*l, 1992). The number of cases has double every year since the accident, with more than 50 reported in 1991. The greatest increases were seen in the Gomel region,. Brest, and Grodno which also received high doses (Hillebrandt e*t al*, 1995).

Children were the most vulnerable to the radioactive Iodine 131 as the thyroid is most active during adolescence in the control of growth and metabolism. Many of the children that developed tumours were tested for p53 mutations. The p53 mutations found were located in three conserved regions of the gene that are involved in the binding of the large T-antigen of the SV40 virus (Hillebrandt *et al,* 1995).

However, conflicting reports (Ito *et al,* 1993 and Zou *et al,* 1993) have been unable to give a clear indication as whether p53 or ras, another gene investigated, really are associated with radiation induced tumourigenesis.

Although enhanced frequencies of p53 mutations are common and have been found in many spontaneous tumours (Levine *et al,* 1994), Wright has suggested that p53 mutations in thyroid carcinomas are rare. In a study of 20 spontaneous tumours from America and Germany only one p53 mutation was found in exon 7 (Wright *et al,* 1991). Thus any thyroid tumours discovered in the wake of the Chernobyl accident that carried a p53 mutations could be used as a helpful diagnostic marker of radiation induced cancer (Taylor *et al,* 1994). It was hoped that radiation induced tumours could then be helped to be distinguished from spontaneous ones by their p53 status.

1.10 Delayed effects of radiation

Puck and Marcus were the first to measure the reproductive potential of single mammalian cells *in vitro* when exposed to ionising radiation. It became possible to measure the quantitative effects of radiation on the ability of cells to divide and form visible colonies in culture. In short, they demonstrated that human epithelial cells were far more radiosensitive then previously thought. Moreover, that ionising

radiation kills epithelial cells in a dose dependant manner probably by lethally affecting the "genetic apparatus" of the cell (Puck and Marcus, 1956).

Further work demonstrated that survivors of a radiation exposure would either die within the first few rounds of division following exposure or they would survive to behave in a similar way to that of control cells (Elkind and Sutton, 1960, Elkind *et al*, 1963, Elkind and Whitmore, 1967). Even though other authors had shown that irradiated progeny formed smaller colonies then that of controls cells (Sinclair, 1964), it had been presumed that this was due to an increased time to division (Gorgojo and Little, 1986) and not an enhanced level of cell death.

1.10.1 Delayed Lethal Damage

It was not until 1986 that it was demonstrated that cells, which survived an exposure of ionising radiation, did not have the same proliferative ability as an unirradiated population. Survival experiments using primary and immortal cultured mammalian cell lines reported the provocative finding that cells which appeared in colony assays, following irradiation, were found lethally defective and unable to sustain a constant reproductive capacity. This phenomenon was termed delayed lethal death. (Alper *et al*, 1986). CHO-KI cells maintained in monolayer and primary sheep and human thyroid cells were irradiated in 6x25cm^2 flasks per dose point. A ^{60}Co teletherapy unit was used to irradiate cells 6 hours after plating at a dose rate ~1.5Gy/min at SSD. Enough cells were seeded into each flask so that approximately 100 colonies would be expected. Immediately after irradiation, the flasks were returned to the incubator at 37°C for 7 days. Three of the six flasks were then stained with carbol fushin and colonies containing an average of 200-300 cells were

scored. The remaining flasks, corresponding to each dose point were maintained for three weeks, then each split at confluence into six fresh flasks labelled B flasks. Three of these B flasks were used to determine the plating efficiency in the usual manner while the remaining three were incubated until confluence was reached. These flasks were likewise handled as before and labelled C flasks. In all cases, confluent cultures yielded total cell numbers of the order of $4x10^6$ (Alper *et al*, 1986).

Initially, it was shown that this state persisted for 15 population doublings after radiation (Seymour *et al* 1989). Though later work has shown the persistence of the DLD phenotype for 30-45 population doublings in many different cell lines (Mothersill and Seymour, 1987, Gorgojo *et al*, 1989, Redpath *et al*, 1989).

The relationship between dose and cancer induction is of great importance. It is necessary to quantify the exact amount of cell kill for a given dose of radiation. The phenomenon of DLD affects this outcome. When the radiation response of epithelial cells is determined by colony assay, it is often characterised by an initial shoulder region at the lower doses. It has been suggested that this shoulder region is composed of cells which are lethally damaged and which will eventually die, but only after many divisions (Seymour and Mothersill, 1989), this has also been referred to as potential lethal damage (PLD). Thus, the number of cells that survive radiation may be overestimated and thus need to be corrected.

An area, which had impact by these findings, was studies that investigated radiation-induced neoplastic transformation frequencies, such as the 10T1/2 assay. These

frequencies are often calculated by dividing the number of foci produced many weeks post-irradiation with the originally number of cells in the population. Lethally damaged survivors which would have eventually died would have been included in the calculation resulting in an under estimation of the transformation frequency of certain radiation qualities and doses (Repath *et al*, 1989).

Further work to investigate the pleomorphic changes in cells expressing DLD was attempted by Chang and Little in 1991. They extended the work of Marin and Bender, 1963 and Thompson and Suit in 1969 who used time lapse photography to form pedigrees of irradiated cells up to 6 doublings post-irradiation. Their work concluded that irradiated progeny had an increased cell cycle time. The mean control cells would take 20hours whereby DLD cells would take 23-31 hours. This appeared to confirm the work of Sinclair who had shown that irradiated cells produced smaller colonies than controls. Sinclairs V79-1 cells also showed a decreased PE during passaging, longer doubling times and increased radiosensitivity to subsequent X-irradiation. The work of Little went on to show that cells expressing DLD in addition had an enhanced frequency of giant cell formation and a reduced ability to attach to dishes.

The delayed lethal damage phenotype has now been confirmed in a number of cell lines. CHO-K1, primary sheep thyroid cells, (Seymour *et al*, 1986, Seymour and Mothersill, 1989) 10T1/2 cells (Mothersill and Seymour, 1987), CHO and BALB/3T3 cells (Gorgojo and Little, 1989) as well as CGL1s (Mendonca *et al*, 1989). It has also been reportedly induced through chemical but not by non-ionising radiation (Chang and Little 1992). The use of restriction endonucleases Hin*f*I, which is able to induce cohesive double strand breaks in DNA, has been found to induce

the DLD phenotype. Though the use of the CHO mutant, xrs-5 which is defective in the repair of dsbs, could not be used to induce the phenotype following X-irradiation (Chang and Little, 1992). However, throughout all of this research little work, so far, appears to have been done on human cells.

1.10.2 Chromosomal Instability

Kadhim and Wright investigated the transmission of chromosomal instability after plutonium alpha particle irradiation (Kadhim *et al*, 1992). Using a clonogenic culture system murine haematopoietic stem cells were irradiated with environmentally relevant doses. Results showed a high frequency of non-clonal chromosome aberrations transmissible to progeny. This work highlighted the possible consequences of low dose exposure to high LET radiation. Later work suggested that delayed apoptotic cell death was also evident after both X and alpha irradiation. Moreover, that the type of genomic instability was dependent on the cell type, its genetic susceptibility and the type of irradiation exposure (Kadhim *et al*, 1995). Radiation induced genomic instability in murine haematopoietic stem cells may be due to an elevated oxidative stress (Clutton *et al*, 1996). Moreover, that pathways for oxidative species production are elevated in irradiated cells and may explain why cells displaying genomic instability appear to have a "memory" of the initial irradiation insult. Little in 1997 published evidence that cells expressing DLD, a slowing growing phenotype, selected sub-population of chromosomally unstable cells. (Little *et al*, 1997).

Evidence presented in 1998 went onto suggest that clones from the same population of irradiated cells, which expressed DLD, not only had different phenotypes but also

genotypes. Previous experimenters had used high LET radiation to induce DLD. However, eloquent experiments by Suzuki using low LET X-rays and primary human embryonic cells confirmed evidence of DLD in human cells. Expression lasted for over 40 population doublings, the presence of giant cells, chromosome bridges and an elevated frequency of micronuclei were also documented. They also provided evidence that genetic instability induced by low LET radiation resulted in a decreased efficiency at the rejoining of broken chromosome ends. (Suzuki *et al*, 1998). Suzuki hypothesised that DLD may be the result of unscheduled recombinational events instigated by radiation induced dsb. Fragile chromosome sites which may include telomere regions which have been associated with bridge-breakage-fusion cycles are thought to result in potentially unstable chromosome regions that may explain the phenomenon of DLD and such indicators as elevated colony micronuclei levels.

Work by Seymour and Mothersill have provided evidence that a cytotoxic substance released into the media from irradiated cells is also responsible for cell death. Moreover, the secreted substance can be used induce immediate and non-recoverable cell death in unirradiated cultures. This bystander effect occurs independent of whether gap junctions are opened or closed, whether cells are in cell-cell contact with doses as low as 0.5Gy inducing the effect. Possible candidates for this substance are AIF (Apoptosis inducing factor) and IL8 (Mothersill and Seymour, 1997).

Some of the latest work on delayed cell death and genomic instability has transferred purely *in vitro* assays to the more realistic *in vivo* systems. Preconceptional paternal irradiation has been implicated as a causal factor in childhood cancer e.g. incidences

of childhood leukaemia clusters in the vicinity of nuclear installations. The system of Luke *et al* was based on a transgenic mouse model, employing a lambda shuttle vector that following irradiation (0.1-4.0 Gy) allowed mutations in the lacI gene to be identified from murine bone marrow. Results showed a two fold increase from 2.39×10^{-5} to 4.26×10^{-5} in the rate of mutations and suggest that a route for the transgenerational transfer of genomic instability exists (Luke *et al* 1997). Other authors have shown that the children of the cleanup workers after the Chernobyl accident have a higher frequency of chromosome aberrations in peripheral blood lymphocytes including HPRT mutations (Vorobtsova et al, 1995, 1997).

In the late 1990's the subject of delayed lethal death was reopened with the application of more sophisticated molecular techniques. Radiation induced genomic instability in murine haematopoietic stem cells may be due to an elevated oxidative stress (Wright *et al*, 1996). Moreover, that pathways for oxidative species production are elevated in irradiated cells and may explain why cells displaying genomic instability appear to have a "memory" of the initial irradiation insult. Little in 1997 published evidence that cells expressing DLD, a slowing growing phenotype, selected sub-population of chromosomally unstable cells. (Little *et al*, 1997).

1.10.3 Quantification of the Delayed Lethal Death Phenotype

Evidence presented in 1998 went onto suggest that clones from the same population of irradiated cells, which expressed DLD, not only had different phenotypes but also genotypes. Previous experimenters had used high LET radiation to induce DLD. However, eloquent experiments by Suzuki using low LET X-rays and primary

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1.10.4 Preconceptional paternal irradiation

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Chapter 2

MATERIALS AND METHODS

2.1 Cell and Media

2.1.1 Cell culture conditions: HTori-3

All cell culture and transformation experiments performed used the human thyroid epithelial cell line HTori-3. Professor Wynford-Thomas of the University of Wales College of medicine kindly donated the original cell line to Dr Riches laboratory in 1989.

HTori-3 was created by transfecting primary thyroid cells removed from a 35-year-old female patient with the replication defective SV40 virus. The SV40 genome is 5.3kb and contains a 6 base pair deletion that inactivates the BgII site at the point of replication, (Lemoine, 1989). The virus encodes two important antigens: the large and the small T-antigen, (Lane and Crawford, 1979). It is the large T antigen that binds to free p53 protein, (Vogelstein, et al 1991), inactivating it and thus preventing cellular arrest. The resulting phenotype of the cell is immortal. The cell has unlimited reproductive capacity. However, karyotype analysis shows the chromosome compliment to be polyploid with no mode. Southern blotting proved the singular integration of a single copy of the SV40 genome, (Lemoine, 1989).

The cells were cultured under standard conditions in a variety of Nunc tissue grade flasks. The most common was the T80 flask with an 80 cm^2 surface area, though the

T25 and T180 were also used. The cells were incubated with Dulbecco's modified Eagles medium and Hams F12, supplemented with 5% foetal calf serum (FCS) and 5% glutamine, 100 units/ml penicillin and 100μg/ml streptomycin. Cultures were gassed with 5% carbon dioxide in air, sealed and kept at a constant 37 °C in a warm air incubator.

2.1.2 Freezing Cells

A subconfluent flask of cells would be trypsinised and counted using an electronic Coulter counter (ZM model). Cells were then spun at 1000 rpm in a cryocentrifuge for 10 minutes. The supernatant was discarded and the pellet resuspended in a volume of antibiotic free media supplemented with 10% FCS. A volume of suspension that gave approximately $1.0x10^6$cells/ml, certainly no less than $7.5x10^5$ and no greater than $1.25x10^6$ was used in addition to 10% dimethyl- sulphoxide (or 100μl) to prevent the formation of ice crystals which would rupture the cells during the freezing process. Later it was discovered the cells recovered more promptly if they were frozen in pure FCS. At Berkeley a Nicool was used to gradually reduce the temperature at the rate of 1°C/min over a period of 90 minutes until the vials were at -80°C. Up to 8 cryovials could be suspended in a rack over an insulated bowl of liquid nitrogen. A fan at the top of the unit drew cold air over the vials at the predetermined rate. Once frozen the vials could be placed directly in liquid nitrogen or a minus 170°C freezer. At St Andrews the vials were prepared as before, with cell line, date, passage number, initials and cell concentration clearly labelled. But cells were placed into a top tray of the cryostat. These upper trays were not in direct contact with the liquid nitrogen but the vapour is sufficiently cold that over a period of several hours a freezing rate equating to approximately 1°C per minute is reached.

Once frozen the vials could be placed into the desired cryostat cheese and recorded in a logbook.

2.1.3 Thawing Cells

For thawing vials, an 80cm^2 tissue culture flask was prepared with 20ml of pre-warmed / pre-gassed media. Though later it was found a T25 flask produced a better recovery. The vial was rapidly thawed in a 37°C water bath, until a pellet the size of a small pea remained. The vial was sterilised with 70% alcohol and then pipetted down the back of the flask to minimise any osmotic shock. Details labelled on the outside of the vial were transferred to the flask. E.g. passage number and date.

2.1.4 Passaging

All the media is removed from the flask and a volume of warmed trypsin sufficient to cover the bottom of the flask was added and used as a wash. A further volume is added and the flask is incubated at 37°C. Once the cells begin to lift off a volume of warmed media equal to or greater than the volume of trypsin is added to neutralise its effects. Adherent cells can be encouraged into suspension by gently tapping the side of the flask. A volume of cell suspension can be returned to a freshly labelled flask, and 1 added to the number of passages made. At Berkeley flasks had vented caps and were placed into Perspex gassed boxes in hot rooms kept at a steady 37°C. At St Andrews all flasks had to be individually gassed using a 5% CO_2 and airline with a sterile Pasteur pipette pushed onto the end on the line. Each flask would take 20-30 seconds to gas. The line would have to swab each time it was used and a fresh pipette

used for each flask. Once gassed the flasks could be returned to a 37°C warm air incubator.

2.2 HTori-3 Characterisation

2.2.1 Growth Curve

Exponentially growing cell were harvested and seeded at appropriate concentrations into small petri dishes. Dishes were incubated @ 37°C for up to three weeks. Two replicate dishes were counted daily and media changed after one week of growth.

Counting the cells involved removing all media and adding a trypsin wash of a couple of mls. This was replaced by a further 2mls and the dishes were returned to the incubator for 5-10minutes. Lightly tapping the dish resulted in adherent cells being released from their substrate, these were pipetted up and down to ensure a single cell suspension resulted. Then, 500µl of this suspension was diluted in 19.5mls of isotonic solution giving a 1:40 dilution. Four Coulter counts would be taken per dish, 500µl was sampled by the Coulter on each count, so the sum of these counts equated to 2mls or $1/20^{th}$ of the total, multiplying the result by this factor gave the cellularity per ml of original suspension.

2.2.2 Plating Efficiencies

Plating efficiencies can be used to determine the number of cells that survive any cytotoxic insult. For example, it may be necessary to determine the percentage of cells that survive following passaging or a dose of irradiation. A sub-confluent S-phase flask is harvested and a number of cells, proportionally to one cell per cm^2, are

re-plated into a new flask. After two weeks of incubation, the media is decanted and the cells are stained. All the cells that were able to reproduce formed colonies and were termed viable cells; i.e. they have reproductive integrity. These cells are counted and divided by the number of cells originally plated. The value obtained is the percentage of viable cells able to survive following the experiment. When determining the plating efficiency for given doses of radiation cells die not only from the re-plating but also directly from the radiation. Thus, both types of cell death have to be accounted for before the surviving fraction can be calculated. All cultures are kept in Perspex boxes gassed with 5% carbon dioxide in air and keep at a constant temperature of 37°C. Below are the equations used to calculate the above variables:

PE = Mean / Actual number of cells plates

VC = Actual number of cells plated x Plating Efficiency

SF = Mean / Viable Cells

PE =Plating Efficiency

VC =Viable cells

SF =Surviving Fraction

2.3 Radiation Protocols

2.3.1 X-Ray Irradiation

The X-ray room at Berkeley contained a shielded Phillips 250 kVp X-ray Unit. Only authorised personnel were allowed to use this apparatus. Prior to irradiations the unit was warmed using a number of sequenced programs. Cells were plated in pre-warmed, pre-gamed media at $2x10^5$ cells/per ml the day before irradiations and

allowed to settle. For collecting survival data a series of dose points were selected and the cells irradiated.

2.3.1.1 Acute irradiations

Acute irradiations required the highest dose rate of 2 Gy/min^{-1}. The flasks were placed in Perspex holder called a phantom, which was pre-warmed to 37°C in the hot room's overnight. A 1.2mm Aluminium and 0.3mm Copper (No2) filter was placed over the aperture of the X-ray tube and the phantom and flask is placed at position (No6) 50.2cm for the correct dose rate. Pre-programmed set-ups were used at 1.31mA and 420kVp. Post-radiation the cells were again harvested and replated at an expected survival, taking into account cell death from the radiation. The cultures were left in the hot room for two weeks for the formation of cfu's (colony forming units). They were then stained with 15% Giemsa v/v or 50% methylene blue in tap water and counted under a light box. The survival and other variables could then be calculated.

2.3.1.2 Chronic irradiation

Chronic irradiations used a 1.2mm Aluminium, 0.3mm Copper and 0.65mm Tin (No4) filter, the phantom was placed in position No12 (105.3cm). The chronic dose rate is 1Gy / hr^{-1}. The required doses were calculated by converting the dose into mGy and using a conversion table for the number of mA required. The current was set at 250kVp.

2.3.2 Strontium Irradiations

2.3.2.1Yittrium/strontium-90

Two Yittrium/strontium-90 sources were available at Berkeley Centre, which had nominal activities of 20 and 140 mCi (30/10/85). Handling of these sources was kept to a minimum while the culture dishes were placed in the units. Dosimetry estimates performed on the 21.11.94 show the low activity source to be 9.8Gy/hr-1 and the high activity source to be 65.5 Gy/hr hr-1 From this it is possible to calculate the doses required for survival experiments. The protocol for this experiment followed the same procedure as for collection of plating efficiency data but included the irradiation of the flask of cells prior to their seeding.

2.3.2.2 Hot Particle Irradiation

Hot particle irradiations were performed at the Physics and Astronomy Department of Birmingham University. 30mm petri dishes were irradiated on a continuous, non-uniform, strontium 60 point source. The dishes were harvested on return to Berkeley Technology Centre and replated at the appropriate concentration. A comparison of these survivals can be seen in the result section.

2.3.3 Gamma Irradiation

All gamma irradiations were performed at the Bute medical buildings at the University of St Andrew. Except for the making of feeder layers all irradiations were performed with the cells in suspension using the ^{137}Cs IBL 437 γ-irradiator at a dose rate of 4.6 Gy /min. Once trypsinised cell were kept on ice for the duration of the experiment, irradiated and then returned to ice.

2.4 Cellular Transformation Assay

HTori-3 cells previously irradiated and frozen in liquid nitrogen at Berkeley Technology Centre (BTC) were now thawed and passaged *in vitro*. Initially cultured in a single T80 flask until sub-confluent, the flask was split into 5 further flasks, which were then kept separate for the duration of the *in vitro* period. The flasks were again grown to subconfluence, and passaged once a week for six weeks. Cells were then harvested at approximately 80% sub-confluence and around 1 million cells were injected sub-cutaneously into nude athymic mice for screening of a transformed clone.

The athymic mice used for all injections were born at the St Andrew animal house, though the originally MF1 parental stock nu/nu were purchased from Charles River and sons in July 1998. The mice were kept under aseptic conditions being fed and watered three times a week and bedding changed weekly. An ambient room temperature of 22.3°C was maintained with a 12-hour diurnal fluorescent light cycle being automatically maintained. Outside air was filtered and warmed before being vented to the room.

The cells to be screened were harvested using standard trypsination protocol. The cells were re-suspended in a 30ml universal tube containing 10mls of media. 500µl of the stock was counted in 19.5mls of Isoton using a ZM series electronic Coulter counter. The required volume to give 1.0×10^6 cells was calculated and removed to a fresh universal. The vial was the spun at 10,000 rpm in a cryo-centrifuge for 10 minutes. The supernatant was discarded and the pellet resuspended in the residual

media (approximately 250μl). The concentrated cell solution was sucked into a sterile 1ml syringe with a 21-gauge needle ready for injection. The procedure was repeated for each flask. The syringes and their content were removed from the tissue culture laboratory to the animal house ready for injection. The mice, which were 4-6 weeks old and were of both sexes were individually swabbed on their backs with 70% alcohol and immediately inoculated with the entire volume of the syringe. Approximately one million cells. The mice were returned to their boxes and checked twice weekly for tumour formation, any swelling was graded with the use of steel ball (Riches and Thomas 1970) bearing as a comparison. The sizes were recorded on data sheets and used to plot tumour growth and volume over time. Once the swellings had reached grade 5 or 6, approximately 1 cm, the mice were sacrificed by cervical dislocation and the lump excised. Half the volume was placed on a small strip of filter paper and placed into a volume of formal saline (1:19 formaldehyde and $NaCl_2$) in a universal tube. This was promptly sent for histological examination and the remaining prepared for return to *in vitro* tissue culture.

2.4.1 Establishment of Tumour cell lines

Mice that had grown tumours of grade 5 or greater were suitable for the tumour to be excised and established *in vitro*. The mice were sacrificed by dislocating the cervical vertebrae. A pinching technique was employed while the mice were stretched on the cage lids. The tumours were swiftly dissected after death and any connective tissue was trimmed away in a petri dish. On occasion the dermal tissues were adherent, these were left *in situ* for histological examination. The tumour, roughly the size of a pea, and crunchy when sliced were first cut in half using a sterile scalpel. One half for histology the other half was for establishment of the cell line. This second half

was cut into small cubes, approximately $1mm^3$, these were placed into a universal with a few drops of standard HTori-3 media. Taken to a laminar flow hood a T25 flask was pre-warmed and pre-gassed with 5mls of media. The cubes of tumour tissue were sucked up using a sterile Pasteur pipette and rubber bulb. This permitted the cubes to be carefully placed inside the bottom of the flask.

Once all the pieces had been arranged the flask was carefully placed into a 37°C incubator to allow the explants to adhere and infiltrate to the floor of the flask. Initially the flask was left untouched for seven days, though observed daily for any signs of infections. After this time the flask could be viewed under a platform microscope for signs of growth. If the explants showed signs of growth then the media could be changed. After 2-3 weeks of incubation subconfluence would be reached and the cultures could be passaged into larger flasks. Chromosome banding and metaphase spreads were then performed to correctly identify the tumours as HTori-3 in origin. Eventually, stocks of the cells could be frozen and stored in liquid nitrogen for future use.

2.4.2 Identification of tumour cell lines

To confirm that the tumours grown in the nude mice were derived from the irradiated HTori-3 cells injected, karyotype analysis of metaphase spreads was performed. The protocol for metaphase spreads is as follows. Glass slides are cleaned to remove any traces of dirt or grease. The are immersed in 1M HCl for 1 hour and then rinsed in molecular grade water and finally dipped in 100% ethanol and stored at 4°C. Log phase cells are treated with 0.04μg/ml of colcemid and left for 4 hours to arrest the cells in metaphase. Colcemid prevents the formation of microfilaments, so as cells progress through the cell cycle the chromosomes condense but once they reach

metaphase they are prevented from progressing any further. Cells are trypsinised as usual and then pelleted at 2,000 rpm in a cryocentrifuge chilled to 4°C. The supernatant is discarded and the pellet re-suspended in the residual volume. 5mls of 0.075M KCl (0.55g/100ml) solution is then added dropwise to the centrifuge tube and then left for up to 5minutes to incubate at room temperature. This solution is hypotonic to the cellular environment and results in the influx of water by osmosis; the cellular compartment expands and eventually ruptures. The suspension is spun again and the pellet of nucleolei is washed with Carnoy's fixative or 1ml of 3:1 ethylalcohol: glacial acetic acid. The procedure is repeated and then remaining pellet is re-suspended in a small volume of fixative. The nucleolei are then draw up into a clean Pasteur pipette and dropped onto the cooled slides. The slides are held at an angle of 45° increasing the shearing force upon the nucleolei as they impact; this improves the chance of complete rupture of the nuclear matrix and favourable deposition of the metaphase chromosomes. Slides are air dried for 15minutes @ 37°C then stained with 5% giemsa in tap water v/v. Once rinsed and air-dried the slides were viewed using oil immersion at x 100 magnification using a light microscope.

2.5 Cellular Characterisation of Tumour Cell Lines

2.5.1 Apoptosis – DNA Fragmentation Detection Kit

Delayed colony assays performed over 6Gy showed the expression of delayed cell death. This was exemplified by

1. Decreased numbers of colony units and /or
2. Small colony formation

The reasons for this phenomenon were not clear. There were two possible reasons;

There had been an increase in the cell cycle time resulting in fewer cell divisions throughout the incubation time.

The duration of the cell cycle was unchanged but there could be an increase in the levels of apoptosis.

Initially, growth curves were produced, these showed that the cells expressing the DLD appeared to have fewer cell division as the growth curves showed a trend down and to the right of the control cultures. However, it was possible that the levels of cell division were similar in both cultures but that there was a higher frequency of cell death in those cells expressing DLD. To prove this hypothesis a DNA fragmentation protocol was employed to investigate differences in the levels of apoptosis between the two cultures.

Although cell death has been shown to occur through necrosis and apoptosis, it has been assumed that necrotic cell death would have occurred almost immediately in the post-irradiated cultures. As necrosis will only occur through structural and mechanical damage resulting in gross deformities and cell lysis, of which the cell has no control over. Whereas apoptosis is a controlled process which occurs over a period of hours with specific stages and is part of normal developmental process involved in cellular differentiation and control of cell numbers. A number of molecular cascades are involved which includes the cleavage of DNA into oligonucleosomes detectable through pulse field gel electrophoresis.

2.5.1.1 Principle of Tunel

Terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments which catalyses the addition of biotin-labelled and unlabeled

deoxynucleotides. Biotinylated nucleotides are then detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Diaminobenzidine reacts with the labelled sample to generate an insoluble coloured substrate at the site of DNA fragmentation. Counter-staining with methyl green aids the morphological evaluation and characterisation of normal and apoptotic cells.

2.5.1.2 Materials Supplied

Proteinase K 2mg/ml Proteinase K in 10mM Tris pH 8.0

5X TdT Equibration buffer 1mM Sodium Cacodylate, 0.15M Tris, 1.5mg/ml BSA, 3.75 mM $CoCl_2$, pH 6.6

TdT labelling reaction mix - 3 vials of labelled and unlabelled deoxynucleotides

TdT Enzyme Terminal Deoxynucleotidyl Transferase

Stop Buffer 0.5M EDTA, pH 8.0

Blocking Buffer 4% BSA in PBS

50X Conjugate Peroxidase streptavidin conjugate 50-fold concentrated solution.

DAB tablets 3,3' Diamionbenzidine (0.7mg/tablet)

H_2O_2/Urea tablets - H_2O_2/urea (0.6mg/tablet)

Methyl green counterstain 0.3% methyl green

Control Slides: HL60 promyelocytic leukaemia cells and HL60 cells incubated with 0.5μg/ml actinmycin D for 19 hours to induce apoptosis; 2 slides per kit.

2.5.1.3 Extra Materials

1 X Phosphate Buffered Saline

4% Formaldehyde (in 1 X PBS)

10mM Tris pH 8.0#

Distilled water

Coplin jars / glass slide holders

Wash bottle for rinsing slides

Humidified chamber

Glass cover slips

Mounting media

Microscope

Pipettes

Microcentrifuge and tubes

Parafilm and wipes

Ice

2.5.1.4 Recommendations

All reagents should be kept at –20°C and should be pulse spun prior to use.

DAB is a potential carcinogen and Cacodylic acid is toxic and a carcinogen

2.5.1.5 Suggested Protocol

The protocol can be broken into these stages:

1 Preparation and pelleting of cells

2 Cytospin cells

2 Permeabilization of specimen

3 Inactivation of endogenous peroxidases

4 Equilibration and labelling reaction

5 Termination of labelling reaction

6 Detection

7 Counterstain

1. **Trypsinise and pellet** cells, 2-3x10E6, then cytospin cells onto slides. To fix the specimen, immerse the slides in 4% formaldehyde (in 1X PBS) for 15 minutes at room temperature. Dry, then immerse in 1X TBS for 15 minutes at room temperature. Mark specimen on slide from underside.
2. **To permeabilse** the cellular membrane dilute 2mg/ml proteinase K 1:100 in 10mM Tris pH 8.0 (mix 1µl of 2mg/ml Proteinase K plus 99µl 10 mM Tris per specimen). Cover entire specimen with 50-100µl of 20µg/proteinase K. Incubate at room temperature for 10 minutes. (Do not over incubate.)
3. Dip slides 2-3 times into a beaker of 1X TBS. Gently tap off excess liquid and carefully dry the slide around the specimen.
4. **To inactivate endogenous peroxidases** dilute 30% H_2O_2 1:10 in methanol (mix 10µl 30% H_2O_2 with 90µl of methanol per solution)
5. Cover entire specimen with 100µl of 3% H_2O_2. Incubate @ room temperature for 5 minutes. Do not over incubate. Then rinse slide with 1X TBS
6. **For equilibration** of labelling of reaction dilute 5X TdT equilibration buffer 1:5 with dH_2O (mix 20µl 5X buffer with 80µl of dH_2O per specimen).
7. Cover entire specimen with 100µl of 1X TdT equilibration buffer. Incubate at room temperature for 10-30 minutes while preparing end labelling reaction mixture. Alternatively slides can be immersed in a Coplin jar containing 1X TdT.
8. **Preparation of the TdT labelling** reaction mixture is as follows: Lightly vortex contents of the TdT labelling reaction mix tube, pulse spin the TdT enzyme tube in a microcentrifuge prior to opening, transfer to a fresh tube for each specimen

57 µl TdT labelling enzyme reaction mix and 3µl of TdT enzyme, mix gently and keep on ice.

9. Carefully blot the 1X TdT equilibration buffer from the specimen, taking care not to touch the specimen. Immediately apply 60µl of TdT labelling reaction mixture (prepared above) onto each specimen and cover with a coverslip and then with parafilm cut larger than the specimen
10. Place the slides in a humidified chamber and incubate @ 37°C for 1.5 hours
11. **To terminate the labelling reaction** remove the parafilm and coverslip and rinse the slide in 1X TBS, however if a precipitate is present first prewarm the Stop Buffer @ 37°C for 5 minutes. Cover the slide with 100µl of Stop Buffer and incubate @ room temperature for 5 minutes. Rinse the slide with 1X TBS and gently dry around the specimen.
12. **For the detection phase** cover the specimen with 100µl of Blocking Buffer and incubate for 10 minutes at room temperature. Then dilute the 50X conjugate 1:50 in Blocking Buffer (min 2µl 50X conjugate with 98µl of Blocking Buffer per specimen.)
13. Carefully blot around the specimen and immediately add 100µl of diluted 1X conjugate to the specimen. Place the slides in a humidified chamber and incubate for 30 minutes.
14. Five minutes prior to the incubation prepare DAB solution by dissolving one tablet of DAB and one tablet of H2O2/Urea in 1ml of tap water. This yields enough Dab solution for 10 specimens. Tap water contains metal ion which aids the staining.
15. Rinse slides with 1X TBS, tap and dry around specimen and cover slide with 100µl of DAB for 10-15mintes before rinsing with distilled water.

16. **To Counterstain** add 100µl of the methyl green counterstain solution and incubate for 3 minutes.
17. Press edge of slide against absorbent paper and dip slide 2-4 times in 100% ethanol
18. Blot and repeat with fresh 100% ethanol
19. Blot and repeat with xylene, wipe away excess from back of slide and mount a coverslip over the specimen.

2.5.1.6 Interpretation of Results

A dark brown DAB signal indicates an apoptotic cell while shade of blue-green to greenish tan signifies a non reactive / normal non apoptotic cell. Morphology as well as the degree of stain can be used to interpret the degree of apoptosis in a cell population. Many apoptotic cells are pyknotic (dark bodies), a blebbing and budding cell membrane morphology is common. Apoptosis is asynchronous and members will be randomly distributed throughout the slide whereas necrotic cells will tend to occur in-groups. Cytoplasmic and diffuse staining is often seen in necrotic cells due to loss of membrane integrity. Oncogene Research Products: *TdT-FragEL™*

2.5.2 MTT Cell Attachment Assay

MTT reduction assay is a widely used technique for the measurement of cell viability (Mosmann, 1983). It is a rapid colourmetric assay for cellular growth and survival and has application to proliferation and cyto-toxicity assays.

MTT is a water soluble tetrazolium dye that is reduced by living but not dead cells to a purple formazan product that is insoluble in aqueous solutions. The assay has been shown to give the same results as the standard colony assay. Cells are incubated in square, flat-bottomed microtitre plates. Once the cells are adherent to the substrate, usually this takes no more than a couple of hours, 50μl of MTT can be added to each well using a multichannel pipette. Plates are wrapped in aluminium foil and incubated in the dark for a minimum of 4 hours and maximum of 8. After the appropriate incubation time the media is removed from the plate with a flicking action into a sink. The water insoluble MTT-formazan crystals are dissolved in DMSO and adjusted to pH 10.5 with Sorensen's Buffer. The optical density of each well are quickly read in an ELISA plate reader.

2.5.2.1 Materials

microtitre plates (Flat bottomed)

Growth Media

Ependorf Tips

Petri dishes

Trypsin

Sorensen's glycine buffer (0.1M glycine, 0.1M NaCl adjusted to pH10.5 with 1M NaOH)

MTT (3-(4-5-dimethyliazol-2-yl)-2,5-diphenyltetrzolium bromide 50mg/ml (Sigma)

Multichannel pipette (Costar)

Dimethyl Sulphoxide (DMSO)

ELISA plate reader (BioRad, ICN–flow)

2.5.2.2 Suggested Protocol

1. A sub-confluent Nunc T80 flask of cells is harvested using the routine method.
2. A sample is counted and the remaining pelleted @1000rpm for 5 minutes
3. The pellet is resuspended in fresh media and serial dilutions are made so that each well contains 200µl of media and cell suspension.
4. All plates are placed in a warm 37°C wet incubator to permit attachment.
5. After a couple of hours the plates are removed from the incubator and a fresh aliquot of 50µl of MTT is added to each well using a multichannel pipette
6. The microtitre plates are then wrapped in aluminium foil and replaced in the incubator for a minimum of 4 hours.
7. Remove the media and add 200µl of DMSO then 25µl of Sorensen's buffer and read immediately, absorbency is recorded at 570nm
8. The first and last wells in the plate are used as blanks and just contain media.

2.5.2.3 Interpretation of Results

All plates were scored using an ELISA plate reader programmed with the appropriate details of plate size and wavelengths and other variable. Plates were automatically shaken for 15 seconds to enhance the distribution of the blue colour produced by the reduction of the MTT dye by the active cells. The plates were then read with the photospectrometer and results printed out for each well. This assay has application for the determination of cellular radiosensitivity as well as the screening of cytotoxic and metabolic drugs in the use of predictive testing e.g. for cancer therapies.

2.6 Molecular Characterisation Techniques

2.6.1 DNA Extraction Protocol

DNA is extracted from samples using puregene reagents. A sub-confluent flask is harvested and centrifuged at 13000g (14000rpm) for 5 seconds to pellet the cells. The supernatant is discarded and the pellet re-suspended in the residual media by vigorously re-suspending it using a vortex. 0.6ml of lysis agent is added. This can be added directly to the flask without trypsination. The samples are stable in lysis solution for up to 18 months. 0.03ml of RNAse solution is added and incubated for 15-60 minutes at 37°C. 0.2ml of protein precipitation solution is then added and vortexed for 20 seconds and centrifuged at 13000g for 3 minutes. The protein pellet is discarded and the DNA supernatant is washed twice with 0.6mls of isopropanol. The sample is inverted 50 times until the DNA threads appear. The sample is then re-spun at 13,000g and washed in 0.6mls of 70% ethanol. This is decanted and the pellet air-dried. 0.1ml of DNA hydration solution is added to allow the DNA to rehydrate, either overnight or for 1hr at 65°C in an oven. DNA concentration was calculated using a spectrophotometer at Berkeley.

2.6.2 Polymerase Chain Reaction: PCR

The first PCR reaction was performed by Saiki *et al* (1985), though others had previously described the reaction in the 1970s. The principle of PCR relies on the intrinsic properties of DNA its ability to form single strands and the properties of the thermal stable polymerase enzyme Taq. The reaction can be split into four distinct stages.

1. Denaturing @ 95°C

2. Annealing @ 55°C

3. Polymerisation @ 72°C

4. Final extension @ 72°C

The Polymerase Chain Reaction is a rapid and relatively simple way to amplify genes. Small amounts of DNA, down to nanogram quantities can be used. Primarily, the sample is denatured by heating to 95°C; double stranded DNA is split into its single stranded form. Short sections of single stranded DNA analogous to the starting and finishing sequences of the gene of interest, called primers, are then used.

Primers can be purchased commercially on a per base basis. The reaction is cooled to 50-60°C the temperature at which the primers will bind to any complementary sequence. In the presence of excess nucleotides, Mg^{++} and the polymerisation enzyme *Thermus aquaticus* (Taq) the temperature increases to 72°C to permit polymerisation from the site of the primer.

Extension occurs continuously along the strand of DNA until the temperature is increased to 95°C and the reaction is arrested. These first copies of the gene will extend beyond the site of the complementary primer; there is no limit to amount of polymerisation on the original template. However, in subsequent cycles complementary primers bind to the newly polymerised strands as readily as the original template. The next new sequences will have a finite length; i.e. will only polymerise as far as the end of the strand. These shorter strands will also act as template for the next round of replication and will predominate exponentially in the

reaction. Far fewer copies of the longer original template will be copied in comparison to these shorter gene sequences.

Through this series of heating and cooling steps the double stranded DNA is broken, primers anneal and polymerisation occurs. In this way a gene sequence may be duplicated many millions of times. At the end of 30-40 cycles the amplified product can be finally extended for 5 minutes at 72°C for double stranded DNA to reform (see table 2.1 and 2.2). The product can then be cooled to ambient and run on a 2-3% agarose gel and its size determined.

2.6.2.1 PCR of p53 gene

All components were rapidly thawed in the hand and pulsed in a microcentrifuge prior to being pipetted onto the side of a clear 1000µl thin walled reaction tube. Thin walled reaction tubes permit rapid temperature exchange to the inside of the reaction vessel and thus its contents. All amplification reactions were placed into the 96 well block of a Techne Gene E thermocycler, a heated lid which prevented excessive evaporation.

Table 2.1 Listed are the components required for PCR amplification of gene fragments amplified from genomic DNA template. Included is the stock concentration as well as the final reaction concentration. The sequence in which each component is added is also included.

Component	[Stock]	Volume	[Final]	Order of addition
PCR Buffer	10X	10µl	1X	2
dATP stock	10mM	2µl	200µM	3
dCTP stock	10mM	2µl	200µM	4
dGTP stock	10mM	2µl	200µM	5
dTTP stock	10mM	2µl	200µM	6
$MgCl_2$	25mM	4µl	1mM	9
Primer A	0.04nm	5µl	400nM	7
Primer B	0.04nm	5µl	400nM	8
DNA template	1µg/10µl	10µl	1µg/100µl	10
Taq	5 Units/µl	0.5µl	2.5U/100µl	11
H_2O	-	57.5µl	-	1
TOTAL		100µl		

When making up a reaction it became helpful to make master mixes where all the reagents were pipetted into a single ependorf. The required volume was removed into a fresh reaction vessel and the separate primers for each exon of the gene were pipetted individually.

Table 2.2 Example of Cycling Constraints. Each pair of primers requires its own cycling constraints. These can be determined in part from equations pertaining to the base sequence of the primer. The size of the fragments is also important in determining the correct ramp rate.

	Initial Denature	Denature	Anneal	Extend	Final Extension
Time	1min	1min	30 Sec	30 Sec	30 Sec
Temperature	94°C	94°C	55°C	72°C	72°C
# Cycles	1	30	30	30	1

Figure 2.1 Below is the entire DNA sequence of the amplified regions of the p53 gene and the Alu repeat sequence. Each pair of primers is coloured red and the amplified section is blue. The size of each exon in base pairs is listed next to DNA fragment. P53 sequences were amplified to test for the presence of DNA mutations, while the Alu repeat sequence was amplified to compare changes in the length of repeat sequence between the different tumour cell lines

Amplified Exon Regions of p53 Gene

```
12061 ccccggacga tattgaacaa tggttcactg aagacccagg tccagatgaa gctcccagaa
12121 tgccagaggc tgctccccgc gtggcccctg caccagcagc tcctacaccg gcggcccctg
12181 caccagcccc ctcctggccc ctgtcatctt ctgtccttc ccagaaaacc taccagggca
12241 gctacggttt ccgtctgggc ttcttgcatt ctgggacagc caagtctgtg acttgcacgg
12301 tcagttgccc tgaggggctg gcttccatga gacttcaatg cctggccgta tcccctgca
12361 tttcttttgt ttggaacttt gggattcctc ttcaccctta ggcttcctgt cagtgttttt
12421 ttatagttta cccacttaat gtgtgatctc tgactcctgt cccaaagttg aatattcccc
12481 ccttgaattt gggcttttat ccatcccatc acaccctcag catctctcct ggggatgcag
12541 aacttttctt tttcttcatc cacgtgtatt ccttggcttt tgaaaataag ctcctgacca
12601 ggcttggtgg ctcacacctg caatcccagc actctcaaag aggccaaggc aggcagatca
12661 cctgagcccc aggagttcaa gaccagcctg ggtaacatga tgaaacctcg tctctacaaa
12721 aaaatacaaa aaattagcca ggcatggtgg tgcacaccta tagtcccagc cactcaggag
12781 gctgaggtgg gaagatcact tgaggccagg agatggaggc tgcagtgagc tgtgatcaca
12841 ccactgtgct ccagcctgag tgacagagca agaccctatc tcaaaaaaaa aaaaaaagaa
12901 aagctcctga ggtgtagacg ccaactctct ctagctcgct agtgggttgc aggaggtgct
12961 tacacatgtt tgtttctttg ctgccgtgtt ccagttgctt tatctgttca cttgtgccct
13021 gactttcaac tctgtctcct tcctcttcct acagtactcc cctgccctca acaagatgtt
13081 ttgccaactg gccaagacct gccctgtgca gctgtgggtt gattccacac ccccgcccgg  Exon 5
13141 cacccgcgtc cgcgccatgg ccatctacaa gcagtcacag cacatgacgg aggttgtgag  274 Bp
13201 gcgctgcccc caccatgagc gctgctcaga tagcgatggt gagcagctgg ggctggagag
13261 acgacagggc tggttgccca gggtccccag gcctctgatt cctcactgat tgctcttagg
13321 tctggcccct cctcagcatc ttatccgagt ggaaggaaat ttgcgtgtgg agtatttgga  Exon 6
13381 tgacagaaac acttttcgac atagtgtggt ggtgccctat gagccgcctg aggtctggtt  199 Bp
13441 tgcaactggg gtctctggga ggaggggtta agggtggttg tcagtggccc tccgggtgag
13501 cagtaggggg gctttctcct gctgcttatt tgacctccct ataaccccat gagatgtgca
13561 aagtaaatgg gtttaactat tgcacagttg aaaaaactga agcttacgag gctaagggcc
13621 tcccctgctt ggctgggcgc agtggctcat gcctgtaatc ccagcacttt gggaggccaa
13681 ggcaggcgga tcacgaggtt gggagatcga gaccatcctg gctaacggtg aaaccccgtc
13741 tctactgaaa aatacaaaaa aaaattagcc gggcgtggtg ctgggcacct gtagtcccag
13801 ctactcggga ggctgaggaa ggagaatggc gtgaacctgg gcggtggagc ttgcagtgag
13861 ctgagatcac gccactgcac tccagcctgg gcgacagagc gagattccat ctcaaaaaaa
13921 aaaaaaaaag gcctccctg cttgccacag gtctccccaa ggcgcactgg cctcatcttg
13981 ggcctgtgtt atctcctagg ttggctctga ctgtaccacc atccactaca actacatgtg  Exon 7
14041 taacagttcc tgcatgggcg gcatgaaccg gaggcccatc ctcaccatca tcacactgga  193 Bp
14101 agactccagg tcaggagcca cttgccaccc tgcacactgg cctgctgtgc cccagcctct
14161 gcttgccgct gaccctggg cccacctctt accgatttct tccatactac tacccatcca
14221 cctctcatca catttccggc gggaatctcc ttactgctcc cactcagttt ccttttctct
14281 ggctttggga cctcttaacc tgtggcttct cctcccacct cctggagctg gagcttaggc
14341 tccagaaagg acaagggtgg ttgggagtag atggagcctg gtttttaaa tgggacaggt
14401 aggacctgat ttccttactg cctcttgctt ctcttttcct atcctgagta gtggtaatct
14461 actgggacgg aacagctttg aggtgcgtgt ttgtgcctgt cctgggagag accggcgcac  Exon 8
14521 agaggaagag aatctccgca agaaagggga gcctcaccac gagctgcccc cagggagcac  223 B
14581 taagcgaggt aagcaagcag gacaagaagc ggtggaggag accaagggtg cagttatgcc
14641 tcagattcac ttttatcacc tttccttgcc tctttcctag cactgcccaa caacaccagc
14701 tcctctcccc agccaaagaa gaaaccactg gatggagaat atttcaccct tcaggtacta
14761 agtcttggga cctcttatca agtggaaagt ttccagtcta acactcaaaa tgccgttttc
14821 ttcttgactg ttttacctgc aattggggca tttgccatca gggggcagtg atgcctcaaa
14881 gacaatggct cctggttgta gctaactaac ttcagaacac caacttatac cataatatat
14941 attttaaagg accagaccag ctttcaaaaa gaaaatagtt aaagagagca tgaaaatggt
15001 tctatgactt tgcctgatac agatgctact tgacttacga tggagttact tctgataact
15061 cgtcgtaagt tgaaatattg aaatattgta agttgaaaat ggatttaata cacctaatct
15121 aaggaacatc atagcttagc ctagcctgct ttttttttt ttttttttt ggagacagag
15181 tctcactctg ctacccaggc tggagtgcag tggcgggatc tcggctcact gcaacctccg
15241 ccttctgggt tcaagcgatt ctcctgcctc agccccactga gtagctggga ttacaggcac
15301 ctgccccgac gcccagctaa ttttttgtta tttatttcct ttttttttag tagagataga
15361 atttcaccat gttggccagg ctagtctcga actcctgacc ttgtgatctg cctgccttgg
15421 cctcccaaag tgctgggatt acaggcgtga gccaccgcac ctggcctgcc tagcctactt
15481 ttatttttatt tttaatggag acagcatctt gctctgttgc ccaggctgga ttacagtgat
15541 gtgatcatag ctcattatac cctcctgggc tcaagcaatc cccctaactc tgcctcccca
15601 gtagctagga ccacaggcat acaccaccat acccagctaa tttttaaaat tttttgtaga
15661 tagatagagt ctcactatgt tgcccaggct ggtctctagc ctacttttttt gagacaaggt
15721 cttgctctgt cacccaggct ggatagagtg cagtagtgca gtcacagctc actgcagcct
15781 ccacctccca ggctccatcc atcctcccag ctcagcctcc caagttgctt caactacagg
15841 cctgcaccac catgcctggc taatttttat ttatttattt ttatttttatt ttatttttatt
15901 ttttgagact cagtctcact ctgtcgcctt aggctggagt gcagtggcat gatctcggct
15961 cactgctaac ctctgcctcc tgggtttcaa gtgattctcc tgcctcagcc tcccgaatag
16021 ctaggactac aagcgcctgc taccacgccc ggctaatttg tgtatttttа gtagagacag
16081 ggtttcacca tgttggccag gctggtctcg aacttctgac catgtgatcg ccgcctcggc
16141 ctcccaaagt gctgggatta caggtgtgag ccaccacgcc cggctaattt ttatttattt
16201 atttaaagac agagtctcac tctgtcactc aggctagagt gcagtggcac catctcagct
16261 cactgcagcc ttgacctccc tgggctccgg tgatttcacc ctcccaagta gctaggacta
```

Alu repeat Polymorphism of p53 gene

```
 7141 aaaaaaaaaa aaaaaaagga aagaaaaaaa ataacgatta gaaaggaaga aatcaaacac
 7201 attcacagcc agtatgattc tatacatacc atggtcctaa tggggccagg cgtggtggct
 7261 catgctgtaa tcctagcact tttaggaggc tgaggcaggt ggcttccctg ggaccagctg
 7321 gccaacatgg tgaaacccca actctaataa aaatacaaaa aatcagccag gcgtggtgag
 7381 ggcacctcta atcccagcta ctcaggaggc tgaggcagga gaattgcttg gacctgggag
 7441 gcagaggttg cagtgagccg agatcgcgct attgcactcc agcctgggca acaagagtga
 7501 aactccggca gggtgtggtc ttacgcctgt aatcccagca cttcgggagg ctgagccagg
 7561 ccgatcacct gaggtcagga gtttgagacc aacctaacat ggtgaaaccc cgtctctact
 7621 aaaaatacaa gaattagctg ggtgtagtgg tgggcgcctg taatcccagc tacttgggag
 7681 gctgagacag aagaattgct tgaacccagg aggtggaggt tgcagtgagc tgagatcatg
 7741 ccattgcaca ccacgccggg caacagagcg agattccgtc tcaaaaaaaa aaaaaagatg
 7801 aaactctatc tcaaaaaaaa aaaaagtcc taatggaaaa tccataaaaa gctaccaaaa
 7861 ctaataaata aatatagcag ggttgcaggt tacagggcaa tatagttatc cctctatctg
 7921 taggggcttg gttctgggac tcctcacaca ccaaacccac agatgtctaa gtcccatata
 7981 taagacggaa tagtatttaa cctacacata tcctcccata tagtttaaat tatctagatt
 8041 acttacatta cccccataca atgaaaatgc taatgtacat gcaagtatgt atgtaagtac
 8101 ttgtactata ttgtttaggg aatcactgga cagataggcc ttcaagactg ataccagcag
 8161 ccactgttaa gattctggtc aggcctgccc ctgtttgggg tctcagttga tctcattgcc
 8221 ttcccaccca gccaagggca cctgcatttc tcttggctcc ctggccattt ggaaggccta
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 8341 ctctgaaaaa cacggagatt ttagttgcta ctgaagattt gagagataaa gacagggaga
 8401 cctgtctgta gacctgtgtc cctccaagtg ggattgagac tttgggcccc ccatttcagg
 8461 acagcacctc ctggcctgtt gactgaatag atccctgaag gaggtgtagt tgcattttag
 8521 gagtgggggt gggagcagta ccactgatcc gcactaacaa tcacacagtt ctctctagaa
 8581 taataatata gaacaagtga aatagaacaa ttgcagaaag agctaacctt tgttgagctc
 8641 ttactgtgtg cccagcactt tcctcaactc tacatttccc ataatacata gagtactagg p53ivs1a
 8701 taggcggggc ttgggggctc acgcctgtaa tcccagcact ttaggaggcc aaggggggtg
 8761 gatcacctga ggtcgggagt tcaagaccag cctgactaac atggtgaaac cccgtctcta
 8821 ctagaagtac aaaattagcc aggtgtggtg gcacatgctt gtagtcctag ctactcagca
 8881 ggctgaggca ggagaatcat ttgaatccgg gaggaggttg cagtaagcgg agatagtgcc ALE3
 8941 actgtactcc agcctgggca ataagagctg agactccgtc tcaaaataaa ataaaataaa
 9001 ataaaataaa ataaaataaa ataaaaaaag aaaagagcct gccattaaag gagctgtttg p53ivs1b
 9061 gtaggggatg ttttgtcagt gcaaacaaca gaaaagtggg ctgggcacag tggttcatgc
 9121 ctgtaatccc agcactttgg gaggccaagg cgggcggatc acctgaagtt gggagttcaa
 9181 gaccagcctg accaatatgg agaaaccccg tctctactaa aaatacaaaa ttagccgggc
 9241 gcagtggccg atgcctgtaa tcccagctac tcgggaggct gaggcaggag aatcgcttga
 9301 acctgggagg cagaggttgc ggtgagccga gatcgcacca ttgcactcca gcctggacga
 9361 gagcaaaact ctgtctcaaa aaaaaaaaaa aacagaaaag tgtaacaaac acttacagta
 9421 ggcatgtttc ttagcaaatc tgatgacaaa tttggcataa agaaagagag catccctgaa
 9481 aaaaaaaaaa agaaaaagaa agagagcatc ctgcctgggc aacatagtga aaccctgcct
 9541 ctacaaaaaa actcaaaaat tggccgggtg cagtggctca cacctgtaat cccagcactt
 9601 tgggagtcgg aggcgggagg atcacctgag gtcaggagtt cgaaaccagc ctggccaaca
 9661 tggcaaaacc ccatctctac taaaaataca aaaaattaat caggcgcatt ggtgggcgcc
 9721 tgtaatccca gctactcagg aagttgaggc aagaggatcg cttgatactg ggaggtggag
 9781 gttacagtga gtcgagatca caccactgca ctctagcctg ggtgacaggg cgagactccg
 9841 tctccaaaaa aaaaaagaaa aagaaaaaga ctaaaaaatt agccaggcag gcctctgtgg
 9901 tcccagctac ttgggaggct gaggcaggag aatcactgag cccaggagtg gcaggctgta
 9961 gtgagccatg attgcaccac tgtaccctag cttgggcttc aaagcaagac cctgcctcaa
10021 aagaaaaaag aaagaaagaa agaacatggc gggccaggca cagtggctca cacctgtaat
10081 cccagcgctt tgagaggccg aggcaggtgg atcacaaggt caggagttcc acaccagcct
10141 ggccaacatg gtgaaaccct gtctctacta aaaatacaaa aaatcagcag gcagggtggt
10201 aggggcctgt aatcccagct actcgggagg ctgaggcagg agaattgctt gaaaccagaa
10261 ggcagaggtt gcagtgagcc tagactgcac cactgcactc cagcctgggc gaaaagagcc
10321 aaactccatc tcaaaaaaca aacaaaaaaa caaaacaaaa aaaaacatgg cagcctttga
10381 aagcttgtct gggagaaggt gcgatgatgg ttgcataact tcgtgcaaga tgctggtcca
10441 cacaggggct gcccctgct ctttctcgct ctcttaacct ctcatataac aggcttgtgt
10501 gttatgcaca tttattgagc ccaagcaggt gcaaggcatt gtgatctaat actttggtca
10561 gcaagacaac aagatagatc actgccctgc ccttaggaag tgtatatgct attagaggaa
```

2.6.3 RFLP - Restriction Fragment Length Polymorphism

RFLP is useful technique that can be used to identify mutations in a known gene sequence. A number of enzymes are available which will restrict DNA at specific nucleotide sequences. DNA is isolated from irradiated cells and then using PCR the gene sequence of interest can be amplified. Then restriction endonucleases can be used to digest the product. A mutation at a known site will prevent restriction and a variance in DNA length compared to a control sample will result when run on an agarose gel. To identify the exact site of the mutation the gene usually has to be sequenced.

2.6.4 DNA Fragment determination and purification by agarose gel electrophoresis

To find the molecular weight of a DNA fragment, especially those amplified by PCR agarose gel electrophoresis is the simplest method. To produce a 3% high definition gel, electrophoresis grade agarose e.g. Metaphor^{TM} is weighed on a balance and dissolved in an appropriate volume of molecular grade water. For example 3g of agar dissolved in 100mlof 1X TBE buffer = 3% gel (see table 2.3). A conical flask heated in a microwave oven, though sealed and then pierced with paraffin wax film provides a safe and rapid way to dissolve the agar. Swirling the flask every 20-30 seconds helps prevent boiling the agar. Once dissolved and cooled, 50µl of ethidium bromide can be added which will later intercalate with the DNA and permit it's resolution when fluoresced under 290nm ultra-violet light. The next step is the casting of the gel into the gel tray. Prior to this step the tray and buffer tank must be thoroughly cleaned with detergent and wiped down with 70% alcohol and the appropriate size comb inserted. Once the gel has become hot to the palm it can be

rapidly cast into the tray on a flat surface. Any bubbles can initially be removed by gently tapping the tray, if this proves unsuccessful 1ml Gilson can be used to remove them so long as the gel has not begun to set. When the gel has firmly set, bearing in mind the higher the % of agar the quicker and more firmly the gel will set, it can be covered in saran-wrap and moved to the lower compartment of a refrigerator. Once the gel has been chilled to 4°C it can be placed in the buffer tank and immersed in pre-chilled 1X TBE buffer. The comb can be removed and any bubbles removed with a Gilson. Pre-running the gel will help remove any concentrated ions from the wells and should ensure a clean run. Once mixed with loading dye (0.25% bromophenol blue in 40% sucrose w/v) samples could be loaded at the electrically positive end of the rig and the gel run at 100 volts for an absolute minimum of 90 minutes to permit differentiation of the bands. ϕX174 was always accompanied when running samples so as to permit the correct determination of the fragments being run.

Table 2.3 Agarose Gel Electrophoresis. Below are listed the requirements for correctly casting and running agarose gels for the separation of PCR fragments amplified from the PCR reactions.

Type	Agarose (High melting point)
Concentration	2.7-3.0%
Format	Horizontal
Length	200mm
Width	5mm
Buffer	1X TBE (Stock of 10X)
Maximum loaded volume	25μl
Voltage	100 (4.5V per cm)

Temperature	> 37°C
Stain	0.5% Ethidium Bromide 50µl/100ml)

2.6.4.1 DNA extraction from agarose gels

After using PCR to amplify your gene of interest (YOGI) is possible to dissect the desired band from the standard agarose gel and purify it with the appropriate reagents. Purified genes products can then be used for direct DNA sequencing. The QIAquick reagents are stored at room temperature (20-25 °C). The protocol is designed to purify single or double stranded DNA PCR products ranging from 100bp to 10kb.

2.6.4.2 Contents and buffers supplied:

Spin columns and collection tubes

Buffer QG – solubilses the agarose gel and provides conditions suitable for the binding of DNA in addition it also contains a pH indicator.

Buffer PB – Permits the efficient binding of small DNA strands and the removal of primers up to 40 nucleotides

Buffer PE – Ethanol wash

Buffer EB – Elution buffer (10mM Tris Cl, pH 8.5), 100% Isopropanol

2.6.4.3 Protocol

1. Using a sterile scalpel blade the band of interest is cut from the agarose gel
2. The slice is weighed and QC buffer in the proportion of 100mg gel/100µl of buffer is added

Gel is incubated @ 50°C for 10 minutes or until the slice has completely dissolved in a column

3. Checking that the colour is orange/violet i.e. pH 7.5. (Otherwise add 10µl of 3M sodium acetate) 100µl of 100% Isopropanol is added in the same ratio as before. The column placed in a collection tube and spun at 10,000g (13,000rpm) for 1 minute
4. 500µl of the QG buffer is then added and spun for 1 minute
5. 750µl of PE buffer is added, and spun for 1 minute
6. Flow through is discarded and column is placed in clean collection tube
7. To elute DNA 50µl of EB buffer is added to the centre of the tube and spun for 1 minute.
8. The flow through is concentrated DNA.

2.6.5 Polyacrylamide gel electrophoresis

Preparation of the glass plates is crucial. Both were washed in detergent and warm water for 10-15 minutes and then allowed to dry. Then sprayed with 70% ethanol and wiped clean. A couple of mls of bind saline were first applied to the short glass plate and wiped in long vertical motions over the entire surface of the plate. The same procedure was repeated using sigma coat on the glass buffer tank plate. Once dry a soft tissue swabbed in 100% methanol was used to wipe away any residues on either of the plates. Once dry the plates were sandwiched together with spacers and the comb inserted upside down, the rig was now clamped together. The same rig was used for denaturing DNA sequencing gels, for ingredients see table 2.6 and non-denaturing SSCP gels, for ingredients see table 2.3.

Table 2.4 Preparation of SSCP non-denaturing acrylamide gel. Ingredients for the preparation of SSCP non-denaturing acrylamide gels. Gels were cast at a variety of concentrations and with a number of additives, as subtle changes in a bands conformation and mobility can be achieved.

Acrylamide (30%)	22ml
Bis-acrylamide (3%)	6.75ml
5X TBE buffer	15ml
Molecular grade water	97.75ml
Glycerol	7.5ml
Ammonium persulphate (10%)	0.8ml
TEMED	0.1ml

2.6.5.1 TBE Buffer

- 5X –54g Tris
- 27.5g Boric acid
- 20ml 0.5M EDTA pH 8.0

2.6.5.2 PCR dilution solution:

- 0.1% SDS and 10mM EDTA

2.6.5.3 Loading buffer:

- 10ml formamide
- 0.2ml 0.5M EDTA @pH 8.0
- 10mg Bromophenol blue and 10mg Xylene cyanol green

2.6.5.4 Sample:

- 90μl of dilution solution,
- 6-10l of loading solution
- 10 l of PCR reaction solution

2.6.6 Silver Staining PAGE gels

This protocol is used for the silver staining of DNA and or RNA in polyacrylamide gels (see table 2.4). When the gel was finished running the rig was carefully dismantled and the buffer discarded. There are huge suction forces binding the buffer tank to the glass plate so a wedge was gently tapped between the plates to help relieve this pressure. When the plates were pulled apart the gel was adherent solely to the thin glass plates. The plate was now lowered into the first buffer. During the incubation the gel was gently agitated. All buffers were prepared an hour or so before the end of the running time of the gel. It was especially important for the developer containing the sodium borohydride.

Table 2.5 Silver Staining buffers. All buffers were made from the following ingredients. While some buffers could be stored e.g. buffer A and others reused e.g. buffer B others had to be made fresh prior to staining.

	Effect	**Constituent**	**Volumes**	**Incubation Time**
Buffer A	Stop	10% Ethanol 0.5% Acetic acid	100ml5mls 895mlddH$_2$O	3 min 2x10 sec ddH$_2$O
Buffer B	Stain	0.1% $AgNO_3$	1g in 1L	10 mins 2 washes
Buffer C	Developer	1.5% NaOH 0.01% $NaBH_4$ 0.015%Formaldehyde	15g in 1L 0.1g of 4mls ddH$_2$O	20 mins
Buffer D	Fixer	0.75% $NaCO_3$	7.5g in 1L	10 mins

A white $AgCl_2$ precipitate may form in gels where heavy concentrations of sodium chloride are present. This leads to heavy silver staining of the background. The precipitate can easily be redissolved by:

1. Washing gel for 30 seconds in ddH$_2$O
2. Incubate the gel in 2.5% Ammonium
3. Wash the gel twice for 30 seconds with ddH$_2$O
4. Incubate the gel with buffer a for 3 minutes
5. Continue staining protocol at buffer B

- 10g of $NaCl_2$ was added to the filtered buffer B. A grey silver chloride precipitate would form which could be then filtered or allowed to settle out with

gravity. The purified water could then be disposed of and the Silver waste safely collected.

2.6.7 DNA sequencing

Automated DNA sequencing was attempted using DNA purified from bands run in agarose gels (500ng of DNA +66ng 20mer primer in 12µl water). This proved unsuccessful as the number of bands that were un-resolvable and designated "x" made the technique inappropriate for further use. Instead manual sequencing using the chain termination method proposed by Sanger was attempted.

2.6.7.1 Dideoxy DNA sequencing – Chain Termination Method

Sequencing DNA involves the amplification of the sequence of interest in four separate reactions (see table 2.5). Each reaction includes the normal PCR reagents but also includes one dideoxy nucleotide, A, T, G or C. The dideoxynucleotides will incorporate into the extending strand at the same rate as any of the other nucleotides, but they will arrest polymerisation once incorporated. At the end of the reaction each tube will contain amplified fragments of all possible lengths, each tubes fragments will terminate in either an A, T, C, or G depending on which ddNTP was added at the start of the reaction. Samples from each reaction can then be run on polyacrylamide gel (PAGE). Four lanes each with numerous bands can then be stained. The smallest and faintest bands will be at the bottom of the gel as these will be the fragments that have migrated the furthest. The darker heavier bands will be those fragments, which were terminated last and have moved more slowly through the gel.

Table 2.6 Reaction contents for a two step genomic DNA sequencing reaction. The second reaction used exactly the same as primers as the first. 1μl of concentrated PCR reaction was used as the template for this second stage. Four reaction tubes were used, one for each ddNTP

Contents	Volumes
Buffer	5μl
DNA	1μl
Primer	5μl
Water	16μl
DideoxyNTPs (ddNTP)	2μl each
Taq	1μl

Table 2.7 Preparation of Sequencing denaturing acrylamide gel. Manual sequencing required a two step PCR reaction followed by electrophoresis using a denaturing polyacrylamide gel

Acrylamide (18%)	18ml
Bis-acrylamide (1%)	1ml
10X TBE buffer	12ml
Molecular grade water	47ml
Glycerol	0 ml
Ammonium persulphate (10%)	0.8ml
TEMED	0.05ml

2.7 MATERIALS

2.7.1 Cell Culture

Dulbecco's Modified Eagles Medium + Hams F12 basal medium (Gibco Life Technology)

200mM L-Glutamine (Gibco Life Technology)

Fetal Calf Serum (PAA)

50mg/ml Gentamicin (Gibco Life Technology)

Trypsin-EDTA).25% trypsin with 0.25% EDTA (Imperial Laboratories)

Dimethyl Sulphoxide (DMSO)

Laminar Flow Hood

Isoton (Coulter Euro Diagnostic Isoton II)

Geimsa stain (BDH)

Methylene Blue stain (BDH)

Tissue culture flasks 25cm^2, 80cm^2, 175cm^2 (Nunclon)

35mm x 10mm Tissue culture petri dishes (Corning)

2.7.2 Chemicals

Methanol / Ethanol (Sigma)

Acetic acid (Sigma)

Silver nitrate (Sigma)

Sodium hydroxide (Sigma)

Sodium Borohydride (Sigma)

Formaldehyde (Sigma)

Ethidium bromide (Sigma)

Cybergreen (Flowgen)

Methylene Blue (BDH)

2.7.3 Equipment

Nicool LM 10 freezing apparatus (Jencons Scientific Ltd)

Cyrostat (Jencons Scientific Ltd)

Philips 420kVp X-ray machine (Ago Installations Ltd)

Caesium 137 IBL 437C gamma irradator (CIS UK Bio International)

Class II Microbiological Safety Cabinet (Envair Ltd)

Coulter Counter (Coulter scientific Instruments), ZM model

PCR Machine (Scotlab GeneE)

Sequi Gene DNA sequencer (BioRad)

Diaphot Microscope (Nikon)

Wifug centrifuge Model 500E

Perspex gas boxes (5% CO_2 and air) maintained @ 37°C.

2.7.4 Reagents and Kits

Puregene DNA extraction kit Puregene	Cat#D-5000A
QIAquick PCR purification kit	Qiagen Cat # 28104
Micro-FastTrack kit	Invitrogen Cat# K5632-01
cDNA Cycle Kit	Invitrogen Cat# K1520-02

Chapter 3

RESULTS

3.1 Transformation HTori-3 Cells

3.1.1 Experimental Procedure

Exponentially growing HTori-3 cells were exposed to single doses of a range of X-rays and beta particles. Doses included: 0.25, 0.5, 0.75, 1.0,2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 Gy. Five flasks were seeded following radiation. Little or no growth was observed in the higher doses of irradiated cells following radiation, however after this initially period, cultures recovered well and grew very rapidly and required only a 1/40 split to sustain the flask. The flasks were passaged independently once weekly for six weeks. All flasks were prepared for injection at a concentration of 1x10E6 cells per flask/per mouse. The three columns below represent the three sets of screenings that were performed:

X-ray Acute 2.0Gy	X-ray Acute 1.0Gy	X-ray Acute 0.5Gy
X-ray Chronic 2.0Gy	X-ray Acute 0.75Gy	X-ray Acute 0.25Gy
Strontium 2.0Gy	X-ray Chronic 1.0Gy	X-ray Chronic 0.5Gy
	X-ray Chronic 0.75Gy	X-ray Chronic 0.25Gy
	Strontium 1.0Gy	Strontium 0.5Gy
	Strontium 0.75Gy	Strontium 0.25Gy

3.1.2 The Results

All but one group developed tumours, while two tumours were found in the second screening control group. A z-test gives a p value of less than 0.001 and thus confirms that there is a significant difference between the radiation treated and the unirradiated control groups. However, due to tumours being found in the control mice this statistic must be viewed with caution. No particular trend was observable, other than that the radiation source was able to produce tumours in all of the 2 Gy treated group, moreover these appeared progressively from the date of injection.

Immediately after injection (19th September 1997) a soft swelling was observed at the site of injection, this disappeared after the first week. The first tumour appeared in the Strontium group on day 56 with others following soon after (23 days). This first tumour grew progressively until day 109 when it was excised and made into a tumour cell line (TCL), using the method previously described. The remaining mice were kept until 27th February 1998. While two mice had no observably tumours a grade 2 and grade 3 were present in the remaining mice. The grade 3 tumour was also made into a TCL. The first tumour was designated ST-1, while the second ST-2. ST-2 was bulked up and frozen for storage, while ST-1 was passaged *in vitro* until it was re-injected on 17 March 1998.

On day 79 the first X-ray acute tumour was found. This grew progressively in just 30 days until its excision at grade 4 on day 109. The remaining 4 mice in this cohort were checked weekly until 1 April 1998, 194 days since injection. Two of the mice had no noticeable tumours, while the remaining two had small grade 1 tumours. The first and largest tumour was re-introduced *in vitro* and made into a tumour cell line.

The cell line was designated XA-1 and was passaged weekly until it too was re-injected on the 17th March 1998.

Three tumours were found in the X-ray chronic cohort. Although injected at the same time as the other group the time taken for these to form suspected tumours was greatly increased. It was over 4 months before the first tumours were seen. Two mice with no observable tumours were killed 30 March, the remaining three all of which had good sized tumours were kept until 12 May 1998 when then they were culled with tumours grade 4, 3 and 3. All were made into TCLs and the best of these was designated XC-1.

All new tumour lines were sent for histological examination (see figure 3.3) and stocks of the newly formed cell lines were frozen in nitrogen for storage. In addition, chromosome analysis (see figure 3.4) was performed on the first tumour from each group so as to confirm the human nature of the cell line. A dose response was plotted and the cells then used to look for the expression of delayed lethal damage at set times post-irradiation. At the end of this period the DNA was extracted and used for molecular biology.

Of the mice in the control group, none were found to have tumours after 192 days since injection. On 30th March they were culled, one mouse was killed on 19th January due to suspected gastro-enteritis. Figure 3.1 gives the tumour grow rates in the nude mice throughout the screening period. The tumour incidence can be viewed in table 3.1 and table 3.2.

3.1.3 Statistical Analysis of Tumour Formation

A Z test was used to test the hypothesis that there was a statistically significant difference between the total number of tumours found in the controls compared to the treated groups. An estimator of the variance was calculated assuming a common population variance. A Z-test is used when n is high i.e. greater than fifty, with an n value less than 50 a t-test would have been more appropriate. Data collected from tumour formation experiments n=80.

$$Z \text{ value} = (x_1 - x_2) \; / \; \hat{\sigma}\sqrt{(1/n_1 + 1/n_2)}$$

Where, x_1 and x_2 are the mean number of tumours from each sample population $\hat{\sigma}$ is the estimator for variance and $1/n_1$ and $1/n_2$ are the reciprocals of n for each sample where n is the number of values for each sample.

The estimator is calculated thus,

$$\hat{\sigma} = (n_1 \times S_1^2) + (n_2 \times S_2^2) / n_1 + n_2 - 2$$

At a 95% confidence interval a critical value (2 tailed) of 1.96 is estimated, at a 99% confidence interval a critical value (2 tailed) of 2.58 is estimated. From the tumour data collected from this study a Z test value of 5.619 is calculated which lies well beyond the critical value interval even at a 99%a C.I. Thus, a significant difference can be confirmed between the two mean data values.

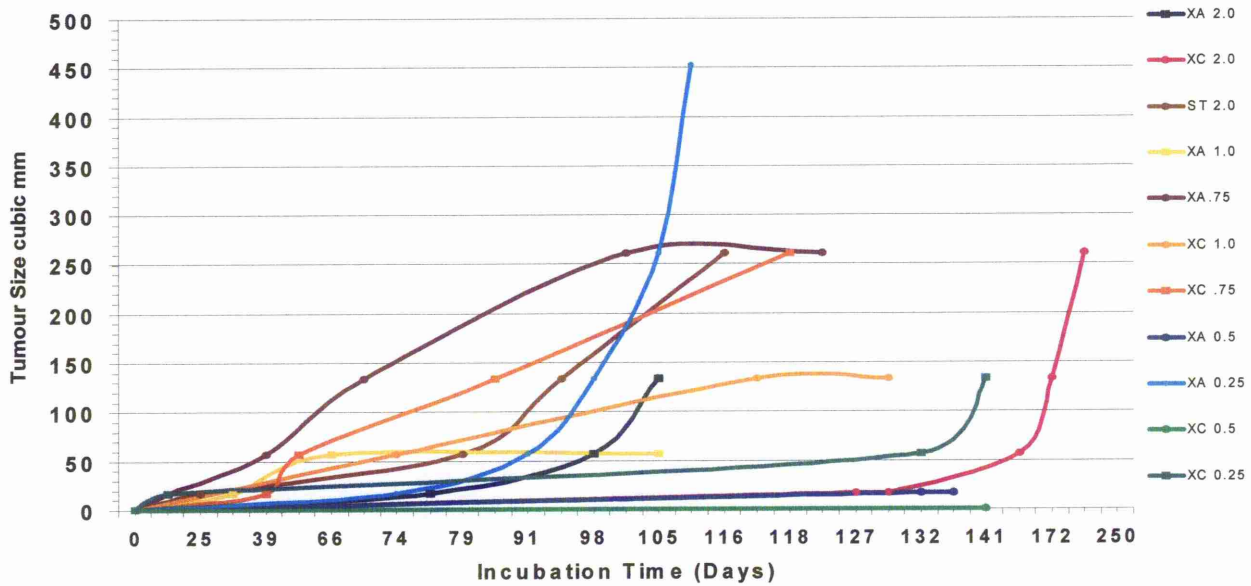


Figure 3.1 Tumour growth curves after transplantation into athymic nude mice. Normal HTori-3 cells injected were treated with different doses of chronic ($1Gy/hr^{-1}$) or acute ($120\ Gy/hr^{-1}$). X-rays doses ranging from 0.25 Gy to 2 Gy. One strontium irradiation @ 2Gy was also screened.

17th March 1998 a second screening of irradiated HTori-3 cells was performed. On this occasion, doses below that of the previous screening were selected to see if a threshold was present. Two doses were selected from the X-ray acute and X-ray chronic groups; 1 Gy and 0.75Gy.

The first tumours appeared in the X-ray acute group in less than a month. By the end of June three suspected tumours were present in the 1 Gy set, while four were present in the 0.75 Gy. These were designated XA-2 and XA-3 respectively. However, two tumours of similar size were found in the controls. Contamination during the culture process was suspected. Extra control mice were injected so as to confirm the non-tumourigenic nature of the parental cell line HTori-3 . In the X-ray chronic three large suspected tumours were found, two variable tumours were seen, these appeared up to a size 2 but then disappeared. The largest of the other three suspected tumours was made into a TCL and designated XC-2. In the lowest doserate set; 0.75Gy over 8 hours size 3 tumours were observed, one of these was made into a TCL and designated XC-3. None of the control mice for this group had any tumours throughout the experimental period.

On 14th July 1998 the last group of X-irradiated cells were injected for screening purposes. The group included 0.5Gy and 0.25Gy chronic and acute, these were the lowest dose available, with greater than a 100-fold difference in their doserate. Extra controls were added into this group to ensure that the induction of tumours in the controls was not due irradiation independent transformation *in vitro*.

Table 3.1 Table of combined tumour incidence in HTori-3 cells for all doses of radiation screening in nude mice. Included are the tumour incidences of secondary tumours formed after cells derived from primary tumours were reinjected.

Dose	Radiation Source	Primary Tumours	Incidence	Secondary Tumours
2 Gy	X-ray Acute	**2/5**	**40%**	2/5 40%
2 Gy	X-ray Chronic	**3/5**	**60%**	1/5 20%
2 Gy	Strontium	**3/5**	**60%**	5/5 100%
Controls	None	**2/10**	**20%**	
1 Gy	X-ray Acute	**4/5**	**80%**	
1 Gy	X-ray Chronic	**3/5**	**60%**	
0.75 Gy	X-ray Acute	**3/5**	**60%**	
0.75 Gy	X-ray Chronic	**2/5**	**40%**	
Controls	None	**3/10**	**30%**	
0.5 Gy	X-ray Acute	**1/5**	**20%**	
0.5 Gy	X-ray Chronic	**0/5**	**0%**	
0.25 Gy	X-ray Acute	**3/5**	**60%**	
0.25 Gy	X-ray Chronic	**3/5**	**40%**	
Controls	None	**1/5**	**20%**	
Total controls		**6/25**	**26%**	
Total Tumours		**27/55**	**49%**	

3.1.3 Re-injection of derived tumour cell lines

On 6th April 1998 the first two cell lines XA-1 and ST-1 were reinjected to confirm their tumourigenecity. With 3 weeks all ST-1 mice had grade four tumours, all were killed 1st May and sent for histology. By the 12th June 2 out of 5 mice in the group XA-1 had grade 5 tumours. The X-ray chronic tumour line was reinjected sometime later but surprisingly no tumours appeared and the mice were culled after 4 months.

3.1.4 Latent Period

Latent period can be defined as the interval between injection and the appearance of the first primary palpable tumours (see figure 3.2). In the experimental group some of the tumours grew progressively from the date of injection e.g. XC 1.0. However, from those tumours that had latent periods before tumour growth the shortest was 56 days for the 2Gy strontium-irradiated cells. Zero latency was observed when these cells were reinjected. The only group of cells screened that did not produce tumours, was the X-ray chronic 0.5Gy. These cells were screened for 143 days, with no visible tumours at the end of the incubation process the mice were culled.

3.1.5 Conclusion

- Gamma radiation can enhance the rate of transformation of thyroid epithelial cell
- All tumours derived are confirmed as from the human thyroid epithelial cell line
- Latent period appears to have no effect on the time to tumourigenesis
- The time to tumourigenecity may be indicative of the rate of instability

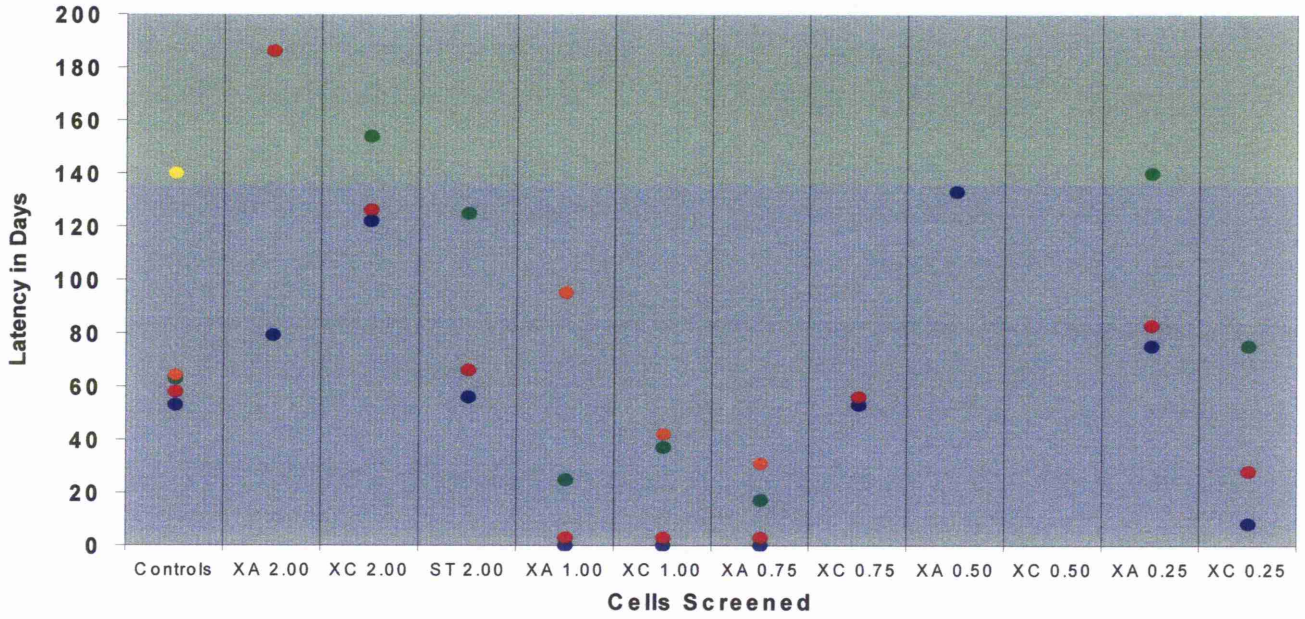


Figure 3.2 Graph of latency of tumour development in athymic nude mice after transplantation of strontium, x-ray acute and x-ray chronic irradiated thyroid epithelial cells after a range of radiation doses. Graph shows all tumours colour coded from each group irradiated, colour coded for appearance of tumours in individual mice.

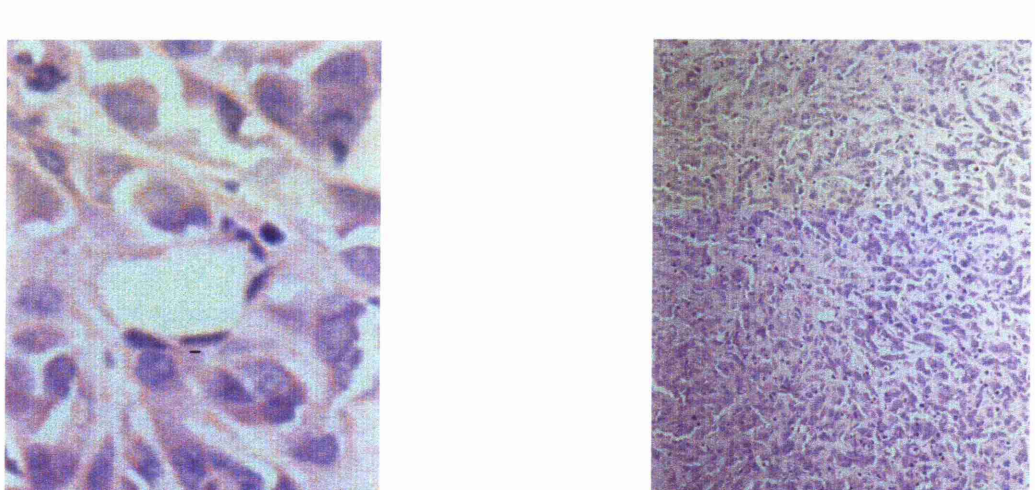
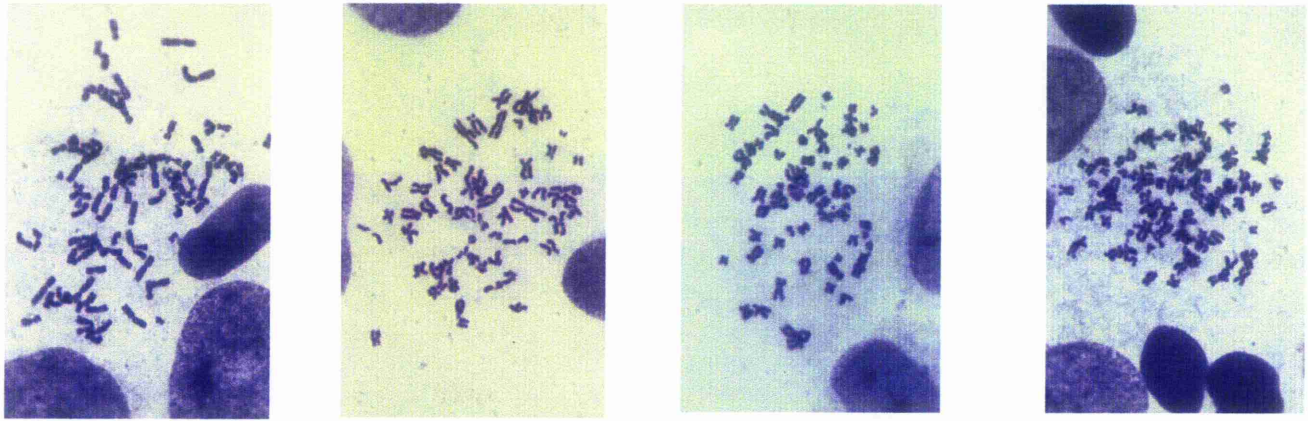


Figure 3.3 Histological appearance of a primary XA-1 (2Gy) tumour at low power (x100) and at high power (x400). Data confirms anaplastic nature of all tumours with little differentiation. Scale bar on left equals 100μm, scale bar on the right equals 25μm, approximately.



A B C D

Figure 3.4 Chromosome spreads of the non-tumourigenic parental control cell line HTori-3 (Panel A). Tumour chromosomes are derived from HTori-3 cells after screening 2 Gy irradiated cells in nude mice. Panel B, X-ray Acute tumour (XA-1), Panel C, X-ray Chronic tumour (XC-1) and Panel D, STrontium tumour (ST-1). Data confirms that all tumours derived from athymic nude mice were human HTori-3 cells and not murine spontaneous tumours.

Table 3.2 Summary of all Tumours produced during this study, including those tumours from the first tumour screening that were reinjected to confirm tumourigenecity

Screenings for Tumourigenecity

First Screening

Group 1	ST 2	XA 2	XC 2	Control
Dose	2	2	2	0
Dose Rate Gy/hr-1	2.286	240	0.25	0
No. of mice	5	5	5	5
Cage 1	3 males	3 males	3 males	3 males
Cage 2	2 males	2 males	2 males	2 males
Passage	5	4	4	10
Date Injected	19.9.97	19.9.97	19.9.97	19.9.97
No of Tumours	4	2	3	0
Grade of Tumours	1 2 3 4	1 4	3 4 4	0
Days in vivo	196	194	237	192
Date Excised	3.4.98	1.4.98	12.5.98	30.3.98
Line Name	ST-1	XA-1	XC-1	N/A
% Tumourigenecity	80%	40%	60%	0%

Second Screening of First Primary Tumours

Cell Line	ST-1	XA-1	Control	XC-1
Passage	4	4	4	4
Date Injected	6.4.98	6.4.98	6.4.98	10.8.98
No. of mice	5	5	5	5
Cage 1	3 males	3 females	3 males	3 males
Cage 2	2 males	2 males	2 males	2 females
No of Tumours	5	2	2	1
Tumour Sizes	4 4 4 4 4	5 5	5 5	3
Days in vivo	21	78	78	57
Date Excised	1.5.98	7.8.98	7.8.98	6.10.98
% Tumourigenecity	100%	40%	40%	20%

Second Screening

Group 2	XA 1	XA 0.75	XC 1	XC .75	Control
Dose	1	0.75	1	0.75	0
Dose Rate Gy/hr-1	2.286	2.286	0.125	0.09375	0
No. of mice	5	5	5	5	5
Cage 1	3 males	3 females	3 females	3 females	3 males
Cage 2	2 males	2 females	2 females	2 females	2 males
Passage	7	7	10	10	8
Date Injected	17.3.98	17.3.98	17.3.98	17.3.98	17.3.98
No of Tumours	4	3	3	2	1
Grade of Tumours	3 2 1 1	5 5 3	1 4 4	5 4	3
Days in vivo	143	143	143	143	143
Date Excised	7.8.98	7.8.98	7.8.98	7.8.98	7.8.98
Line Name	XA -2	XA-3	XC-2	XC-3	N/A
% Tumourigenecity	80%	60%	60%	40%	20%

Third Screening

Group 3	XA 0.5	XA 0.25	XC 0.5	XC 0.25	Control
Dose	0.5	0.25	0.5	0.25	0
Dose Rate Gy/hr-1	2.286	2.286	0.0625	0.03125	0
No. of mice	5	5	5	5	5
Cage 1	3 males	3 males	3 females	3 females	3 females
Cage 2	2 females	2 males	2 females	2 females	2 females
Passage	9	10	10	10	11
Date Injected	14.7.98	14.7.98	9.7.98	9.7.98	14.7.98
No of Tumours	1	3	0	3	1
Grade of Tumours	2	3 2 1	0	3 6 3	2
Days in vivo	89	93	94	94	89
Date Excised	7.12.98	7.12.98	7.12.98	7.12.98	4.12.98
Line Name	XA-4	XA-5	XC-4	XC-5	N/A
% Tumourigenecity	20%	60%	0%	60%	20%

3.2 Growth and Survival

3.2.1 HTori-3 Growth Curves

Growth curves for the HTori-3 cell line were the first to be undertaken in an attempt to understand the kinetics and dynamics of the cells reproductive cycle in culture.

3.2.1.1 Experimental Procedure

Plates for the growth curve were prepared in duplicate to cover the experimental period. Each was filled with 2mls of culture medium. Each dish was seeded at a total concentration of 12,000 cells. Cells were incubated for 72 hours with two dishes being sampled daily. Repeat experiments were staggered to account for weekends.

3.2.1.2 Results

As the cells recovered from the plating procedure, an initial lag period, characteristic of all growth curves was observed which lasted for 2-3 days (see figure 3.5, 3.6 and 3.7). Steady growth was then observed over the next 10-14 days. If media was withheld after one week in culture a characteristic nutrient plateau was observed similar to that seen in cultures at confluence. Eventually, the surface area of the dishes became carpeted in cells and confluence was reached. Growth in cultures of tumour cells would also eventually arrest, though cells became highly concentrated and tightly packed. Little or no piling of cells occurred so contact inhibition is likely to have contributed to their arrest. An estimated doubling time could be made from the gradient of the exponential phase of growth; for HTori-3 cells this was around 22 hours per cell division.

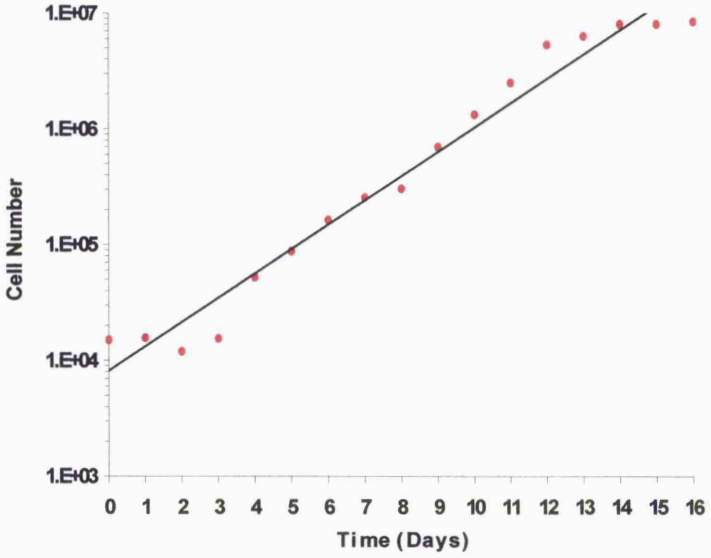


Figure 3.5 Growth curve of HTori-3 cells *in vitro* culture, with an average doubling time of 22 hours.

An initial lag period is observed over the first few days as the cells recover from the plating procedure. Exponential growth is then observed throughout the next 10 days until day 12. Even after feeding on day 7 the cells were unable to continue exponential growth indefinitely. Saturation is reached after day 12 and the cells begin to senesce as confluence is reached.

3.2.2 Tumour Growth Curves

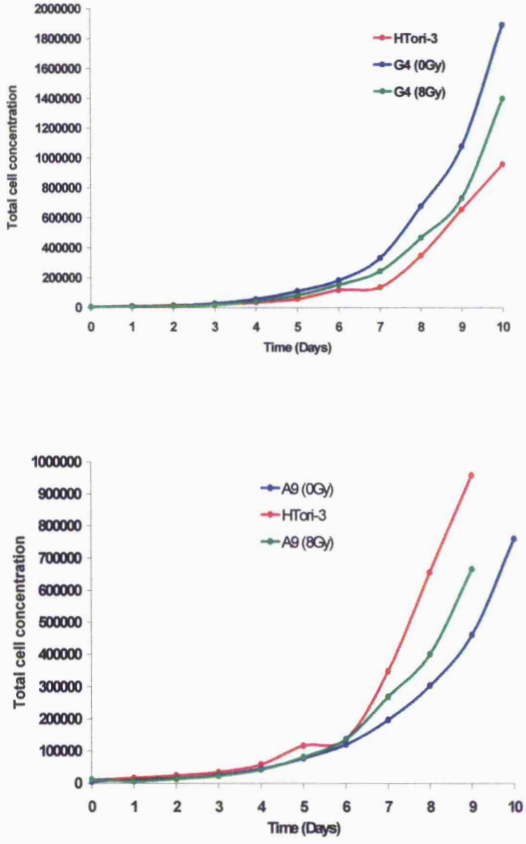
Ten and fourteen day growth curves were made for selected alpha and gamma tumour cell lines. These tumour cell lines had previously been derived in our laboratory from alpha and gamma irradiation of HTori-3 cells (see appedix). These lines were now characterised by exposure to gamma particles (Riches et al, 1994, 1997). The first X-ray acute, X-ray chronic and strontium tumour cell lines were also included in this study. Normal tumour cell growth curves were compared against those expressing the characteristics of the delayed lethal damage (DLD) phenotype. These data could then be directly compared to that of the parental cell line HTori-3.

3.2.2.1 Experimental procedure

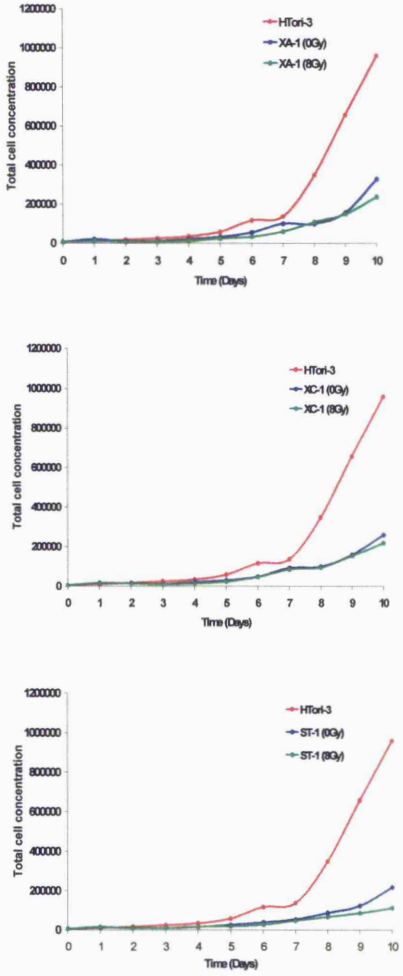
The cell line of choice was seeded at a concentration of $6x10^3$ cells per ml. A total of 2mls of cell suspension in standard Dulbecco's media was added into a small $8cm^2$ Nunc dishes.. Duplicate dishes were seeded, e.g. 20 dishes for a 10-day growth curve. Over time, three repeats were made at staggered intervals.

3.2.2.2 The Results

On the whole, normal HTori-3 cells grew faster then any of the newly derived X-ray tumour lines (see 3.6 and 3.7). However, no significant difference was noted for any of the cell lines when compared in the exponential phase of the cell cycle. However, general trends could be noted: 1) The new X-ray and Strontium tumours had growth rates below that of HTori-3 .2) Tumours that expressed DLD after 8Gy of gamma radiation had an apparent growth rate less than their parental tumour. 3) The long established tumours G4 and A9 appeared to have a faster rate of growth.



Figures 3.6 Comparison of *in vitro* growth curves of HTori-3 control cell and the tumour cell lines; A9 and G4. Growth rates of the tumour cell lines were compared to the same tumour expressing the DLD phenotype. Both are plotted with comparison to the HTori-3 cell line. Cell cycle times was calculated as 22 hours per cell division. An initial lag period was observed during the first few days, this lead to the exponential growth phase which lasted over a week until confluence was reached.



Figures 3.7 Comparison of *in vitro* growth curves of HTori-3 control cell and the tumour cell lines; XA, XC and ST. All lines were also compared to their DLD expressing counterparts which had previously been irradiated with 8Gy and cultured for 4 weeks.

3.3 Dose Response of HTori-3 and Tumour Cells Lines.

These experiments were initially used to investigate the relative radiosensitivity of the different cell lines. Later they were used to collect cells to investigate the expression of Delayed Lethal Damage (DLD) after a variety of doses of gamma radiation.

A series of dose response experiments were performed using 9 alpha, (see figure 3.9), 12 gamma (see figure 3.10), one x-ray acute, (see figure 3.11) one x-ray chronic (see figure 3.12) and one strontium tumour cell line (see figure 3.13). The dose response of HTori-3 cells irradiated with ^{131}I can be seen in figure 3.14.

3.3.1 Experimental Procedure

An exponentially growing culture (T80cm^2 sized flask) was harvested and re-suspended in a universal tube with an equal or greater volume of media. This volume was then split into separate universal tubes, labelled with the appropriate dose of radiation they were to receive; 0, 2, 4, 6, or 8 Gy. All tubes were then placed on ice and taken to the radiation room were the Cs 137 gamma source was housed. Irradiation times were calculated and programmed into the source. Each universal was placed inside a special container and irradiated before being placed immediately back on ice. The tubes were returned to the laboratory and serially diluted at the appropriate concentrations so to yield 50 colonies per plate.

3.3.2 Results

All cells lines showed a clear dose response to gamma irradiation. Direct comparisons of the surviving fraction at SF8 (8Gy) have been made (see table 3.3). Not only are there obvious differences between the individual tumour cell lines dose response but differences in colony size were clearly noted, as can be seen below.

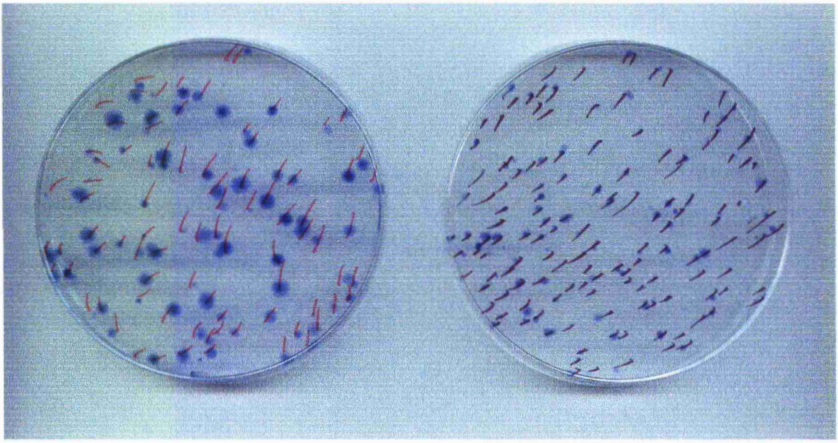


Figure 3.8 Photograph of the variations found in colony sizes. All 90cm^2 petri dishes had previously been filled with 10ml of Dulbecco's media and had been pre-warmed and pre-gassed in a 5% CO_2 incubator.

Once seeded dishes were returned to the CO_2 incubator for 14 days to allow for the formation of colonies. After this time dishes were stained with giemsa and counted. At the same time fresh flasks were seeded at 10X the plating dose to study the effects of delayed lethal damage phenotype (DLD). These flasks would be then be used to investigate plating efficiencies at T=2, T=4, T=8 and T=12 weeks post-irradiation. Cells were also collected during this stage for the micronucleus and apoptosis assays. Clear differences can be observed between the control plate on the

left and the DLD expressing dish on the right. The scoring of colonies was subjective and open to some interpretation. However, as a rule, colonies over 50 cells were termed reproductively viable and were scored.

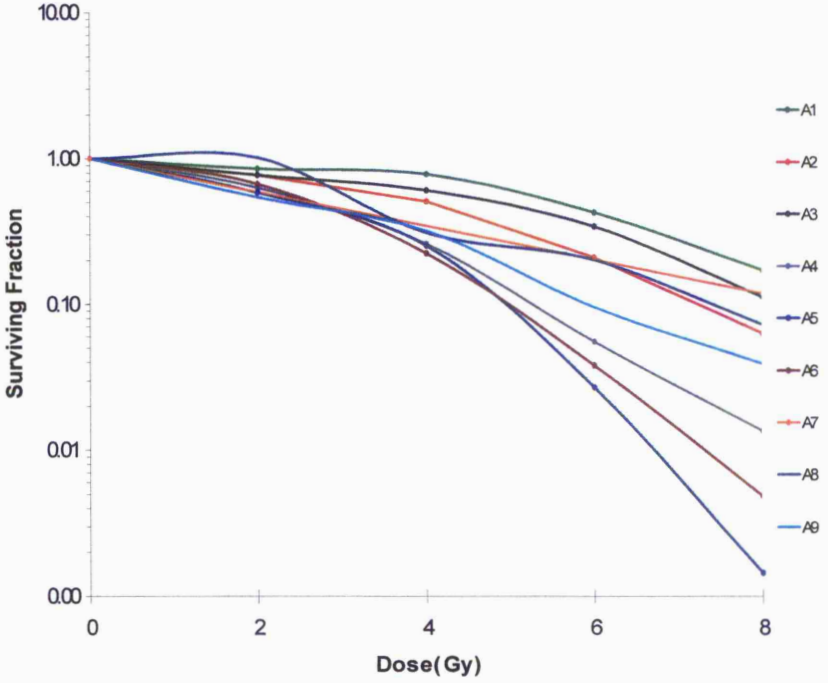


Figure 3.9 Comparison of the radiation dose-response of the alpha tumours A1-A9, all tumours were derived from the parental cell line HTori-3 after different doses of alpha radiation. Each tumour cell line is derived from the explant of the primary tumour that grew in the nude mice.

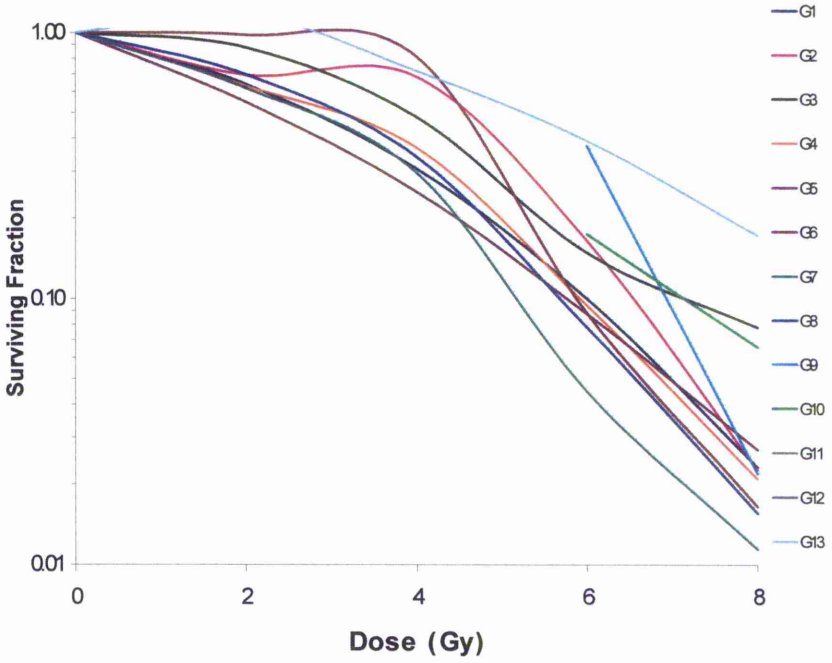


Figure 3.10 Comparison of the radiation dose-response of the gamma tumours G1-G13, all tumours were derived from the parental cell line HTori-3 after different doses of gamma radiation. Each tumour cell line is derived from the explant of the primary tumour that grew in the nude mice.

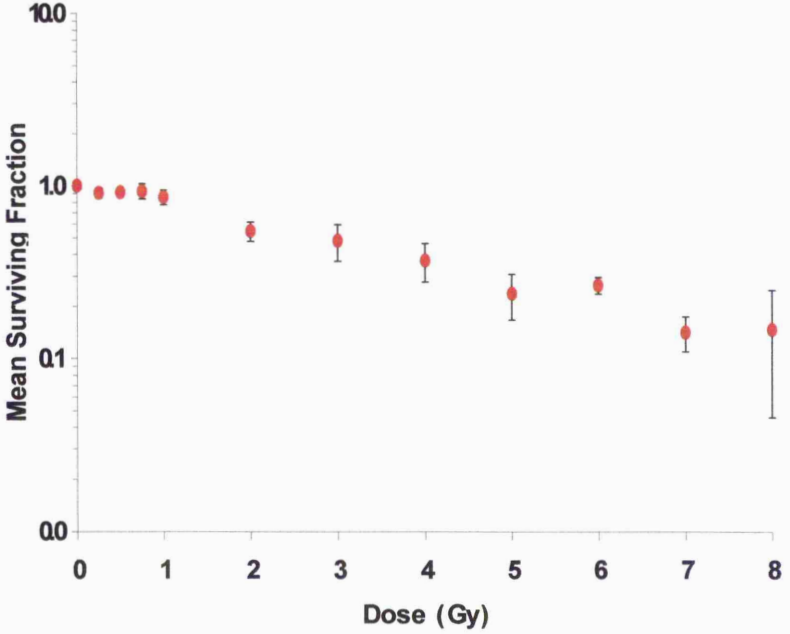


Figure 3.11 The mean radiation dose-response of the X-ray tumours XA-1, the tumour was derived from the parental cell line HTori-3 after a 2 Gy dose of X-ray radiation. Each tumour cell line is derived from the explant of the primary tumour that grew in the nude mice. Dose points for dose-response curve included: 0.25, 0.50, 0.75, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 Gy

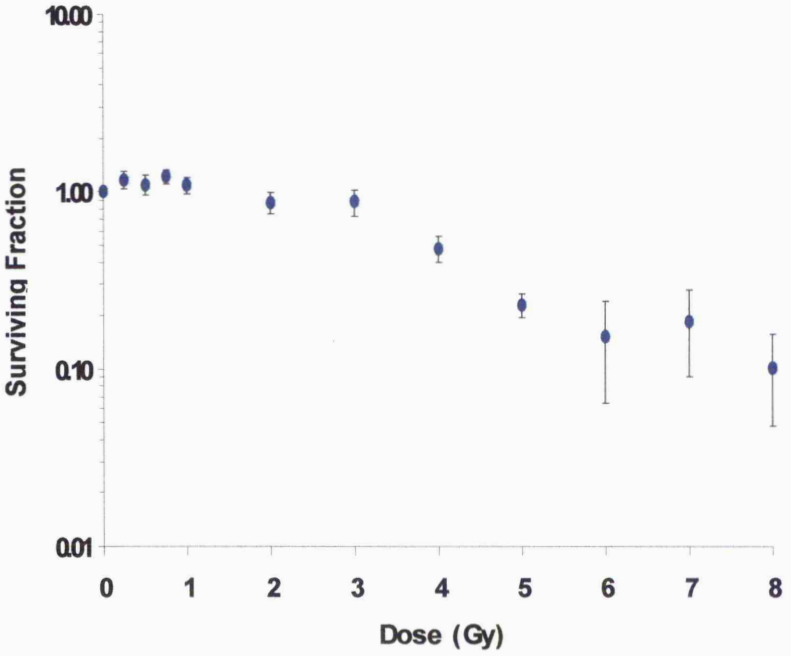


Figure 3.12 The mean radiation dose-response of the X-ray tumours XC-1. The tumour was derived from the parental cell line HTori-3 after a 2 Gy dose of X-ray radiation. Each tumour cell line is derived from the explant of the primary tumour that grew in the nude mice. Dose points for dose-response curve included: 0.25, 0.50, 0.75, 1.0, 2.0, 3.0 ,4.0, 5.0, 6.0, 7.0, 8.0 Gy

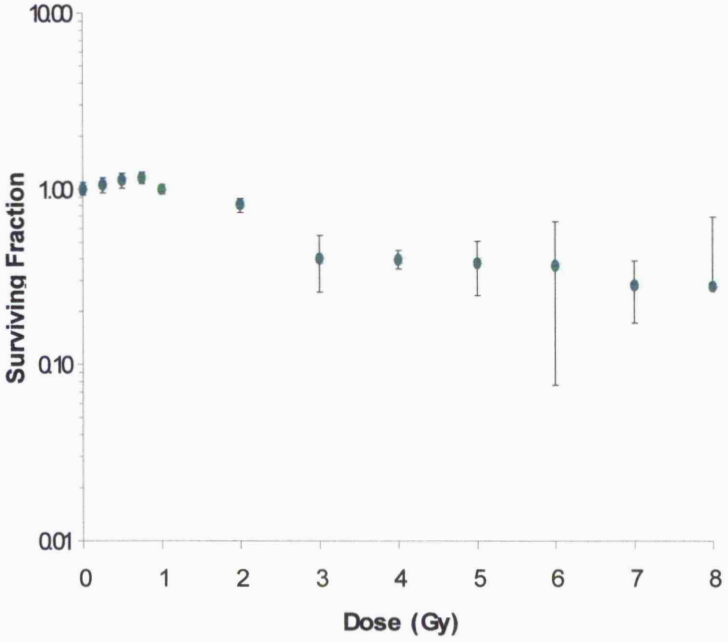


Figure 3.13 The mean radiation dose-response of the strontium tumour ST-1, the tumour was derived from the parental cell line HTori-3 after a 2 Gy dose of X-ray radiation. Each tumour cell line is derived from the explant of the primary tumour that grew in the nude mice. Dose points for dose-response curve included: 0.25, 0.50, 0.75, 1.0, 2.0, 3.0 ,4.0, 5.0, 6.0, 7.0, 8.0 Gy

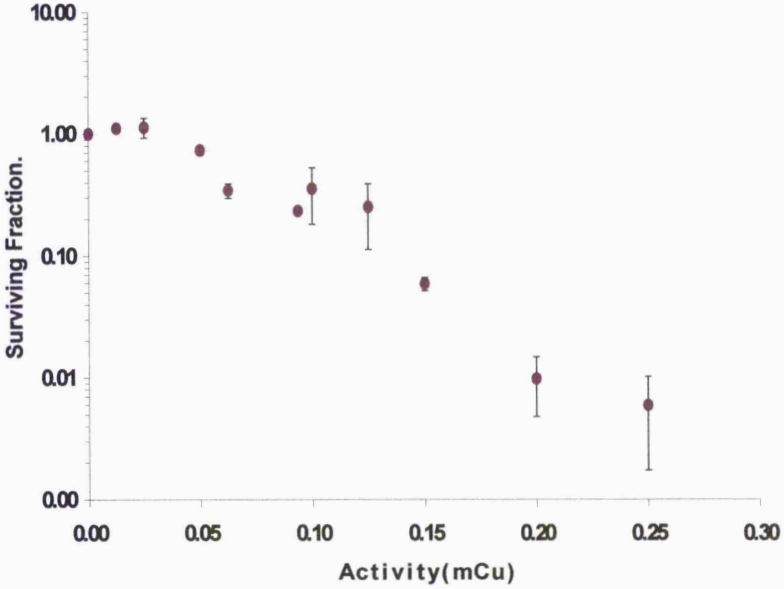


Figure 3.14 The mean radiation dose-response of HTori-3 cell irradiated with Iodine 131. Intra-cellular irradiation with iodine 131 was hoped to induce tumours and mimic cellular response similarities to those observed from the radiation induced tumours from children surrounding Chernobyl. (Combined data from Dr Clare Peddie).

Table 3.3 A comparison of the surviving fraction after 8Gy of all tumours analysed for dose responses and plating efficiencies after gamma radiation. While some tumour cell lines reacted well to the radiation, being relatively radio-resistant e.g. ST-1, G13 and A7. Others reacted poorly, being more sensitive to the damage sustained e.g. G11, A4 and A6.

Cell Line	Mean SF8	Cell Line	SF8	Cell Line	SF8
HT	0.09	**G1**	0.02	**A1**	0.17
		G2	0.023	**A2**	0.06
XA1	0.118	**G3**	0.08	**A3**	0.11
XC1	0.101	**G4**	0. 02	**A4**	0.01
ST1	0.281	**G5**	0.03	**A5**	0.00
		G6	0.02	**A6**	0.00
		G7	0.01	**A7**	0.12
		G8	0.02	**A8**	0.07
		G9	0.02	**A9**	0.04
		G10	0.07		
		G11	0.03		
		G12	0.09		
		G13	0.17		

3.4 Studies into the expression of the delayed lethal death phenotype

Plating efficiencies measure reproductive integrity following a given procedure. The ability of a culture to adhere to a surface and form a visible colony of 50 cells or more is used as confirmation. Measurement post-irradiation gives an insight into the cells ability to repair or pass on a defective karyotype to its progeny over time.

3.4.1 Experimental Procedure

Initially, HTori-3 cells were used for the standard colony assay, later this assay was extended to include all tumour cell lines. Doses of 2, 4, 6, and 8 Gy were chosen to give a broad range of response. Later some of the repeats included doses up to 16 Gy where cell survival was greatly reduced. Time intervals of two weeks from the date of irradiation and up to 8 weeks post-irradiation were chosen to observe the cells delayed response to an ionising insult. Repeats were performed with a control at the 8 Gy dose; as this was the dose at which the induction of DLD was most clearly observed.

3.4.2 The Results

All cell lines tested expressed the DLD phenotype as detailed by Alper et al, 1989. A dose dependant decrease in cell survival and therefore colony forming potential was observed with all cell lines (see figures 3.15, 3.16 and 3.17). The extent of recovery in all lines was found to increase over the study period. At 8 weeks post-irradiation, colony forming potential in 8Gy irradiated HTori-3 cells was equal to that of their control, i.e. the cultures could be termed reproductively recovered.

The in ability of the cells to prevent the passage of defective material into progeny is termed "Genomic Instability" and was used as a measure of delayed lethal death. Figure 3.18 plots the ratios of normal to DLD expressing plating efficiencies of all the tumour cell lines (see also table 3.4).

3.4.3 Conclusions

- Using a decreased plating efficiency as an indicator, all tumour cells lines investigated confirmed the expression of the DLD phenotype
- Doses of 6Gy and above were required to induce DLD expression
- An incubation period of around four weeks was required
- Recovery of the PE appeared after 8-12 weeks post radiation

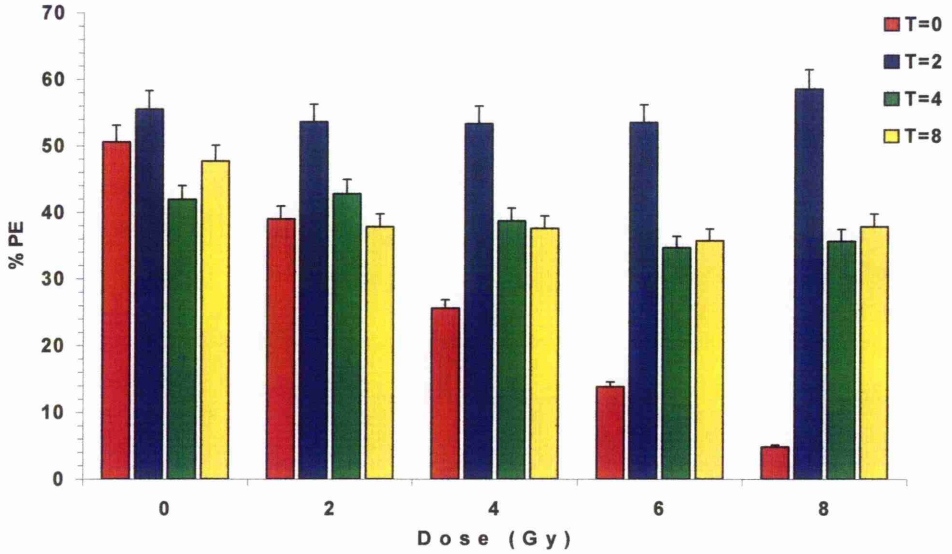


Figure 3.15 HTori-3 plating efficiency at two weekly intervals post irradiation. There appears to be a dose dependant decrease in plating efficiency at T=0.. An effective recovery is apparent at 2 weeks post-irradiation. Although there is a slight decrease in plating efficiency over 4 and 8 weeks from cultures irradiated at doses over 6 Gy the HTori-3 cell line can not be said to express the DLD phenotype as expressed in the tumour cell lines.

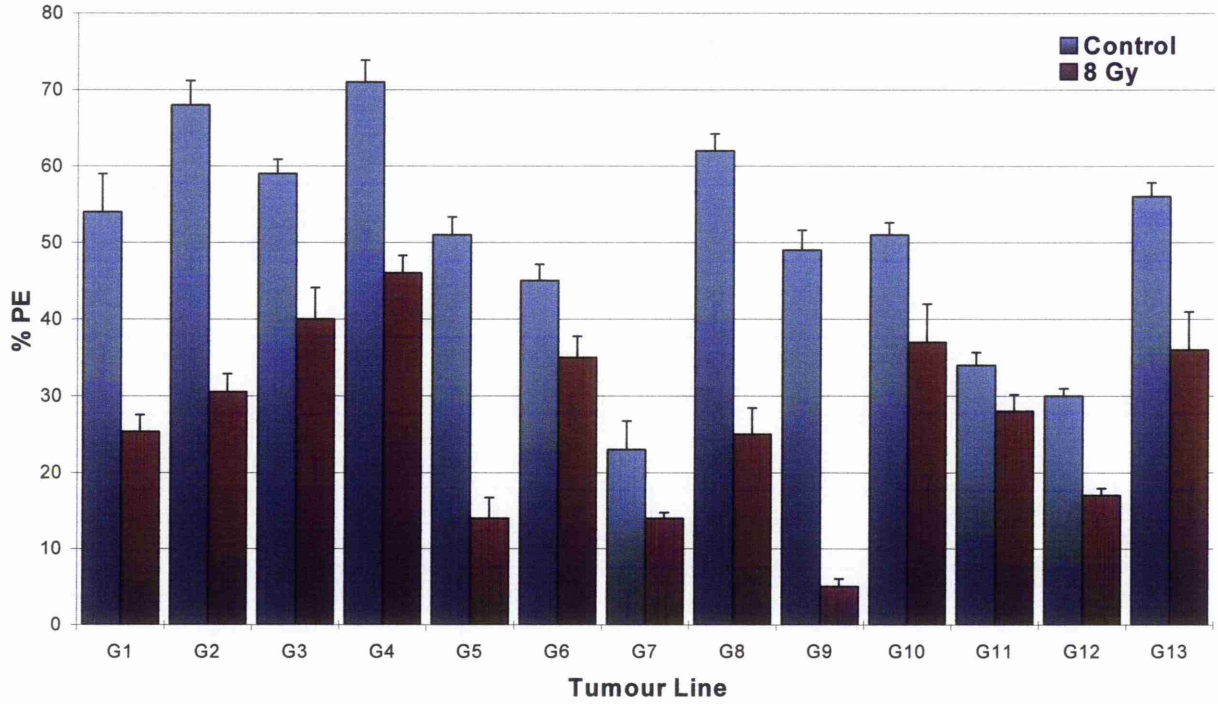


Figure 3.16 Plating efficiencies of all 13 gamma tumour cell lines. Each control line is compared to the 8Gy irradiated DLD expressing group that had been irradiated four weeks earlier and passaged *in vitro*.

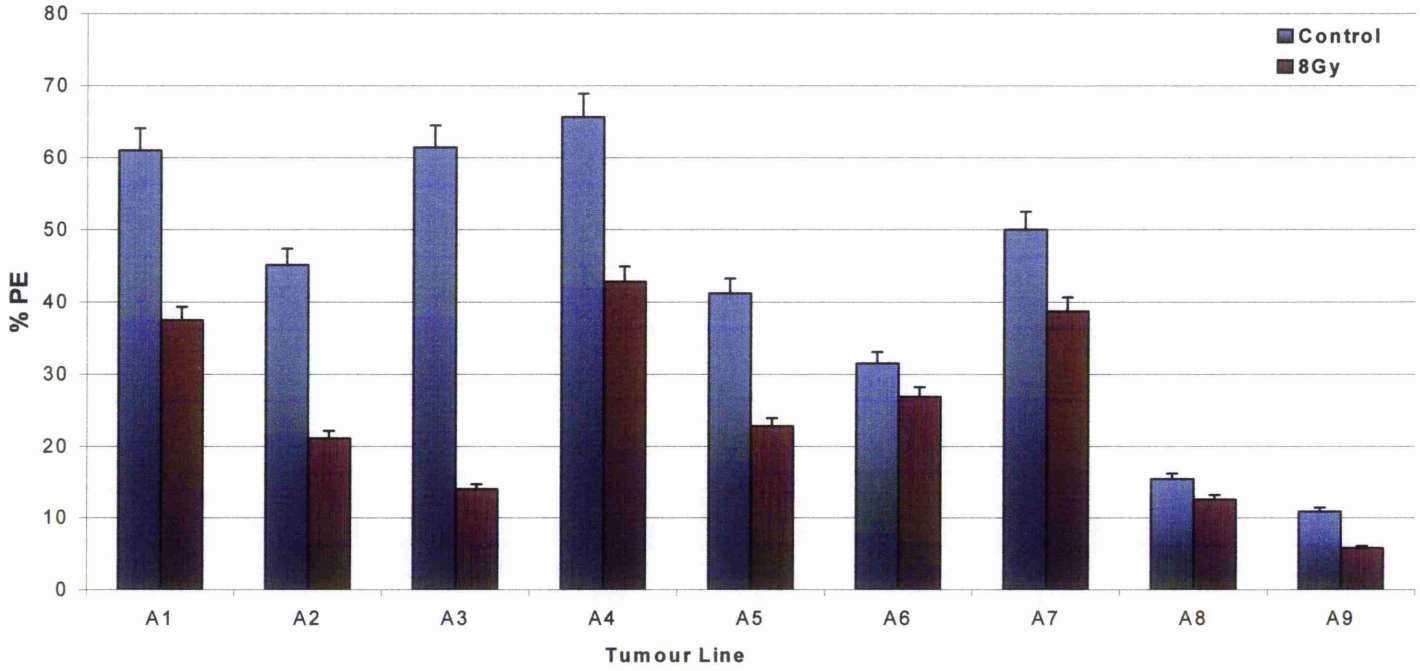


Figure 3.17 Plating efficiencies of all 9 alpha tumour cell lines. Each control line is compared to the 8Gy irradiated DLD expressing group that had been irradiated four weeks earlier and passaged *in vitro*.

Table 3.4 The plating efficiencies from the all the *in vitro* colony assay experiments can be converted into ratios so to clarify the observed differences between the control and the treated tumour cell lines (TCL). i.e. The plating efficiency of each cell line at T=4 weeks was divided by its own control plating efficiency at T=0 weeks. The larger the ratio, the greater the difference in plating efficiency. While some lines appeared to recover well, back to near control levels e.g. A5. Others showed a marked decline in colony forming ability during the experimental period, e.g. ST-1, XA-1, G13, A1 A3 and A7 showing the most dramatic loss of colony forming ability.

Cell Line	Ratio	Cell Line	Ratio
A1	0.48	**G1**	0.77
A2	0.49	**G2**	0.44
A3	0.75	**G3**	0.67
A4	0.64	**G4**	0.65
A5	0.32	**G5**	0.28
A6	0.85	**G6**	0.79
A7	0.53	**G7**	0.11
A8	0.81	**G8**	0.41
A9	0.53	**G9**	0.11
XA-1	0.69	**G10**	0.72
XC-1	0.68	**G11**	0.79
ST-1	0.85	**G12**	0.58
HP	0.98	**G13**	0.57

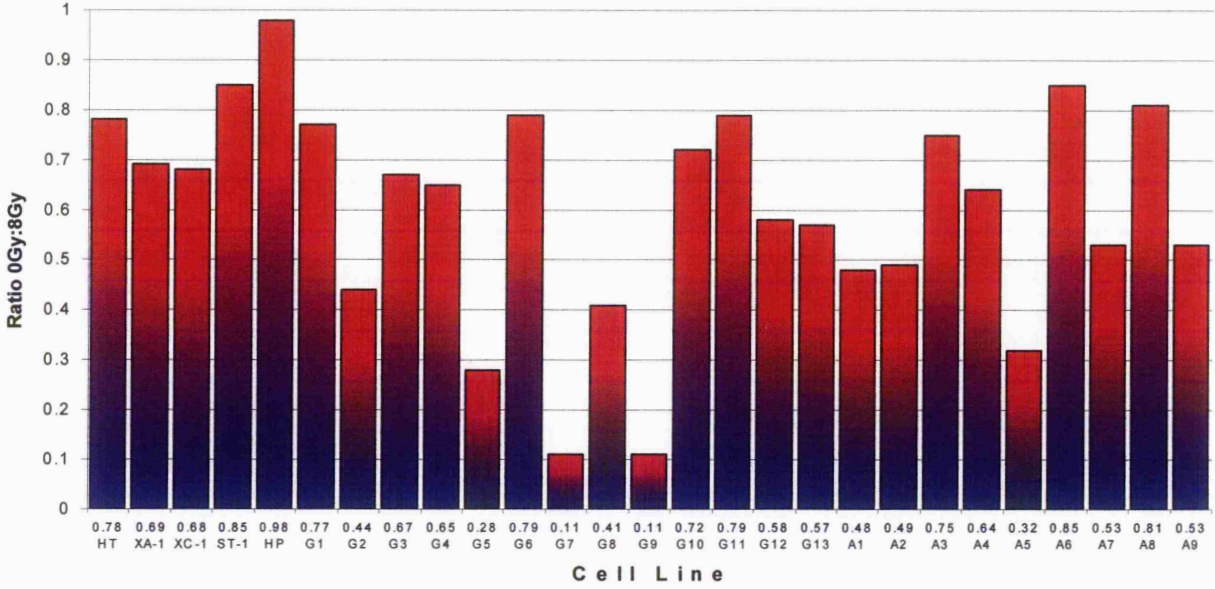


Figure 3.18 Ratios of the delayed plating efficiency at 4 weeks (T=4) post-irradiation, compared to each control PE. Graph includes the new tumour cell lines XA, XC, ST as well as the 13 gamma and 9 alpha tumour cell lines previously established from our laboratory.

3.5 Molecular Analysis of Tumour Cell Lines

3.5.1 Experimental Procedure

DNA was extracted from all the x-irradiated cell lines including XA-1, XC-1 and the strontium tumour ST-1. Using a p53 database the most commonly mutated alpha and gamma cell lines, (which had previously been sequenced) were also selected to confirm their p53 mutational status. Primers were prepared to amplify exons 5-8 of the human p53 gene using each tumour DNA as an independent template (see figures 3.19, 3.20 and 3.21).

Exon 5= 282 Bp Exon 6 = 207 Bp Exon 7 = 193 Bp Exon 8 = 231 Bp

A HaCaT cell line, known to be a p53 positive mutant in exons 5 and 8 was also grown in culture. DNA from the HaCaT cell line was extracted and used as a template to amplify its mutant exons, this was used as a positive control. When this sample was run with others of unknown status, a characteristic double band appeared which helped identify unknown samples.

3.5.2 Results

Initially the PCR-SSCP technique yielded no positive mutants (see figure 3.24). However, when the work was extended and hetroduplex analysis was used (see figures 3.22 3.23 and 3.25), apparent mutations were found in XA-1 tumour exon 7, XC-1 tumour exon 7 and the fast growing ST-1 tumour in exons 5 and 7. Confirmation of the p53 mutations in the most commonly mutated alpha and gamma tumours could not be verified by sequencing.

3.5.3 Conclusions:

- p53 mutations are likely to contribute to genome instability but moreover the expression of the DLD phenotype.
- Variations in the length of Alu repeat sequences in tumour cell lines may be indicative of genome instability and contributory to the expression of the DLD phenotype.

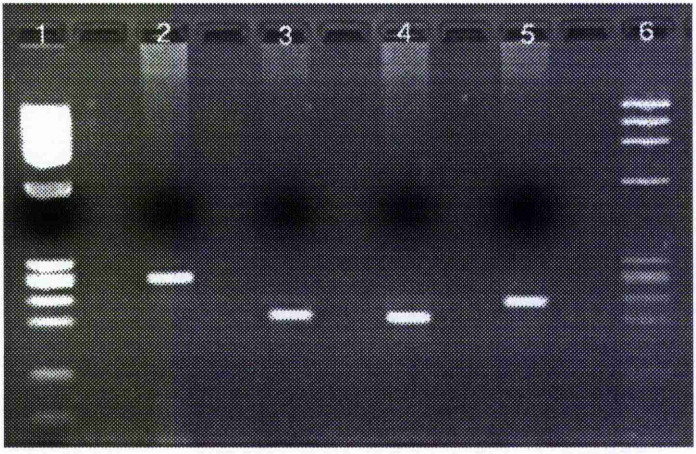


Figure 3.19 PCR amplification of p53 tumour suppressor gene, using HTori-3 DNA as template. Confirmation that all exons were successfully amplified. The bands were excised, purified, and used for re-amplification and mutation analysis.

Lane 1: ϕX174 marker. Lane 2: Exon 5. Lane 3: Exon 6.

Lane 4: Exon 7. Lane 5: Exon 8. Lane 6: ϕX174 marker

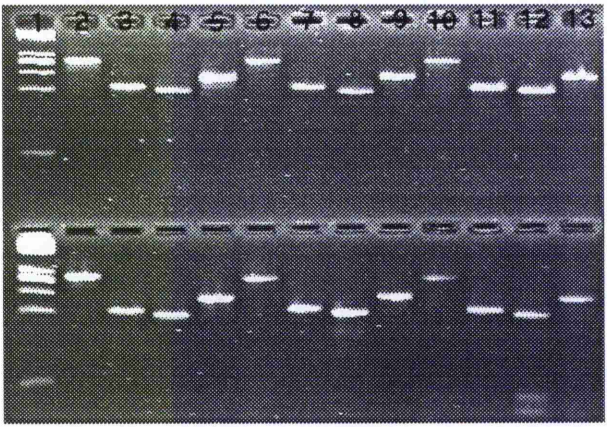


Figure 3.20 PCR amplification of exons 5-8 of the p53 tumour suppressor gene from a number of different tumour DNA templates. Amplified exons 5-8 of each of the tumour lines was stored for later use in mutation analysis assays.

Top gel:: Lane 1: OX174 marker, Lanes 2-5: XA Exons 5-8. Lanes 6-9: XC tumour DNA template Exons 5-8. Lanes 10-13: ST tumour DNA template. Bottom gel: Lane 1: ϕX174 Marker. Lanes 2-5: XA-8 Exons 5-8. Lanes 6-9: XC-8 tumour DNA template Exons 5-8. Lanes 10-13: ST-8 tumour DNA template.

Figure 3.21 Confirmation of the amplification of the most frequently mutated exons of the alpha and gamma tumour cell lines. Each product could be isolated and used to look for mutations within the individual exon.

Lane 1: ϕX174 Marker,

Lane 2 A3 Exon 5, Lane 3: A3 Exon 7 Lane4: A4 Exon 7,

Lane5: A4 Exon 5, Lane 6: A5 Exon 8 Lane 7: G13 Exon 5,

Lane 8: G1 Exon 8, Lane 9: G2 Exon 7 Lane 10: G9 Exon 7,

Lane 11: G10 Exon 7, Lane 12: MarkerϕX174

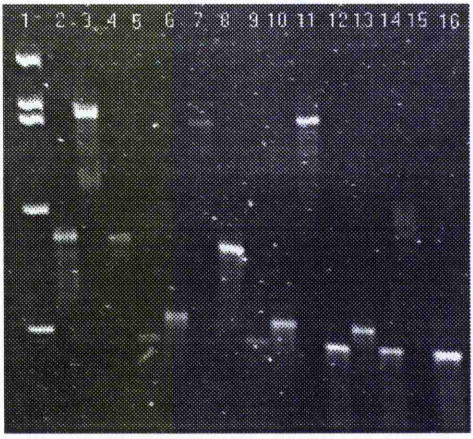


Figure 3.22 Hetroduplex analysis of p53 gene. Included are amplified exons 5-8 of DNA from HTori-3 control cells and HTori-3 cells expressing the DLD phenotype. Selected exons from alpha and gamma tumour DNA templates are also included. Gel confirms that the p53 status is not altered between normal and DLD expressing templates. Any mutants would appear as a double band, e.g. positive control lane 3. No mutants were found.

Lane 1: ϕX174 Marker, **Lane 2**: HaCaT Exon 8, **Lane 3**: HaCaT Exon 5,

Lane 4: HT Exon 8, **Lane 5**: HT Exon 7, **Lane 6**: HT Exon 6,

Lane 7: HT Exon 5, **Lane 8**:HT-8 Exon 8, **Lane 9**: HT-8 Exon 7,

Lane 10: HT-8 Exon 6, **Lane 11**: HT-6 Exon 5, **Lane 12**: Alpha 1Exon 6,

Lane 13: Alpha 4 Exon 7, **Lane 14**: Alpha 9 Exon 6, **Lane 15**: Gamma 13 Exon 5

Lane 16: Gamma 9 Exon 6.

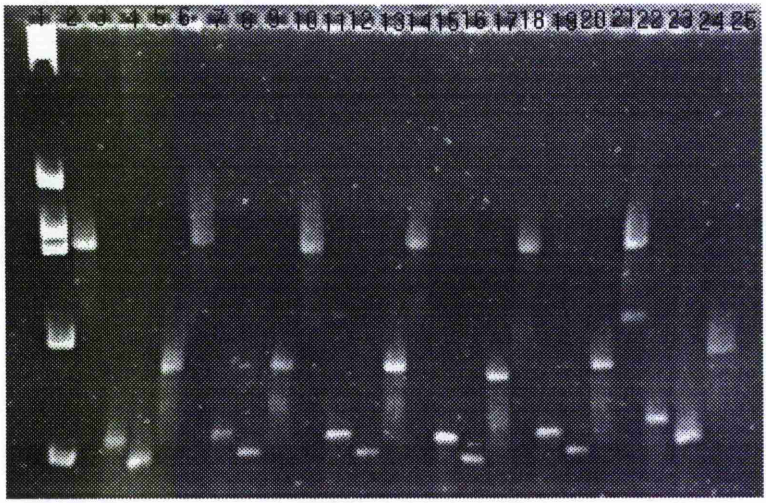


Figure 3.23 Hetroduplex analysis of p53 gene, exons 5-8 on a high resolution spreadex gel. Mutant hetroduplexes form an extra band(s) and are usually retarded in the gel. No mutants were found.

Lane 1: ØX174 marker, Lanes 2-5: HT template, exons 5-8. Lanes 6-9: XA tumour DNA template, exons 5-8. Lanes 10-13: XC tumour DNA template, exons 5-8. Lanes 14-17: ST tumour DNA template, exons 5-8. Lanes 18-21: ST tumour expressing DLD phenotype, exons 5-8. Lanes 22 and 25: HaCaT positive controls, exons 5 and 8. Lane 22-24 negative controls, exons 6 and 7.

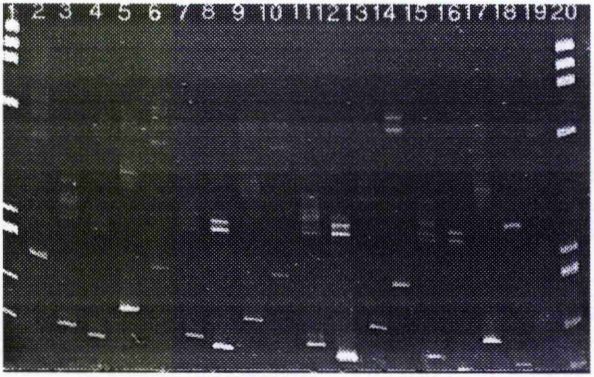


Figure 3.24 SSCP gel of p53 exons 5-8 of HT and tumour templates XA, XC and ST. Possible mutations were found in lane 8, lane 12, lane 14 and lane 16. However, due to the high number of background bands it is difficult to confirm these as true mutants without sequencing.

Lane 1: ØX174,

Lane 2: HT Exn 5,

Lane 3: HT Exn 6,

Lane 4: HT Exn 7,

Lane 5: HT Exn 8,

Lane 6: XA Exn5,

Lane 7: XA Exn 6,

Lane 8: XA Exn 7,

Lane 9: XA Exn 8,

Lane 10: XC Exn 5,

Lane 12: XC Exn 6,

Lane 13: XC Exn7,

Lane 14: XC Exn 8,

Lane 15: ST Exn 5,

Lane16: ST Exn 6,

Lane 17: ST Exn7,

Lane 18:ST Exn 8,

Lane 19: HaCaTExn 5

Lane 20: ØX174.

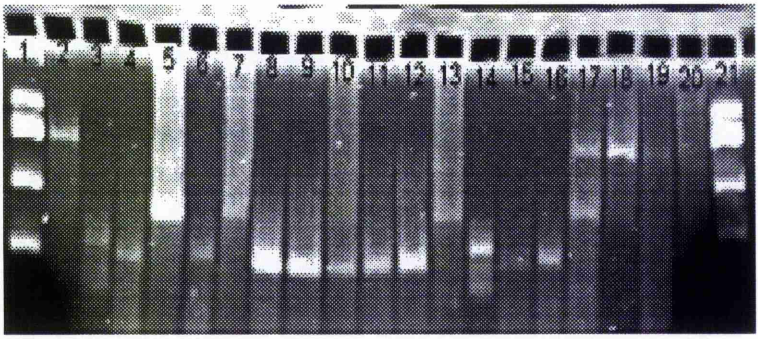


Figure 3.25 Alpha and gamma tumour template hetroduplex analysis of p53 exons 5-8 including positive controls. No mutants were found.

Lane 1: ØX174 marker **Lane 2**: HT Exn 5, **Lane 3**: HT Exn 6,

Lane 4: HT Exn 7, **Lane 5**: HT Exn 8, **Lane 6**: G1 Exn 7,

Lane 7: G2 Exn 8, **Lane 8:** G9 Exn 7, **Lane 9**: G10 Exn 7,

Lane 10: A3 Exn 7, **Lane 12**: A4 Exn 7, **Lane 13**: A5 Exn 8,

Lane 14: A6 Exn 6, **Lane 15:** A9 Exn7, **Lane 16:** G10 Exn 7,

Lane 17: HaCaT Exn 5, **Lane 18:** HaCaT Exn 8, **Lane 19:** blank

Lane 20: Blank. **Lane 21**: ØX174 marker.

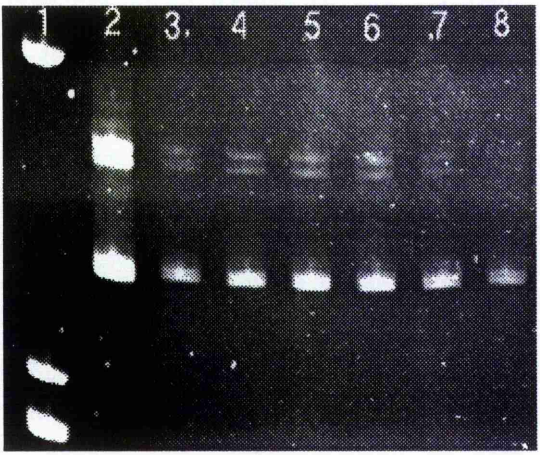


Figure 3.26 Alu repeat polymorphism on 8% acrylamide gel. 406 Bp products all appear the same size. Any variation in product size may indicate genomic instability within each cell line tested. No variation was found at this resolution.

Lane 1: ϕX174 marker. **Lane 2**: HT Alu **Lane 3**: XA Alu

Lane 4: XC Alu **Lane 5**: ST Alu **Lane 6**: XA-8 Alu

Lane 7: XC-8 Alu **Lane 8**: ST-8 Alu

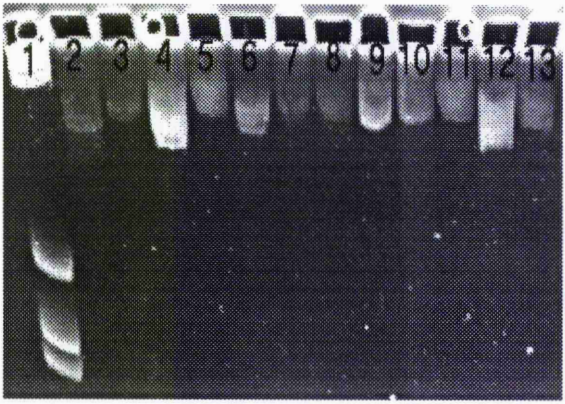


Figure 3.27 High resolution spreadex gel of Alu repeats. Includes normal and DLD phenotypes for new tumour cell lines. Each band represents a 406 base pair product from an early intronic sequence of p53. Up to 5 bp variations could be resolved, possibly indicating genomic instability.

Lane 1: Marker ØX174

Lane 2. HT	Lane 6.XC	Lane 10. A3 Exn 5
Lane 3.HT-8	Lane 7.XC-8	Lane 11. A4 Exn 7
Lane 4.XA	Lane 8.ST	Lane 12. G1 Exn 8
Lane 5.XA-8	Lane 9.ST-8	Lane 13. G2 Exn 7

3.6 Investigations into the delayed induction of Micronuclei

Micronuclei are small distinct fragments of nucleus that may be used to measure the effects of radiation damage and therefore the induction of genomic instability. In these experiments, micronuclei are scored immediately after irradiation (72 hours permitted for colony formation) or at intervals of four weeks post-irradiation. Scoring colonies of cells grown on slides overcomes the limitations associated with cytochalasin block that only permits the scoring of binucleated cells, as described by Fenech *et al*, 1985.

3.6.1 Experimental Procedure

For analysis of immediate, (T=0) and delayed, (T=4) formation of micronuclei, tumour cells were harvested either immediately following irradiation and/or following four weeks of *in vitro* incubation.

Cells were seeded onto coverslips and incubated for 72 hours to permit the formation of colonies. Irradiated cultures were seeded at 10X the dose of controls to compensate for radiation induced cell killing. Following incubation, dishes were giemsa stained and mounted on glass slides ready for evaluation.

Selected alpha and gamma tumour lines were selected on either being highly or poorly transforming. X-ray and strontium lines were also included to provide a range of data covering a variety of radiation transforming qualities. Cell colonies from 4-16 were scored and the number of micronuclei per cell within each colony was scored. Binuclear and pyknotic cells were also scored.

3.6.2 The Results

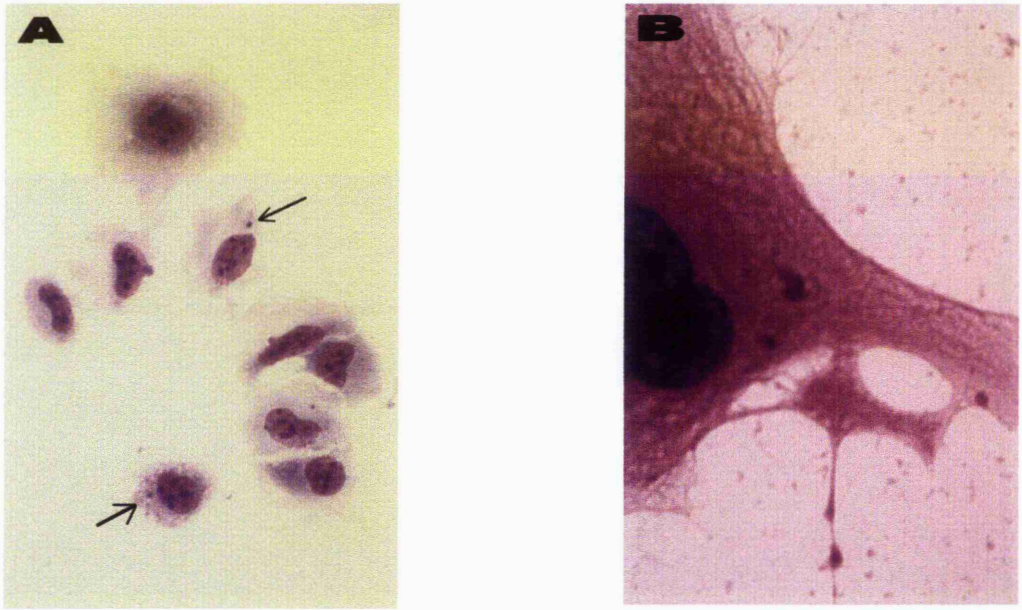
Micronuclei formation was found in all cell lines tested. Figure 3.28 shows a typical colony of HTori-3 cells and a high power picture of a micronucleus. All cell lines tested at T=0 gave a characteristic dose response.

However, data were collected from those cells expressing the DLD phenotype at four weeks post-irradiation produced more diverse results. While the gamma tumour cell line G2 appeared relatively radioresistant to the formation of micronuclei G1 did not. It must be remembered that although each TCL was originally transformed with either alpha or gamma or x-rays each was then irradiated with a Cs137 gamma source to induce the DLD phenotype. As such, it is difficult to find trends between the different radiation sources used. However, in all cases cells irradiated were never able to recovery to levels observed in control cultures.

HTori-3 cells scored at 72 hours post-irradiation showed that a dose of 8 Gy was able to induce the highest frequency of micronuclei. At T=4 an elevated frequency of micronuclei formation was observed that remained dose dependant and elevated above that of its control (see Figure 3.29). Figure 3.30 represents the micronuclei frequency in selected tumour cell lines. It is interesting to note that the new tumour cell lines XA-1 and ST-1 have the highest frequencies of micronuclei when compared to the well established alpha and gamma tumour cell lines. Intra-colony variations were noted and are displayed graphically in Figure 3.31.

3.6.3 Conclusions

- Dose dependant increase in micronuclei formation is observed immediately after irradiation
- Levels of micronuclei remain elevated upto 4 weeks after irradiation
- Fluctuations in the frequency of colony size and micronuclei numbers at up to 6 Gy may be indicative a generalised genome instability.
- At doses above 6 Gy most cells are lethally damaged and are unable to form colonies. Those 8 Gy irradiated cells that form colonies are true survivors and thus plate/divide normally with reduced frequency of micronuclei.
- Enhanced levels of colony micronuclei may contribute to the expression of the DLD phenotype and may be a good indicator of cellular instability post-irradiation.



Figures 3.28 Panel "A" shows a low power (x400) HTori-3 colony as observed when scoring micronuclei. High power micronuclei within a single cell (x800) can be seen in panel "B". The non-clonal distribution of micronuclei observed suggests *de novo* mutations arising in the population independent of colony size. This is indicative of an unstable population.

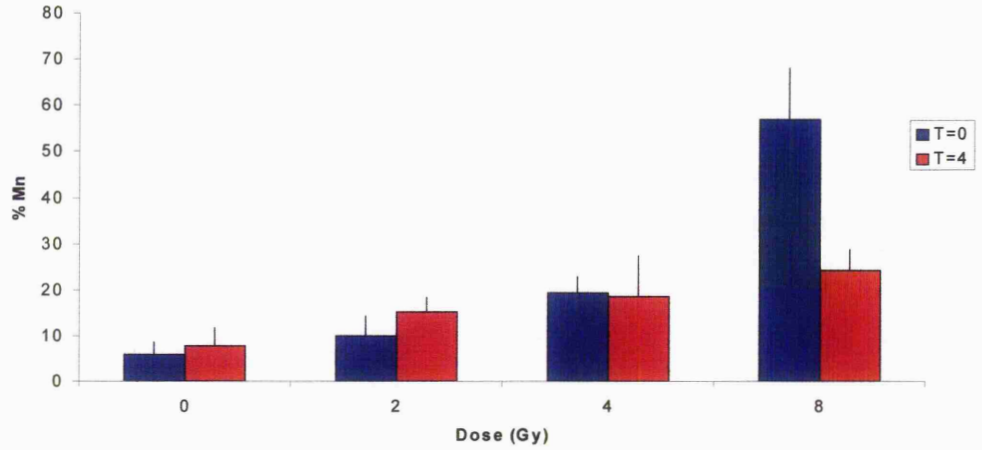


Figure 3.29 Comparison of micronuclei formation in HTori-3 cells immediately following radiation (blue bars) and in those cells cultured for 4 weeks after the radiation dose and then tested (red bars). Although both show a dose dependant increase, the levels after 4 weeks in culture remain elevated above that of the control.

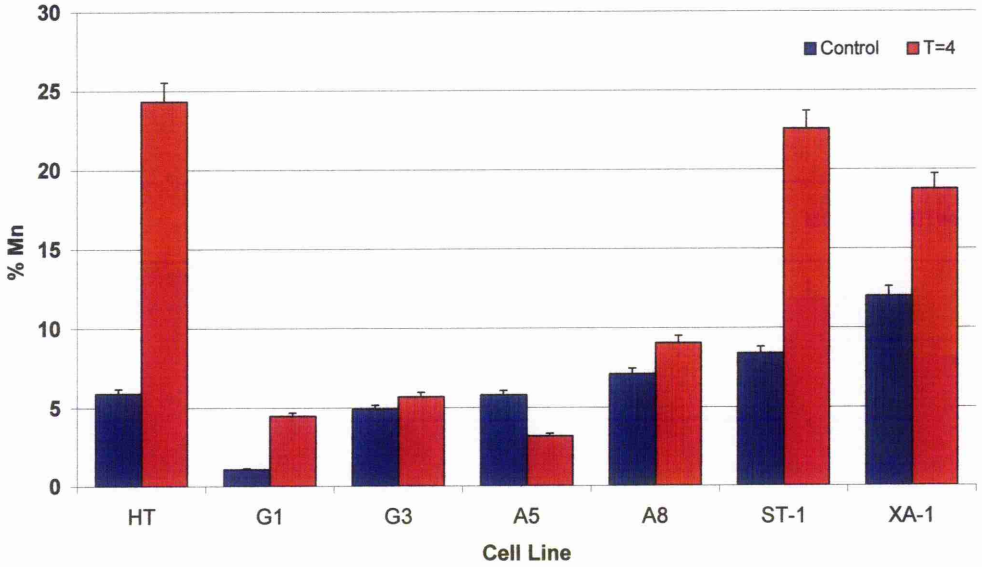


Figure 3.30 Frequency histogram of micronuclei found in HTori-3 control cells, selected alpha and gamma lines as well as a strontium and x-ray acute tumour cell lines were examined. All but A5 show an elevated frequency of micronuclei at 4 weeks post-irradiation.

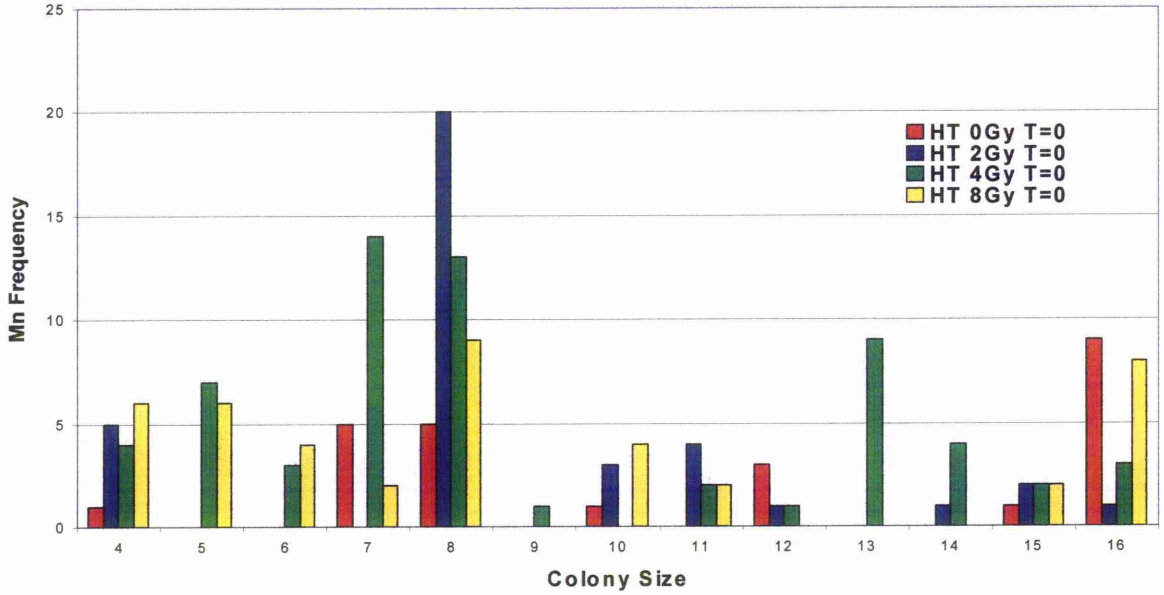


Figure 3.31 Colony micronuclei frequencies in HTori-3 cells as a function of colony size over a range of doses. Eight cell sized colonies are the most frequently recorded due to 72 hr incubation period. A high level of odd number sized colonies indicate high levels of instability, as not all cell divisions are being completed successfully.

3.7 Analysis of Attachment Ability in radiation transformed cells

The HTori-3 cell line and the radiation induced thyroid tumours derived from it were examined for their ability to attach to a substrate at two weekly intervals post-irradiation. Variations in the cells' ability to attachment to a plastic surface could be a sign of early transformation events and skew any colony assay data. It may also implicate cell surface attachment proteins in the DLD phenotype.

3.7.1 Experimental Procedure

Initially, the parental HTori-3 cell line and HT cells expressing DLD after 8Gy and two weeks incubation were chosen, designated HT and HT-8 @ T=2 (see figure 3.32). Shortly after the experiment was developed to include the tumour cell lines XA, XA-8 and XC, XC-8. These were tested at time intervals of T=2, T=4, T=8 and T=12 weeks post radiation (see figures 3.33 and 3.34).

Each cell line to be tested was passaged at sub-confluence and then serially diluted from a stock of 200,000 c/ml. The following concentrations were made: 40,000 c/ml; 20,000c/ml; 15,000c/ml; 10,000c/ml; 5,000c/ml; 2500c/ml; 1250 c/ml and 625c/ml. All concentrations were seeded in a volume of 200µl per well, in a 96 well flat bottom microtitre plate. Each cell concentration had eight replicates per dish and each dish was repeated three times. Each experiment was completed within the minimum time constraints allowed, to prevent dividing cells having an influence on the outcome.

3.7.2 Results

An small decrease in attachment ability was observed over the lower range of cell concentrations used at four weeks post-irradiation(see figure 3.32). The data collected indicates that at low cell concentrations attachment ability may play a contributory factor in the expression of delayed lethal damage phenotype up to four weeks post irradiation. However, after this time and at high cell concentrations the cells' ability to attach to a surface appears to diminish or becomes uncertain.

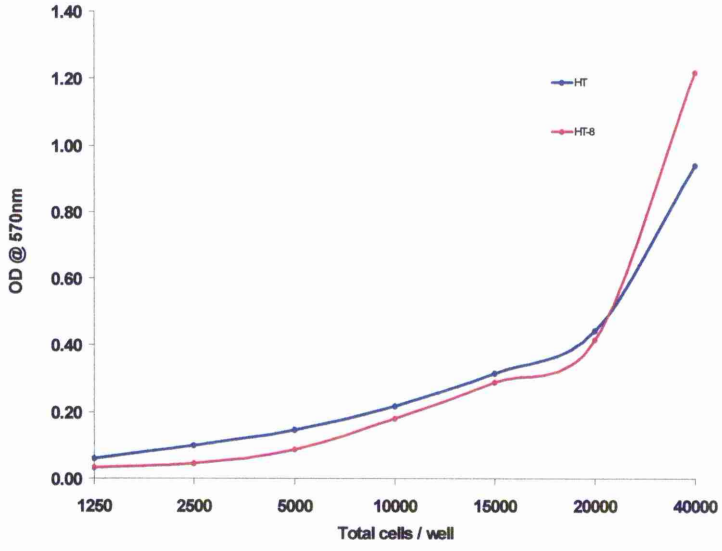
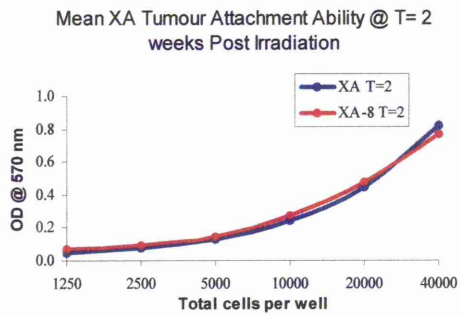
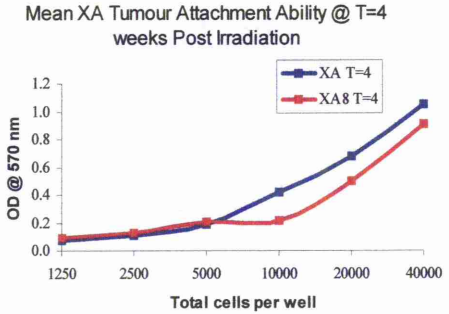


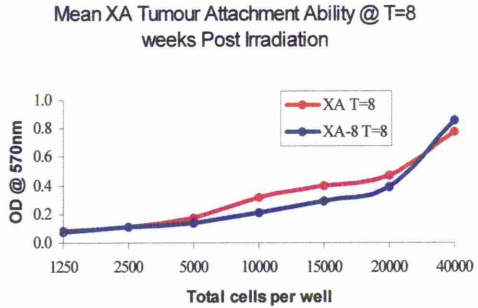
Figure 3.32 HT and HT-8 attachment ability as measured through the MTT dye exclusion assay at four weeks post-irradiation.

3.7.3 Conclusion:

- Anchorage independence may be expressed in the DLD phenotype and help to explain the decreased plating efficiencies in colony assays.
- Lack of cell surface adhesion in DLD expressing cell lines could be investigated at the molecular level by looking for mutations in known genes involved in the attachment process.







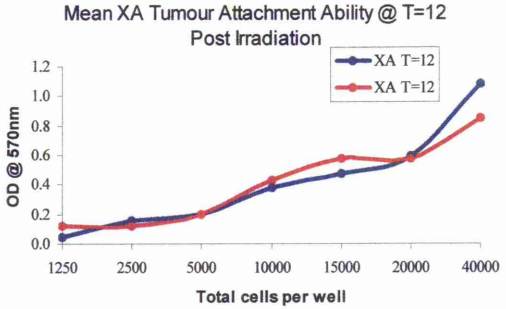


Figure 3.33 MTT mean attachment ability for XA and XA-8 tumour cell lines after 2, 4, 8 and 12 weeks of *in vitro* incubation post irradiation.

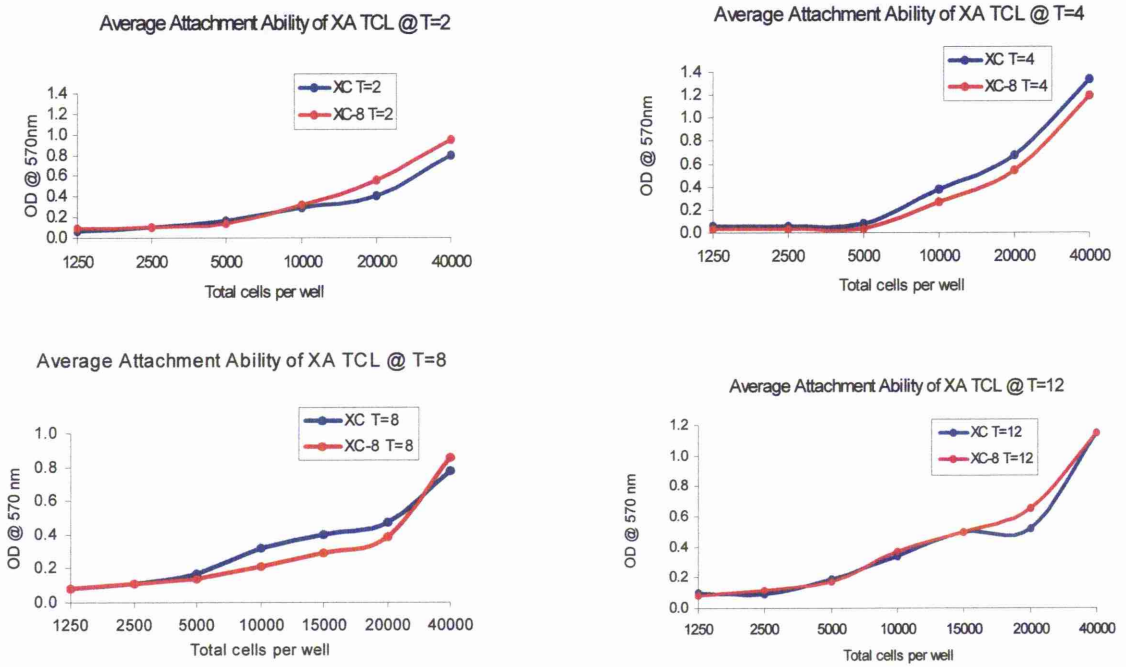


Figure 3.34 MTT mean attachment ability for XC and XC-8 tumour cell lines after 2, 4, 8 and 12 weeks of *in vitro* incubation post irradiation.

3.8 Measurement of Apoptosis and Necrosis in anchorage dependent and independent cells

Apoptosis has been implicated as a cause of the delayed lethal death phenotype in non-tumourigenic cells but not tumour cells (O'Reilly *et al* 1994, Mothersill, *et al* 1995, 1996). It has been suggested that DLD is a "cleansing mechanism" signalled externally and used to eradicate lethally damaged cells before they become tumourigenic. This author wanted to investigate if the radiation induced thyroid tumours produced in this study were also expressing a delayed form of apoptosis.

3.8.1 Experimental Protocol

Adherent and non-adherent cells were collected from exponentially growing cultures of the parental, non-tumour, cell line HTori-3 and the tumour line XA-1, these were designated HT and XA. To induce the DLD phenotype, cells were irradiated with 8 Gy of gamma rays and passaged for 4 weeks *in vitro*, these cells were designated HT-8 and XA-8.

The media was decanted and spun for the collection of floating cells, while the culture flask was passaged and samples of adherent cells aliquoted. All cells were diluted to 20,000 c/ml and then a single drop of dye was added (acridine orange/ethidium bromide).

3.8.2 Results

Distinction between transformed and non-transformed, floating and adherent could be made. As expected, the relative percentage of necrotic cells in the floating

fraction was higher than that of the adherent population in both groups. However, levels of apoptosis appeared higher in the cells expressing delayed lethal damage. The tumour cell line had an increased percentage of apoptotic cells over the non-tumour line (See table and figure 3.35). Internucleosmal degradation could be subdivided into early apoptotic, late apoptotic, early necrotic or late necrotic (Compton *et al*, 1992, Eastman, 1995).

3.8.3 Conclusions

Although it is likely that apoptosis may play a role in the expression of DLD and explain the decrease in plating efficiency found in tumour cell line colony assays the data presented here is insufficient to confirm these findings. There are always uncertainties in scoring apoptotic and necrotic cells, as such an alternative assay would be required to confirm these results e.g. TUNEL.

Table 3.5 Fraction of apoptotic and necrotic cells in floating and adherent fractions of non-tumour and tumour cell populations

Non Tumour	**HT Adherent**	**HT Floating**	**HT-8 Adherent**	**HT-8 Floating**
% Apoptotic	*3*	*4*	*4*	*5*
% Necrotic	*5*	*8*	*6*	*9*
Tumour	**XA Adherent**	**XA Floating**	**XA-8 Adherent**	**XA-8 Floating**
% Apoptotic	*5*	*7*	*6*	*7*
% Necrotic	*7*	*9*	*8*	*10*

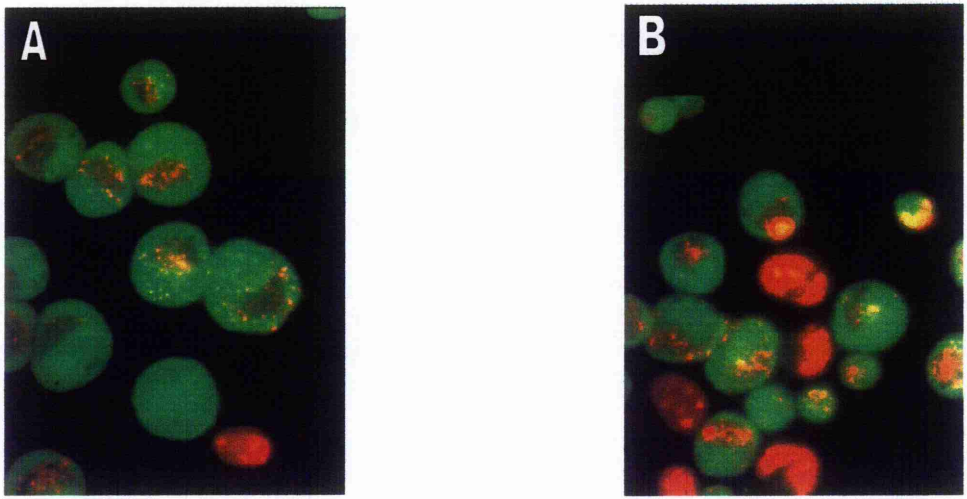


Figure 3.35 X-ray acute tumour (control) cells can be seen in panel "A", while panel "B" shows the same tumour cell line that expresses the DLD phenotype (i.e. previously irradiated with 8Gy and incubated *in vitro* for 4 weeks). An enhanced frequency of necrotic cells are present in panel "B" with an elevated apoptotic population. Green cells are viable, orange are necrotic. Apoptotic cells are green but with eclipse shaped chromatin.

Chapter 4

DISCUSSION

4.1 General Discussion

Transformation of human cells *in vitro* has proven difficult. Models of the past have relied on murine and rodent cell lines. The HTori-3 system hoped to overcome these problems with the use of an immortalised human epithelial cell line. An advantage of this type of system was, not only could cells be cultured indefinitely, but any tumours produced could be directly correlated with the non-transformed parental cell line. Phenotypic and genotypic comparisons could then be made. It was anticipated that this model would be more relevant than other animal cell systems and permit the correlation of *in vitro* carcinogenesis more closely with that of human carcinogenesis.

The use of nude mice allows the screening of large numbers of cells from different experimental procedures in a relatively short time period e.g. 3-6 months, compared to human carcinogenesis of 30-40 years. It becomes experimentally feasible to produce tumours using a variety of ionising radiation sources and directly compare a number of variables e.g. the effects of dose, cell number, passage.

The importance of developing a model of thyroid carcinogenesis is clear. The ability to understand the mechanisms involved would impact both on cancer

research and radiation biology. The major findings from this work will now be discussed in the light of the work of other key authors.

4.1.1 Major Findings

The human thyroid epithelial cell line HTori-3 has been transformed by ionising irradiation. These newly derived tumour cell lines have been used in a number of assays to test for the expression of the delayed lethal death phenotype.

The colony assay has shown the induction of this phenotype after doses of gamma radiation above 6 Gy and after four weeks but not two weeks of incubation *in vitro*.

The colony micronuclei assay has been used as an alternative assay for measuring the same biological endpoint. This method has shown that levels of micronuclei, giant cell formation and pyknotic bodies remain elevated many weeks after the initial radiation insult.

Using the MTT cell attachment assay it has been shown that although it is likely, that cell adherence and apoptosis may also play an important role the expression of the DLD phenotype it is difficult to confirm this with the data presented here. It is likely that all the endpoints tested in this thesis do play a critical role in the expression of genomic instability and assist in the expression of transformation. However, further detailed investigations would be required to define their roles conclusively.

4.2 Transformation Studies

The results of this study show that the human thyroid epithelial cell line, HTori-3 can be transformed by a single exposure to low LET radiation. Moreover, that

comparison of this induction can be made between the chronic and acute delivery of the radiation and over a range of low doses.

In culture, the tumour cell lines (TCLs) grew in a similar fashion to the parental HTori-3 cell line forming continuous monolayered sheets of cell. Morphological appearances were identical though when growth rates were measured clear differences in some tumours growth curves were noticed. Experiments were performed to compare the radiation response of HTori-3 cells a variety of low LET radiation sources. These included chronic and acute x-rays, beta particles from both a strontium/yttrium uniform and strontium particle source as well as gamma rays from the Csl37 source.

The data presented clearly shows that irradiated thyroid epithelial cells express genomic instability in several forms after exposure to radiation e.g. decreased plating efficiency. Genetic changes have undoubtedly occurred in exposed cells, which directly affect their ability to repair DNA damage and thereby complete stable divisions. Moreover, these cells are more capable in forming tumours than unirradiated control cells. It seems likely the expression of the genomic instability in the form of the delayed lethal death phenotype and the formation of tumours are directly related and dependent.

All cultures exposed to radiation were passaged once weekly for six weeks. This step has been regarded as indispensable for the fixation of damage (Borek & Hall, 1966; Borek & Sachs, 1967; Reznikoff *et al,* 1992). Whether this period is required for the expansion of the transformed cell(s) is unlikely, as it would mean that

transformation occurs almost instantaneously or within the short period of time that the cells are passaged in culture. It is more likely that this period of passaging provides time for the fixation of damage. Damaged cells are then able to replicate and accumulate more mutations, which makes cells increasingly unstable. It is a high concentration of unstable cells, which are then screened in mice. Selection, which has occurred to an extent in vitro, is likely to continue in vivo.

However, other reports have shown that this step may not be necessary. Some suggest that the effects of radiation may linger for some time after the initial delivery of the dose Clutton *et al,* 1996). Persistent oxidative stress and the release of stored free radicals from the cytoplasmic endoplasmic reticulum have all contributed to this theory, (Clutton *et al,* 1996; Kadhim *et al,* 1995, 1996, 1998; Watson 1997).

A relationship for the radiation dose delivered and the subsequent transformation in *vivo* can be made. Although a range of low doses were screened for their tumour forming potential they do not show a dose response, tumours were found in all but one group. This data is consistent with other reports (Borek and Hall, 1973). Moreover, the single transforming dose is also supported by others (Martin *et al,* 1993 and Hei *et al,* 1994).

To expand, all groups of cells screened, except the X-ray chronic 0.5 Gy, produced progressively growing tumours. Although a few tumours were found in the control groups these remain insignificant when tested statistically. The reasons for control tumours maybe the result of a general instability within the HTori-3 cell line. This is difficult if not impossible to control for. The original paper by Lemoine in 1989

provides data on the unstable karyotype of the cell line. Data from studies in our lab shows an increase in chromosomal aberrations with passage number. Indeed as the cell line has been immortalised with the SV40 virus the p53 protein status is uncertain. As such it is not known whether the cells are able to induce a G2 or even a G1 arrest. There may well be an accumulation of damage over time in HTori-3 cells, which remain unexposed to experimental doses of radiation. It is possible that background levels of radiation could conceivable induce a mutator phenotype along the lines of that suggested by Loeb (1991). HTori-3 must be regarded as a partially transformed cell line thus the work described in this thesis has used several related cell and molecular endpoints e.g. plating efficiency, micronuclei, formation, cell adherence, gene mutations as indicators of genomic instability. These variables were first studied in the parental cell line HTori-3 to provide a definitive background level of spontaneous genomic instability. Samples of cells were then irradiated and screened for their tumourigenic potential in nude mice. Tumours derived from these experiments were then used to further investigate and compare the levels of instability with controls

It is likely that the tumours created in this study have defective repair pathways. In humans lack of these pathways have been found in a number of hereditary diseases, these syndromes predispose individuals to higher rates of stabilised damage and an increased risk of cancer. The genetic diseases that result include xeroderma pigmentosum which is characterised by extreme sensitivity to sunlight, ataxia telangiectasia which predisposes individuals to elevated sensitivity to x-rays with immune and blood vasculature abnormalities. Others include Fanconi's anaemia, Bloom's and hereditary retinoblastoma and the germline p53 minus syndrome Li-

Fraumeni. Underlying all these genetic diseases and syndromes are mutations; hereditary mutations, mutations induced by ionising radiation or any other deleterious sources.

This study demonstrates that human thyroid epithelial cells can be transformed by exposure to chronic or acute x-rays as well as strontium beta particles. It is likely that the SV40 immortalisation of these HTori cells is a crucial first step along the path to transformation. Not only does SV40 immortalised cells and permit indefinite cell cycling but as p53 function is compromised, it is likely to lead to an enhancement in the accumulation and stabilisation of mutations. The cell, primed by the radiation becomes incapable of preventing the further accumulation of mutations. This multi-step hypothesis is supported by several authors (Reznikoff *et al,* 1992, Rhim, 1990, Loeb, 1991)

4.2.1 Characterisation of Tumour Cell Lines

The production of tumour cell lines (TCL) was a primary concern after irradiated HTori-3 cells formed tumours in nude mice. Once TCLs were established it was crucial to confirm their human phenotype. Previous studies have highlighted the importance of confirming the nature of derived tumours from *in vitro* experiments. *In vitro* assays of this kind can easily be contaminated with other cell lines which out compete the growth of the original leading to erroneous results and conclusions (McCormick *et al,* 1988).

Half of each tumour was sectioned and taken for histological analysis. The paraffin embedded sections revealed that the tumours were anaplastic and classified as undifferentiated anaplastic carcinomas. Reports after the Chernobyl disaster

indicated that papillary thyroid tumours dominated the childhood cases, (Baverstock *et al* 1992). Of the primary tumours retransplanted in to nude mice, all formed progressively growing secondaries from the time of injection with significantly reduced latent periods. Growth rates were much faster and the time to excision of the tumour was greatly reduced, thus confirming their tumourigenecity. This is confirmed by the work of Reznikoff (1992). Karyotype analysis of selected tumours was performed by preparing giemsa stained chromosome spreads. Although the number of chromosomes varied considerably and chromosome aberrations were frequently encountered the human nature of all spreads was unmistakable.

Dose response curves from the different tumour cell lines created showed an initial shoulder region characteristic of low LET radiation. Gamma and x-irradiation is relatively sparsely ionising and thus relatively ineffective at killing cells at low doses. The intrinsic repair capacities of the cell are able to overcome the damage sustained, especially during chronic irradiations. However, the results presented in this thesis show how latent damage can lead to cell death many generations after the initial dose response.

4.2.2 Dose Response Data

To compare the parental dose response with that of the other TCLs survival curves were constructed. Where appropriate the linear quadratic, model which assumes that there are two components to cell killing by radiation was used. While some of the lines appeared relatively radiosensitive e.g. A9 others showed an elongated shoulder region suggestive of increased radioresistance e.g. A3. A similar pattern was observed for the gamma tumour dose response. While G10 appeared radiosensitive

G13 dose response up to 8Gy was more suggestive of a relatively radioresistant cell line with an extended shoulder region.

To investigate this further a number of comparison were made to see if any patterns could be observed between the cell lines dose response and a number of collected variables which could characterise each cell line as an individual. Unfortunately, no substantial correlation could be drawn between the cell lines p53 status, mutation type, passage number, doubling time or growth in culture. While some of the cell lines grew extremely rapidly others were relatively slow growers, this could not be correlated with either the irradiation regime, radiation quality or the cell lines subsequent dose response.

4.2.2 Radiation Induced Tumourigenesis and the Mutator Phenotype

It has been suggested that p53 mutations may be indicator of radiation induced tumourigenesis (Taylor *et al*, 1994). Cancer progression is thought to be a multistep process (Rhim, 1990). Certain cancers e.g. HNPCC have rate limiting steps involving 6-12 key rate limiting genes that directly or indirectly affect cell proliferation. Loeb (1991) has predicted that the known mutation rate in somatic body cells = 10E7 per cells per generation. This rate is inadequate to account for the large number of chromosome rearrangements found in some cancer cells. Thus, the chance of a single cell accumulating six or more mutations in its lifetime are very unlikely. Yet carcinogenesis occurs and chromosome spreads of tumour cells derived from this study show an array of chromosome abnormalities.

However, Loeb's early calculations did not take into account an increased mutation rate. Such an increase could be induced by a single mutation in genes that repair damage. Under these circumstances a mutator phenotype could be induced, hastening the chances of a transformation. One indication of a cell with a mutator phenotype is the appearance of variations in microsatellite sequence lengths. Losses at specific gene loci, which are not matched on the complementary strand, are termed LOH or loss of hetrozygosity. Thus an Alu repeat sequence from the early intronic region of the p53 gene (Lengauer *et al* 1992) was used in the current study as a potential marker of a) mutator phenotype b) presence of genomic instability. However, it is more common for other types of repetitive sequences to be used in these types of studies, such as mini or microsatellite.

4.2.3 P53 and Alu repeats

The Alu repeat sequence was amplified using the PCR technique and then size was determined by electrophoresis. Results indicate that the size of Alu repeat sequence does vary in the tumour lines investigated. Moreover a precedent for variations by Dubrova *et al* (1996) confirms that the human minisatellite sequences do vary after exposure to ionising radiation. The mutation rate in the area surrounding Chernobyl was measured using a tandem repeat minisatellite was found to be twice that of the control group.

Estimates of doses received after the Chernobyl accident have been calculated at about 0.185 Gy per person (Dubrova *et al* 1996). It has been suggested that these low chronic doses of radiation are more effective in the induction of tumourigenesis and enhancing the mutation rate (Dubrova *et al* 1996). Suggestions of how radiation

can induce alterations in the mutation rate have been linked to mutator phenotype. The initiating lesion includes the loss of regulatory repair pathways.

In this study PCR amplified DNA from selected alpha and gamma, strontium and x-ray radiation induced TCLs were tested for the presence of p53 mutations. Exons 5-8, known to be hotspot regions involved predominately in the DNA binding domain were amplified. DNA extracted from XA TCL exon 8, XC exon 7 ST exon 5 and ST exon 7 appeared to have mutations, though DNA sequencing would be required to confirm this. No mutations were detected in any of the selected alpha and gamma TCLs previously established in this laboratory. There are conflicting reports on the association of p53 mutations in thyroid carcinogenesis Ito *et al* (1992) reported mutations at exon 5 and 8, suggesting that p53 mutations have an important contribution in the development of thyroid cancer. However, Wright *et al*, (1991) appears to conflict with this argument after investigating p53 mutations in primary thyroid cell lines. However, as only a small sample of cell were tested for mutations it is difficult to come to a firm conclusion regarding its role in the pathogenesis of thyroid carcinogenesis.

4.2.3 Repair Pathways and Genomic Instability

The mismatch repair and polymerase genes sustain the careful balance between the need for replication and restoration of the DNA helix. If either of these genes which code for the proteins of this mechanism are damaged then the likelihood of other sequences being disrupted are increased. This in turn leads to the phenomenon of the mutator phenotype (Loeb *et al,* 1991), whereby a positive feedback loops which results in the increased accumulation of mutations.

If a cell loses mismatch control and checkpoint control e.g. loss of p53, the mutation rate would raise dramatically, increasing the chance of uncontrolled cell division. As the frequencies of un-repaired mutations rise the likely hood of one arising in other important genes may also rise exponentially. Cahill *et al,* (1998) has shown how mutations in genes controlling the mitotic spindle apparatus that prevent chromosome attachment will accelerate the formation of aneuploidy and or polyploidy cells.

Under normal circumstances, chromosomes are attached by their kinetechore to the microtubule spindle apparatus. Chromosomes poorly attached will produce a signal that induces a checkpoint preventing the transition to anaphase until the correct alignment is achieved. The genes responsible for this signal, which were initially found in yeast, have now been identified in humans. Three BUB (budding uninhibited by benomyl) and 3 MAD genes (mitotic arrest-deficient) and the protein kinase Msplp also has a contributory function. A mutation in these genes results in chromosome instability and acquired aneuploidy.

The tenet of much cancer research is based on the belief that if mutant alleles of a gene are consistently detected in tumours, than these mutations are likely to confer a selective advantage on cancer cell clones and progression of the disease. In this study, PCR amplification of a number DNA transcripts for possible mutations proved largely unsuccessful. From the few possible mutations found in the strontium and x-ray lines it is difficult to conclude the effectiveness of p53 mutations as a potential instigator of the later phenotypic changes. However, there

is little doubt that radiation is able to induce a specific phenotype change, which can be studied in detail in using this thyroid model.

4.2.4 Correlation with Chernobyl

After the Chernobyl accident the incidence of childhood thyroid tumours increased dramatically (Baverstock *et al* 1992) and as previously mentioned so did the mutation rate (Dubrova *et al* 1996). Many of the childhood tumours were screened for mutations found in normal adult tumours e.g. ras and p53 mutations. The results of that study showed that less than 20% of childhood thyroid tumours analysed had p53 mutations (Hilldebrandt *et al,* 1996). However, this data correlates with the lack of p53 mutation found in this study. In a recent paper by Suchy *et al,* 1998 none of an additional 34 children sampled for mutations in these genes proved positive either. They conclude, "ras or p53 mutations do not play a role in childhood thyroid carcinogenesis after Chernobyl". Results presented in this thesis confirm this result as both Iodine 131 and gamma particles were used throughout this study and should produce effects similar to those found after the release of radiation from the Chernobyl accident.

Other data from childhood thyroid tumours after the Chernobyl disaster states that most were papillary carcinomas or PTC, solid and follicular variants predominated (Palle and Salomaa, 1994). Any adult tumours found after the Chernobyl accident were mostly undifferentiated, anaplastic variants. In this study the largest tumour from each group of each screening was excised. Half was used to produce a new tumour cell line (TCL), while the other half was taken for histological purposes. The sections showed that all the tumours were anaplastic in nature, showing little sign of their tissue of origin. Occasionally, a section would show signs of

differentiation i.e. the formation of small follicular areas but these were sparse. The larger tumours showed signs of vascularisation.

High frequencies of stable and unstable aberrations in lymphocyte of people exposed to Chernobyl fallout have been reported (Salomaa *et al,* 1997) and chromosome painting techniques using primary thyroid tumours (Lehmann *et al,* 1996) have revealed translocations on chromosomes 1, 4 and 12. However, only a high incidence of RET rearrangements have been reported in thyroid tumours of children from Belarus. Mutations commonly observed in adult thyroid tumours are of the cRET/HGF receptor gene (diRenzo *et al* 1992) and activating ras mutations (Bos, 1989). Ito *et al,* (1993) has shown that these rearrangements can be induced *in vitro* in a human thyroid cell line but very high doses are required, in excess of 50Gy. Screening current tumour lines for RET rearrangements would be able to confirm their presence in tumours induced *in vitro* and confirm their importance in thyroid tumourigenesis.

cRET encodes a receptor type tyrosine kinase whose ligand is unknown, it is a proto-oncogene that becomes oncogenic in one of three intronic splice variants with the unknown H4 gene or ubiquitously expressed ELE 1 gene. Two thirds of childhood PTC tested showed RET rearrangements, this is very high when compared with adult PTC. Possibly a time dependant mutation is occurring whose expression is reduced over time and whose expression may vary with age, it is possible that RET rearrangements are only active during childhood. It is hoped that RET mutations maybe a radiation induction mutation signature of papillary thyroid tumours, especially in children. Screening current tumour lines for RET

rearrangements would be able to confirm their presence in tumours induced *in vitro* and confirm their importance in thyroid tumourigenesis.

So it can be concluded that although the Chernobyl tumours found in children are now thought to be radiation induced, the specific lesions original anticipated to be involved e.g. p53 are now not thought to play a role in their pathogenesis. However, other genetic markers such as variations in minisatelites and Alu repeat sequences as well as other genes e.g. RET involved in growth regulation are being investigated. Moreover, this study has been able to produce a model of radiation induced tumourigenesis and provide a valuable source of radiation induced tumours cells which are required to continue investigations in this area of radiobiology.

4.3 Delayed Lethal Damage

The original premise in early sixties was that radiation induced damage would result in cell death within the first few population doublings; And that radiation survivors were indistinguishable from unirradiated cells (Elkind and Whitmore, 1967). Work by Nias (1965) and Grote (1981) attempted to explain the reduced growth rates in radiation cell survivors in terms of an increased cell cycle time and this author would agree that this is a contributory factor. Variations found in colony sizes in this study and growth curve data suggest that cells expressing DLD have an elongated cell cycle time. However, O'Reilly, (1994) believed that the failure of irradiated cells to produce viable colonies was in a proportion of the cells failing to produce two viable progeny, rather than this elongated cell cycle time.

However, work published principally by Seymour and Mothersill proposed that the reasons for a reduced ability for a successful reproductive life could include:

1 Damage to a Mutator gene

2 Damage to a Lethal mutation gene

3 Induced form of apoptosis

Their experiments for the reassessment of cloning efficiency (delayed plating efficiency) in post-irradiation cultures allowed the correction of survival curves resulting in a new residual curve, i.e. a fraction of cells originally termed reproductively viable at the first plating efficiency are subsequently lost from the population when the plating efficiency is reassessed sometime after the initial radiation dose is received.

Loss of the initial shoulder resulted in a series of experiments to investigate the effects of repair (Mothersill *et al,* 1989). An inhibitor of repair (sodium arsenate, which reduces levels of ATP) was added to cultures post-irradiation. It was found that inhibition of repair prevented the expression of DLD, as all lethality occurred with the first few population doubling. It was only repair occurring in the first 2 hours after irradiation that lead to the expression of DLD (Seymour and Mothersill, 1989) i.e. that is a subpopulation of sub-lethally miss repaired cells is what leads to the expression of the DLD phenotype.

The increased frequency of chromosome aberrations found in irradiated HTori-3 cells would tend to support this hypothesis. Experiments by Alper, (1988) and Chang and Little, (1992) proved that double strand break repair deficient cell lines e.g. xrs-1 mutants did not express DLD. Fractionation of dose resulted in reduction

of DLD and the first dose proved the most important in DID induction. Metabolic inhibitors or dsb inducers would lead to DLD, while cytotoxic agents like cisplatin did not (Mothersill, 1991). However, Mothersill states that lethal mutations are either reduced or do not exist in tumour cell lines. Lastly the irradiation of cells with gap junctions for extracellular communication resulted in fewer lethal mutations than single cells without (Mothersill, 1997).

Further work by Mothersill has shown the release of a chemical into the media of irradiated cells that can be added to unirradiated cultures resulting in the effective expression of DLD. This work suggests that although gap junctions may prevent the transmission of a secreted substance preventing the induction of increased cell death unirradiated cells can still be induced into cell death.

All these results suggest that fast but defective repair of dsb was the main cause of DLD, which this author agrees with. In this study delayed cell death has been shown to predominate, but not exclusively from apoptosis, this is also supported by (Mothersill and Seymour, 1997).

Thus, apoptosis is an important process involved in the expression of DLD. Investigations into the processes involved in apoptosis have shown that the active prevention of programmed cell death is caused by the upregulation of the anti-apoptosis gene Bcl2. Evidence suggests that rather than cells dying from a p53 mediated apoptotic pathway, it is the reduction in the levels of Bcl2 in the progeny many divisions after the initial radiation that finally allows the cells to die of

apoptosis. As p53 function is undetermined in HTori-3 cells, this method of cell death may be a mechanism active in HTori-3 tumours.

Mothersill *et al,* have been shown that the levels of Bcl2 fall to 50% at 10-15 population doublings after irradiation and this correlates well data presented in this thesis, (Seymour and Mothersill, Unpublished). The expression of DLD appears to be independent of p53 status as mutant human keratinocytes, which are p53 mutants are still able to express DLD. Again, this correlates well with lack of observed p53 mutants found in this study.

However, Mothersill *et al* 1996 claim that lethal mutation are only found in the non-tumourigenic cell lines. The DLD phenotype is induced only to act as a "mechanism for ridding the population of unstable cells rather than being an expression of instability" (Mothersill *et al* 1996, page 719). On the basis of the work presented in this thesis, this author would fundamentally disagree with this hypothesis. Instead, I propose that delayed lethal damage is an expression of *de novo* genomic instability induced by ionising radiation. Results in this thesis also suggest that the presence of micronuclei and cellular adherence are also factors.

In addition, reports by Mothersill *et al* express terminal differentiation in the form of giant cell formation, formation of vacuoles and lipid accumulation and even, some senescence. Giant cell formation was found to be a common feature in the post-irradiation cultures in this present study. *In vivo* it is likely that a complex combination of these processes all contribute to the expression of the DLD

phenotype. This study sets a precedent in that the DLD phenotype has been induced in thyroid tumour cell lines using a broad range of radiation qualities.

During the current studies I have attempted to induce tumour cell lines through a variety of ionising radiation sources. Having established their tumourigenic potential I wanted to investigate if the DLD phenotype could be expressed. The results from the series using tumour cell lines support the hypothesis that delayed lethal death phenotype is the expression of genomic instability induced through doses of low LET radiation.

4.4 Further Work

Further work that would like to have been undertaken was to investigate the mismatch repair genes MSH2 and MLH1 using the reverse transcriptase PCR method to look at protein transcripts. Loss of mismatch repair and MIN would be a good indication of why these cell lines express DLD. Repeats of these assays using a non partially transformed cell line would also be advantageous.

Multiple roles have been discovered for mismatch repair genes. In addition to correcting the genetic anomalies that occur during normal cell replication, these genes fix environmentally induced DNA damage and have also been implicated in regulating the cell cycle. Mismatch repair proteins are known to repair a variety of environmentally induced lesions, including those from ultraviolet radiation, ionising radiation and oxidation. Defective DNA results in defective transcripts and an altered protein function. Malformed proteins are likely to have deranged functions and an inability to identify mismatches. (Burke, 1990, Bignami, 1994).

Any lack of proof reading and mismatch repair is crucial and potentially lethal to the cell. Transcripts are at a far higher risk from environmental free radical and other base damage attack than at any other event of the cell cycle.

Work with mutant MLH-1 has shown that neither cell cycle arrest nor repair will occur without it. It is believed that the important implications for this work are the refinement of chemotherapy regimes. For example, HNPCC patients often do better than others because the damage induced by the drugs are hampered by the lack of mismatch repair genes and thus more cells die. (Hoguen, Kim *et al,* 1998).

Mismatch repair errors can *in vivo* through replication errors, hetroduplex formation, deamination of 5-methicytosine and the presence of DNA adducts, such as those induced by U.V. Cells cope with this damage by the recruitment of specialised proteins able to recognise, excise and correctly match mismatched bases. In addition to this, mismatch repair genes have been implicated in cell cycle arrest, microsatellite instability and proto-oncogene instability. (Hoguen Kim, *et al,* 1998, Wind,- *et al,* 1995, Mac Phee, 1995 and Modrich, 1994). Of the many genes found four have been directly implicated. hMSH2, hMLH1, hPMSI and hGTBP hMSH2 and hGTBP form a dimer that is able to recognise mismatched base pairs and directly bind to the region, hPMSI and hMLH1 are able to recognise the DNA/protein complex and also bind. Once the protein complex is complete ATP is exchanged for ADP and in doing so a number of other ATP dependant exonuclease, polymerases and ligases are recruited into the protein scaffold to complete the repair

CHAPTER 5

5.1 CONCLUSIONS

The partially transformed human thyroid epithelial cell line HTori-3 can be transformed *in vitro* following ionising radiation to produce detectable tumours *In vivo*

- Tumours produced were confirmed e.g. derived from the human thyroid cell line HTori-3 Re-injection of these tumours confirmed the, tumourigenecity and progressive growth from the day of injection

- Survival studies show that all cell lines investigated had individual radio-sensitivities, some deviating considerable from the parental line. This was also observed in the screening of irradiated cells in nude mice. Each sampled screened having a variable time to the formation of a tumour. This may be indicative of the stochastic nature of radiation inducing indiscriminate damage and a unique sequence of mutability.

- Doses of gamma radiation are able to induce a de-stabilisation of the genome resulting the Delayed Lethal Damage (DLD) phenotype. Induction of the DLD phenotype appears most significant at doses above 6Gy of radiation and is seen to persist for up to eight weeks in the parental cell line. However, data suggests

that doses less than 6Gy may also be involved. After this period phenotypic stability in the form of reproductive integrity is re-established.

- High numbers of non-adherent cells, (floaters) were observed throughout the incubation period. A fraction of these were later established as an apoptotic population

- Measurement of cell adherence using the MTT assay indicates that this variable may be important in the, reduced cloning efficiencies of cells observed in this study

- Colony micronuclei formation has proven that nuclear damage has been sustained in a dose dependant manner and is a useful marker for delayed lethal damage.

- Variable sequence length of Alu repeats may be indicative of a mutator phenotype and subsequent DLD expression.

- P53 mutations, although difficult to confirm in this study, remain likely to enhance the expression of genome instability.

- Although suitable in providing information about the delayed lethal damage phenotype, this cell line could not be recommended for *in vitro* transformation studies due to a level of radiation independent tumourigenesis

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Appendix

Human Thyroid Tumours Coding Information

Alpha Irradiated

New Code	Old Code ZH	Old Code SG	Dose (Gy)	Source	P53 Status
A1	2	8	0.125	0.125 a2	
A2	4	2	0.25	0.25 a4	
A3	7	7	0.25	0.25 a7	Exon 5 & 7
A4	2	10	0.5	0.5a2	Exon 7
A5	8	3	0.5	0.5 a8	Exon 5 & 8
A6	1	17	0.5	0.5 a1	Exon 7
A7	6		0.5	0.5 a6	
A8	4	16	1.0	1.0 a4	Exon 7
A9	9	18	1.5	1.5 a9	

Gamma Irradiated

New Code	Old Code ZH	Old Code SG	Dose (Gy)	Source	P53 Status
G1	T	6	0.5	0.5 GT	Exon 8
G2	C	1, 11	1.0	1.0 GC	Exon 7
G3	1	23	2.0		
G4	6	13	2.0	1x2 G6	
G5	7	9	2.0	1x2 G7	
G6	9	12	2.0	1x2 G9	Exon 6
G7	6	4	4.0	1x4 G6	
G8		19	3x1	3x1	Exon 6
G9			3x2	3x2	Exon 6 & 7
G10	X	22	3x3	3x2	Exon 7 & 8
G11		21	3x2	3x3	
G12		14	6x1	6x1	
G13		15	6x3	5x3	Exon 5 & 6

MICRONUCLEI ANALYSIS
G1 T=0 weeks

T=0 weeks

10/12/99

Dose:	Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	10	11	12	13	14	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	4	4	6	4	2	6	4	4	6	6	8	4	4	6	8	76	2	1
	Silde B	6	4	4	6	4	4	4	4	4	4	4	2	4	4	2	60	0	0
	Silde C	4	4	4	8	4	4	4	6	4	6	4	2	2	2	4	62	0	0
	Silde D	2	2	2	4	6	4	4	4	2	6	6	2	6	4	4	58	1	3
	Totals																256	3	4
																		1.17%	

T=0 weeks

Dose:	8 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	10	11	12	13	14	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	4	4	2	2	6	4	4	4	4	6	4	9	2	4	4	63	4	2
	Silde B	8	4	6	4	4	4	7	6	4	4	5	6	6	8	4	80	6	0
	Silde C	4	4	4	4	4	4	4	6	5	4	3	8	5	4	4	67	7	0
	Silde D	4	4	5	4	6	4	8	2	4	4	6	8	4	4	6	73	7	1
	Totals																283	24	3
																		8.48%	

MICRONUCLEI ANALYSIS
G1 T=4 weeks

T=4 weeks

Dose:	Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	10	11	12	13	14	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	6	8	15	8	10	12	6	14	16	6	4	6	4	10	6	131	4	3
	Silde B	6	6	6	4	14	8	9	4	7	6	4	4	8	6	6	98	6	2
	Silde C	4	10	8	7	7	6	6	8	10	6	8	6	8	8	6	108	7	5
	Silde D	8	14	6	5	10	16	4	4	8	4	4	8	6	4	10	111	7	1
	Totals																448	24	11
																		5.36%	

T=4 weeks

Dose:	8 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	10	11	12	13	14	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	6	8	8	15	18	8	7	14	14	8	14	12	4	9	10	155	7	10
	Silde B	10	18	25	12	8	8	10	4	8	5	8	9	10	12	8	155	6	6
	Silde C	12	9	6	8	8	11	9	8	12	14	8	12	4	12	12	145	7	7
	Silde D	9	14	4	12	8	14	14	10	10	10	7	12	9	9	7	149	7	5
	Totals																604	27	28
																		4.47%	

MICRONUCLEI ANALYSIS
G3 T=0 Weeks

T=0 weeks

10/12/99

Dose	Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	9	16	8	8	16	8	16	6	15	14	10	16	12	14	9	177	3	7
	Silde B	16	10	8	12	8	15	14	16	6	4	8	16	14	10	8	165	4	17
	Silde C	14	8	8	10	14	10	5	10	8	8	8	13	16	5	9	146	8	10
	Silde D	9	7	7	10	14	12	8	14	10	11	8	12	6	16	8	152	8	15
	Totals																640	23	49

T=0 weeks

Dose	2 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	16	16	12	8	16	15	10	16	16	8	14	9	8	8	10	182	3	15
	Silde B	15	16	12	4	14	8	8	16	13	8	16	7	8	13	12	170	18	14
	Silde C	8	14	8	8	12	6	4	5	5	14	7	8	15	11	12	137	6	10
	Silde D	14	8	7	14	9	15	8	8	14	8	6	15	8	15	14	163	17	7
	Totals																652	44	46

T=0 weeks

10/12/99

Dose	4 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	13	10	15	15	16	4	7	9	7	8	8	9	4	8	7	140	11	12
	Silde B	6	15	16	12	8	8	6	8	14	13	7	14	10	6	15	158	4	18
	Silde C																		0
	Silde D	15	12	8	16	8	11	11	14	12	10	16	8	5	8	13	167	16	9
	Totals																465	31	39

T=0 weeks

Dose	8 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
	Silde A	4	4	4	4	6	4	16	4	7	6	8	4	6	4	6	87	8	8
	Silde B	16	6	10	4	7	4	8	5	8	4	8	10	6	5	4	105	5	5
	Silde C	4	8	4	8	8	7	8	6	4	8	7	8	8	10	8	106	15	2
	Silde D	5	12	8	6	4	5	7	4	6	8	6	4	8	6	6	95	10	10
	Totals																393	38	25

MICRONUCLEI ANALYSIS
G3 T=4 weeks

T=4 weeks

10/12/99

Dose Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
Silde A	12	4	10	16	16	8	12	14	16	16	16	6	8	8	9	171	9	1
Silde B	16	6	14	14	6	4	10	12	16	12	6	13	14	8	8	159	15	4
Silde C	4	16	8	10	16	10	7	4	16	16	14	16	13	6	14	170	7	8
Silde D	12	4	8	4	4	8	12	8	8	16	8	16	4	12	6	130	0	0
Totals																630	31	13

T=4 weeks

Dose 2 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
Silde A	16	14	4	14	10	12	16	14	15	16	14	8	4	12	6	175	13	4
Silde B	5	8	12	8	16	6	16	16	8	16	6	7	6	6	12	148	7	8
Silde C																0		
Silde D																0		
Totals																323	20	12

T=4 weeks

10/12/99

Dose 4 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
Silde A	16	12	7	12	4	4	9	6	7	4	4	10	8	14	16	133	12	10
Silde B	13	6	8	10	16	12	12	15	16	13	10	16	8	4	6	165	10	8
Silde C	16	6	12	10	8	10	8	8	16	8	7	11	7	11	9	147	15	6
Silde D	12	15	4	16	9	4	8	6	10	16	8	14	10	15	8	155	6	7
Totals																600	43	31

T=4 weeks

Dose 8 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
Silde A	10	12	10	8	8	6	10	12	8	16	14	16	7	12	9	158	4	4
Silde B	9	9	8	9	16	7	8	16	16	14	15	6	14	16	12	175	10	5
Silde C	9	12	8	13	16	8	8	12	14	8	16	8	13	9	6	160	11	9
Silde D	12	8	13	8	6	8	13	12	6	14	12	12	4	8	4	140	11	1
Totals																633	36	19

MICRONUCLEI COLONY FREQUENCIES

G3 0Gy T=0

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	1	4	0	0	0	0
5	2	10	0	0	0	0
6	3	18	1	0	0	0
7	2	14	1	0	0	0
8	15	120	6	0	0	0
9	4	36	1	0	0	0
10	8	80	6	0	0	0
11	1	11	0	0	0	0
12	4	48	2	0	0	0
13	1	13	0	0	0	0
14	8	112	1	0	0	0
15	2	30	1	0	0	0
16	9	144	2	0	0	0
Colonies sampled	60					
Total cells sampled		640				
Micronuclei			21	0	0	0
Total micronuclei	3.28%		21			
Micronuclei per cell	3.28%					
SD			2.063107	1.224899		
SE			0.572203	0.169863		
%SE 1 MN			0.120163			
%SE total MN				0.025234		

G3 2Gy T=0

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	2	8	0	0	0	0
5	2	10	1	0	0	0
6	2	12	0	0	0	0
7	3	21	1	0	0	0
8	17	136	6	1	0	1
9	2	18	0	0	0	0
10	2	20	0	0	0	0
11	1	11	2	0	0	0
12	5	60	1	0	0	0
13	2	26	3	0	0	0
14	7	98	6	1	1	0
15	7	105	4	0	0	0
16	8	128	4	0	0	0
Colonies sampled	60					
Total cells sampled		653				
Micronuclei			28	4	3	4
Total micronuclei	4.29%		39			
Micronuclei per cell	5.97%					
SD			2.230327	1.430122		
SE			0.618581	0.198322		
%SE 1 MN			0.173203			
%SE total MN				0.067549		

G3 4Gy T=0

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	2	8	0	0	0	0
5	1	5	1	0	0	0
6	3	18	1	0	0	0
7	4	28	0	0	1	0
8	10	80	5	1	0	0
9	2	18	1	0	0	0
10	3	30	2	0	0	0
11	2	22	1	0	0	0
12	3	36	1	0	0	0
13	3	39	4	0	1	0
14	3	42	2	0	0	0
15	5	75	2	0	0	0
16	4	64	3	0	0	0
Colonies sampled	45					
Total cells sampled		465				
Micronuclei			23	2	6	0
Total micronuclei	4.95%		31			
Micronuclei per cell	6.67%					
SD			1.480644	1.057188		
SE			0.410657	0.146606		
%SE 1 MN			0.094451			
%SE total MN				0.02928		

G3 8Gy T=0

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	19	76	10	1	0	0
5	4	20	3	0	0	0
6	12	72	5	2	0	0
7	5	35	2	0	1	0
8	14	112	5	0	0	0
9	0	0	0	0	0	0
10	3	30	1	0	0	0
11	0	0	0	0	0	0
12	1	12	3	0	0	0
13	0	0	0	0	0	0
14	0	0	0	0	0	0
15	0	0	0	0	0	0
16	2	32	0	0	0	0
Colonies sampled	60					
Total cells sampled		389				
Micronuclei			29	6	3	0
Total micronuclei	7.46%		38			
Micronuclei per cell	9.77%					
SD			3.00427	1.760446		
SE			0.833235	0.24413		
%SE 1 MN			0.241638			
%SE total MN				0.091822		

MICRONUCLEI COLONY FREQUENCIES

G3 0Gy T=4

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	8	32	0	0	0	0
5	0	0	0	0	0	0
6	6	36	1	0	0	2
7	1	7	0	0	0	0
8	11	88	3	0	0	0
9	1	9	1	0	0	0
10	4	40	1	0	0	0
11	0	0	0	0	0	0
12	7	84	2	0	0	0
13	2	26	0	0	0	0
14	5	70	3	1	0	0
15	0	0	0	0	0	0
16	15	240	3	0	0	0
Colonies sampled	60					
Total cells sampled		632				
Micronuclei			14	2	0	8
Total micronuclei	2.22%		24			
Micronuclei per cell	3.80%					

SD	1.255756	0.8097725	
SE	0.348284	0.1122952	
%SE 1 MN	0.0487598		
%SE total MN		0.0117023	

G3 4Gy T=4

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	7	28	2	0	0	0
5	0	0	0	0	0	0
6	5	30	1	0	0	0
7	4	28	1	0	0	0
8	10	80	4	0	0	0
9	3	27	1	0	0	0
10	7	70	7	0	0	0
11	2	22	1	0	0	0
12	6	72	3	2	1	0
13	2	26	3	0	0	0
14	2	28	3	0	0	0
15	3	45	4	0	0	0
16	9	144	5	0	0	0
Colonies sampled	60					
Total cells sampled		600				
Micronuclei			35	4	3	0
Total micronuclei	5.83%		42			
Micronuclei per cell	7.00%					

SD	1.9741925	1.5225802	
SE	0.5475425	0.2111439	
%SE 1 MN	0.1916399		
%SE total MN		0.0804887	

G3 2Gy T=4

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	2	8	0	0	0	0
5	1	5	0	0	0	0
6	5	30	0	0	0	0
7	1	7	0	0	0	0
8	4	32	0	0	0	0
9	0	0	0	0	0	0
10	1	10	1	1	0	0
11	0	0	0	0	0	0
12	4	48	2	0	0	0
13	0	0	0	0	0	0
14	4	56	4	0	0	0
15	1	15	2	0	0	0
16	7	112	4	2	0	0
Colonies sampled	30					
Total cells sampled		323				
Micronuclei			13	6	0	0
Total micronuclei	4.02%		19			
Micronuclei per cell	5.88%					

SD	1.5275252	0.8974574	
SE	0.4236593	0.124455	
%SE 1 MN	0.0550757		
%SE total MN		0.0104644	

G3 8Gy T=4

Colony size	Frequency	Cells sampled	Number of Micronuclei 1	2	3	>4
4	2	8	3	0	0	0
5	0	0	0	0	0	0
6	5	30	1	0	0	0
7	2	14	0	0	0	0
8	14	112	3	0	0	0
9	6	54	4	0	0	0
10	3	30	2	0	0	0
11	0	0	0	0	0	0
12	10	120	4	0	0	0
13	4	52	4	0	0	0
14	5	70	4	1	0	0
15	1	15	0	0	0	0
16	8	128	6	1	0	0
Colonies sampled	60					
Total cells sampled		633				
Micronuclei			31	4	0	0
Total micronuclei	4.90%		35			
Micronuclei per cell	5.53%					

SD	2.0223114	1.4284071	
SE	0.5608883	0.1980844	
%SE 1 MN	0.1738754		
%SE total MN		0.0608564	

MICRONUCLEI ANALYSIS
G4

T=0 weeks

10/12/99

Dose: Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	10	11	12	13	14	15	16	17	18	[19]	[20]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
Silde A	7	4	6	4	7	4	6	7	4	8	8	9	4	4	5	7	8	8	4	4	118	14	7
Silde B	7	11	4	8	12	8	4	4	8	6	4	8	4	8	6	5	8	6	9	6	136	7	4
Silde C	5	5	5	5	4	4	5	5	4	4	12	4	6	4	4	7	8	5	4	10	110	6	6
Slide D	8	6	4	5	9	12	8	4	4	5	7	7	4	5	4	4	4	4	4	8	116	7	5
Silde E	5	4	9	6	6	6	7	6	4	5	4	6	12	8	8	5	4	8	4	8	125	9	5
Totals																					605	43	27
																						7.11%	

T=4 weeks

10/12/99

Dose: 8 Gy	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	10	11	12	13	14	15	16	17	18	[19]	[20]	Total Cells Scored	Total Micronuclei Scored	Total Pyknotic Scored
Silde A	4	4	4	12	4	4	4	12	6	5	8	10	12	6	6	6	4	10	4	6	131	6	10
Silde B	6	4	6	4	4	6	10	4	8	5	4	8	5	4	4	4	4	4	4	5	103	4	15
Silde C	4	4	6	4	4	4	8	6	4	10	6	6	6	9	8	6	6	6	12	5	124	3	7
Slide D	6	8	6	5	8	9	4	6	5	4	6	6	4	4	5	4	4	8	4	4	110	4	5
Silde E	9	8	4	4	4	8	8	4	5	11	4	6	4	6	9	4	8	12	5	4	127	2	2
Totals																					595	19	39
																						3.19%	

Miconuclei Analysis
A5

A4 Control T=4 weeks	Colony Size	Frequency	Mn	
	12	4	1	48
	11	1	0	11
	10	1	0	10
	9	4	4	36
	8	18	5	144
	7	9	6	63
	6	13	3	78
	5	15	5	75
	4	35	12	140
Total cells				605
Total Colonies Scored		100		
Total Micronuclei			35	
Total Pyknotic cells			14	
% Mn				5.79%

A4 8 Gy T=4 weeks	Colony Size	Frequency	Mn	
	12	5	0	60
	11	1	0	11
	10	4	1	40
	9	4	0	36
	8	12	4	96
	7	0	0	0
	6	24	6	144
	5	9	0	45
	4	41	8	164
Total Cell Scored				596
Total Colonies Scored		100		
Total Micronuclei			19	
Total Pyknotic cells			31	
% Mn				3.19%

Miconuclei Analysis
A8

A8 Control T=8 weeks	Colony Size	Frequency	Mn	
	12	0	0	0
	11	1	0	11
	10	3	2	30
	9	2	2	18
	8	20	5	160
	7	2	0	14
	6	11	9	66
	5	6	2	30
	4	55	19	220
Total Cell Scored				549
Total Colonies Scored		100		
Total Micronuclei			39	
Total Pyknotic cells			7	
				7.10%

A8 8 Gy T=8 weeks	Colony Size	Frequency	Mn	
	12	2	1	24
	11	3	0	33
	10	3	5	30
	9	7	4	63
	8	12	9	96
	7	9	9	63
	6	19	15	114
	5	15	7	75
	4	30	7	120
Total Cell Scored				618
Total Colonies Scored		100		
Total Micronuclei			56	
Total Pyknotic cells			12	
				9.06%

MICRONUCLEI ANALYSIS
XA-8 Tumour line

T=0 weeks

10/12/99

Dose: Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	Total Cells Scored	Total Mn Scored	Total Pyknotic Scored
Silde A	6	4	8	6	8	4	6	4	6	8	8	10	4	8	4	4	4	8	4	4	118	19	3
Silde B	4	7	8	8	4	4	4	5	8	6	4	4	4	4	10	4	6	4	9		107	12	4
Silde C	4	8	4	8	4	4	9	4	4	4	6	6	4	7	4	4	7	9	7		107	14	2
Silde D	8	5	4	8	4	4	8	8	12	5	8	4	4	4	4	8	4	8	4		114	11	4
Silde E	4	6	4	4	4	7	4	4	4	4	7	6	11	4	4	9	8	6	4	4	108	15	1
Totals																					554	71	14
																						12.82%	

T=4 weeks

10/12/99

Dose: Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	Total Cells Scored	Total Mn Scored	Total Pyknotic Scored
Silde A	4	8	6	4	4	8	4	4	4	4	6	10	8	4	7	8	4	4	4	8	113	26	10
Silde B	4	4	4	4	5	8	10	4	8	6	10	12	4	4	4	4	4	6	6	4	115	22	4
Silde C	4	9	4	4	4	4	8	4	10	4	7	5	5	4	4	4	4	4	8	4	104	24	0
Silde D	4	8	3	4	4	4	4	10	4	4	4	4	9	8	8	4	7	6	8	4	111	12	0
Silde E	6	7	7	4	4	6	10	8	8	4	4	12	4	4	4	4	7	4	4	6	117	28	0
Totals																					560	112	14
																						20.00%	

MICRONUCLEI ANALYSIS
ST-8 Tumour line

T=0 weeks

10/12/99

Dose: Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	Total Cells Scored	Total Mn Scored	Total Pyknotic Scored
Silde A	12	8	4	8	8	6	9	4	4	8	5	8	8	7	8	8	5	11	8	11	150	9	3
Silde B	7	7	4	8	8	9	8	12	6	8	6	8	6	9	8	4	4	4	4	4	134	17	2
Silde C	8	8	8	4	8	8	4	8	8	5	4	4	4	8	6	4	4	8	4	8	123	5	1
Silde D	4	8	8	7	10	10	5	7	8	4	4	4	5	4	4	8	5	4	8	7	124	7	5
Silde E	4	8	4	6	10	4	4	8	9	4	4	7	8	110	8	6	5	8	4	5	226	9	6
Totals																					757	38	17
																						5.02%	

T=4 weeks

10/12/99

Dose: Control	Colonies Counted per Slide [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]	[20]	Total Cells Scored	Total Mn Scored	Total Pyknotic Scored
Silde A	6	4	6	8	4	4	4	4	4	8	10	6	2	9	7	4	6	4	5	5	110	16	8
Silde B	4	4	6	4	6	4	4	4	5	4	4	4	3	4	4	6	7	4	4	4	89	48	5
Silde C	6	7	6	4	4	7	4	4	4	8	6	8	4	4	4	4	10	4	4	4	106	14	4
Silde D	6	4	4	6	4	6	4	6	4	6	4	4	4	4	4	4	4	4	5	5	92	13	1
Silde E	4	4	4	8	6	6	7	5	4	6	6	4	4	4	4	8	4	6	4	5	103	28	12
Totals																					500	119	30
																						24%	

MTAssayRawHTData

HTF2 1:1298

Plated	Vol	OD@570nm								Mean
4000	200	0.701	0.621	0.592	0.615	0.623	0.705	0.667	0.687	0.651
2000	100	0.601	0.428	0.487	0.648	0.668	0.579	0.678	0.718	0.635
1500	75	0.453	0.401	0.466	0.439	0.494	0.466	0.394	0.504	0.451
1000	50	0.316	0.272	0.314	0.294	0.389	0.328	0.281	0.325	0.315
500	25	0.215	0.312	0.216	0.271	0.239	0.237	0.201	0.226	0.240
250	12.5	0.101	0.104	0.151	0.154	0.135	0.143	0.134	0.184	0.138
125	6.25	0.065	0.645	0.053	0.064	0.051	0.071	0.067	0.054	0.133

HTF2 1:1298

Plated	Vol	OD@570nm								Mean
4000	200	0.713	0.735	0.764	0.686	0.744	0.626	0.644	0.609	0.690
2000	100	0.494	0.399	0.415	0.5	0.428	0.516	0.514	0.481	0.466
1500	75	0.333	0.355	0.316	0.325	0.321	0.355	0.377	0.352	0.341
1000	50	0.33	0.28	0.263	0.251	0.257	0.291	0.244	0.317	0.279
500	25	0.233	0.229	0.276	0.268	0.24	0.225	0.237	0.251	0.247
250	12.5	0.133	0.163	0.125	0.132	0.14	0.168	0.176	0.135	0.147
125	6.25	0.054	0.053	0.059	0.051	0.054	0.053	0.052	0.058	0.054

HTF2 1:1298

Plated	Vol	OD@570nm								Mean
4000	200	0.717	0.651	0.682	0.645	0.657	0.797	0.667	0.997	0.715
2000	100	0.455	0.464	0.448	0.438	0.458	0.419	0.475	0.552	0.464
1500	75	0.349	0.348	0.375	0.287	0.45	0.296	0.459	0.371	0.361
1000	50	0.281	0.251	0.273	0.236	0.257	0.256	0.232	0.287	0.260
500	25	0.181	0.125	0.172	0.184	0.131	0.124	0.205	0.141	0.158
250	12.5	0.101	0.102	0.15	0.143	0.125	0.995	0.108	0.128	0.232
125	6.25	0.051	0.054	0.051	0.41	0.068	0.058	0.054	0.064	0.101

MIT Assay Raw HIT Data

HP8T=2 1.1298

Plated	Vol	OD@570nm								Mean
40000	200	1.448	1.194	1.501	1.139	1.289	1.29	0.981	1.213	1.257
20000	100	0.394	0.482	0.392	0.479	0.558	0.4	0.583	0.478	0.471
15000	75	0.412	0.395	0.379	0.397	0.326	0.328	0.356	0.271	0.358
10000	50	0.21	0.204	0.211	0.323	0.256	0.258	0.188	0.211	0.235
5000	25	0.099	0.138	0.24	0.124	0.178	0.2	0.167	0.083	0.154
2500	125	0.063	0.072	0.102	0.124	0.103	0.057	0.016	0.085	0.078
1250	6.25	0.028	0.067	0.04	0.003	0.022	0.015	0.046	0.025	0.031

HP8T=2 1.1298

Plated	Vol	OD@570nm								Mean
40000	200	1	0.873	0.987	0.928	1.115	1.138	1.022	1.024	1.011
20000	100	0.439	0.389	0.443	0.372	0.346	0.403	0.467	0.525	0.423
15000	75	0.363	0.287	0.489	0.319	0.359	0.399	0.336	0.253	0.351
10000	50	0.231	0.196	0.231	0.256	0.298	0.258	0.222	0.236	0.241
5000	25	0.117	0.109	0.096	0.081	0.106	0.109	0.121	0.123	0.108
2500	125	0.057	0.075	0.082	0.054	0.082	0.083	0.058	0.034	0.066
1250	6.25	0.082	0.041	0.044	0.054	0.066	0.061	0.07	0.08	0.062

HP8T=2 1.1298

Plated	Vol	OD@570nm								Mean
40000	200	1.04	1.344	1.233	1.739	1.43	1.641	1.368	1.488	1.410
20000	100	0.618	0.651	0.636	0.637	0.763	0.633	0.707	0.625	0.659
15000	75	0.421	0.37	0.424	0.535	0.507	0.597	0.39	0.406	0.456
10000	50	0.311	0.359	0.326	0.312	0.406	0.367	0.276	0.261	0.327
5000	25	0.164	0.198	0.133	0.168	0.196	0.103	0.124	0.112	0.150
2500	125	0.076	0.097	0.067	0.075	0.063	0.061	0.082	0.029	0.069
1250	6.25	0.008	0.014	0.048	0.031	0.035	0.036	0.05	0.073	0.037

MTT Assay Raw HT Data

HT T=2 26.11.98

Plated	Vol	OD @ 570nm								Mean
40000	200	1.617	1.65	1.982	1.792	1.561	1.605	1.609	1.572	1.674
20000	100	0.774	0.822	0.885	0.935	0.825	1.022	0.819	0.692	0.847
15000	75	0.469	0.503	0.595	0.551	0.519	0.537	0.594	0.531	0.537
10000	50	0.377	0.376	0.369	0.45	0.38	0.453	0.36	0.34	0.388
5000	25	0.191	0.118	0.142	0.127	0.171	0.247	0.216	0.177	0.174
2500	12.5	0.162	0.09	0.073	0.075	0.048	0.065	0.073	0.037	0.078
1250	6.25	0.157	0.108	0.04	0.082	0.056	0.06	0.024	0.096	0.078

HT T=2 26.11.98

Plated	Vol	OD @ 570nm								Mean
40000	200	1.187	1.117	1.003	1.188	1.071	1.246	1.278	1.181	1.159
20000	100	0.587	0.555	0.487	0.598	0.557	0.567	0.612	0.625	0.574
15000	75	0.344	0.338	0.35	0.359	0.358	0.388	0.38	0.409	0.366
10000	50	0.288	0.275	0.298	0.324	0.327	0.314	0.348	0.355	0.316
5000	25	0.092	0.159	0.162	0.178	0.158	0.13	0.104	0.099	0.135
2500	12.5	0.163	0.069	0.087	0.074	0.052	0.056	0.077		0.083
1250	6.25	0.063	0.072	0.045	0.049	0.033	0.057	0.025	0.029	0.047

HT T=2 26.11.98

Plated	Vol	OD @ 570nm								Mean
40000	200	1.463	1.033	0.942	0.976	0.919	1.165	1.244	1.309	1.131
20000	100	0.519	0.63	0.678	0.648	0.586	0.59	0.676	0.771	0.637
15000	75	0.37	0.392	0.386	0.391	0.39	0.397	0.452	0.396	0.397
10000	50	0.252	0.277	0.278	0.285	0.332	0.284	0.288	0.324	0.290
5000	25	0.146	0.134	0.152	0.181	0.174	0.145	0.171	0.125	0.154
2500	12.5	0.039	0.056	0.026	0.06	0.041	0.054	0.073	0.04	0.049
1250	6.25	0.046	0.074	0.02	0.03	0.026	0.028	0.05	0.0193	0.037

MTT Assay Raw HT Data

HT-8 T=2 26.11.98

Plated	Vol	OD @ 570 nm								Mean
40000	200	1.366	1.189	1.07	1.037	1.08	1.177	1.034	1.351	1.163
20000	100	0.298	0.238	0.245	0.207	0.218	0.2	0.21	0.248	0.233
15000	75	0.203	0.169	0.166	0.129	0.133	0.149	0.142	0.152	0.155
10000	50	0.11	0.083	0.063	0.061	0.067	0.066	0.08	0.09	0.078
5000	25	0.03	0.026	0.037	0.026	0.032	0.045	0.038	0.018	0.032
2500	12.5	0.006	0.011	0.005	0.006	0.004	0.015	0.004	0.004	0.007
1250	6.25	0.006	0.027	0.013	0.016	0.04	0.029	0.033	0.007	0.021

HT-8 T=2 26.11.98

Plated	Vol	OD @ 570 nm								Mean
40000	200	1.301	1.075	1.185	1.325	1.375	1.431	1.151	1.517	1.295
20000	100	0.335	0.265	0.203	0.255	0.268	0.227	0.251	0.275	0.260
15000	75	0.316	0.178	0.159	0.145	0.15	0.18	0.188	0.161	0.185
10000	50	0.094	0.067	0.063	0.079	0.073	0.08	0.094	0.088	0.080
5000	25	0.044	0.042	0.019	0.041	0.021	0.024	0.056	0.019	0.033
2500	12.5	0.027	0.012	0.013	0.023	0.017	0.009	0.023		0.018
1250	6.25	0.007	0.011	0.004	0.021	0.014	0.02	0.017	0.022	0.015

HT-8 T=2 26.11.98

Plated	Vol	OD @ 570 nm								Mean
40000	200	1.098	1.207	1.097	1.142	1.203	1.198	1.126	1.277	1.169
20000	100	0.531	0.388	0.383	0.449	0.408	0.488	0.428	0.574	0.456
15000	75	0.263	0.235	0.188	0.258	0.218	0.208	0.205	0.219	0.224
10000	50	0.131	0.097	0.091	0.129	0.116	0.111	0.122	0.131	0.116
5000	25	0.016	0.025	0.024	0.074	0.045	0.019	0.026	0.051	0.035
2500	12.5	0.028	0.048	0.035	0.029	0.041	0.033	0.026	0.021	0.033
1250	6.25	0.031	0.023	0.002	0.03	0.028	0.035	0.037	0.014	0.025

MTT Assay Raw XA Data @T=2

XA T=4

Vol	Cmt	OD@570nm								Mean
200	40000	0.972	0.842	0.87	0.775	0.783	0.804	0.833	0.663	0.818
100	20000	0.465	0.402	0.46	0.448	0.406	0.431	0.466	0.449	0.441
50	10000	0.194	0.16	0.194	0.274	0.264	0.306	0.276	0.236	0.238
25	5000	0.098	0.136	0.153	0.133	0.154	0.122	0.106	0.129	0.129
12.5	2500	0.049	0.073	0.068	0.093	0.081	0.081	0.075	0.111	0.078
6.25	1250	0.043	0.028	0.041	0.047	0.042	0.047	0.051	0.042	0.044

XA8 T=4

Vol	Cmt	OD@570nm								Mean
200	40000	0.65	0.714	0.718	1.024	0.854	0.741	0.74	0.703	0.768
100	20000	0.412	0.454	0.494	0.506	0.444	0.458	0.406	0.615	0.474
50	10000	0.241	0.219	0.297	0.293	0.314	0.256	0.316	0.237	0.272
25	5000	0.123	0.164	0.162	0.18	0.195	0.081	0.124	0.135	0.146
12.5	2500	0.057	0.083	0.08	0.107	0.098	0.085	0.084	0.083	0.088
6.25	1250	0.056	0.057	0.072	0.059	0.095	0.076	0.066	0.069	0.070

MTT Assay Raw XA Data @T=4

XA T=4

Vol	Cmt	OD@570nm								Mean
200	40000	0.958	1.117	1.293	1.004	1.132	0.992	1.015	0.88	1.049
100	20000	0.636	0.76	0.771	0.762	0.645	0.651	0.696	0.5	0.678
50	10000	0.351	0.38	0.505	0.403	0.483	0.371	0.43	0.401	0.416
25	5000	0.154	0.183	0.18	0.183	0.211	0.192	0.205	0.18	0.186
12.5	2500	0.11	0.129	0.094	0.096	0.093	0.108	0.118	0.106	0.107
6.25	1250	0.063	0.064	0.085	0.059	0.062	0.081	0.075	0.06	0.069

XA8 T=4

Vol	Cmt	OD@570nm								Mean
200	40000	1.205	1.103	0.885	0.883	0.765	0.703	0.813	0.873	0.904
100	20000	0.565	0.615	0.475	0.464	0.513	0.484	0.474	0.376	0.496
50	10000	0.242	0.239	0.185	0.21	0.181	0.226	0.206	0.241	0.216
25	5000	0.207	0.187	0.211	0.192	0.218	0.225	0.195	0.221	0.207
12.5	2500	0.1	0.126	0.131	0.134	0.129	0.14	0.130	0.119	0.126
6.25	1250	0.101	0.093	0.077	0.094	0.088	0.078	0.086	0.091	0.089

MTT Assay Raw XA Data @ T=8

XA T=8

Vol	Cmt	OD@570nm								Mean
200	4000	0.665	0.655	0.758	0.869	0.798	0.77	0.83	0.813	0.772
100	2000	0.419	0.407	0.504	0.449	0.471	0.499	0.492	0.518	0.470
75	1500	0.353	0.346	0.421	0.429	0.411	0.397	0.395	0.41	0.395
50	1000	0.286	0.276	0.339	0.357	0.338	0.359	0.299	0.331	0.319
25	500	0.182	0.166	0.163	0.192	0.152	0.157	0.149	0.154	0.169
12.5	250	0.098	0.086	0.119	0.14	0.119	0.112	0.092	0.098	0.107
6.25	125	0.081	0.092	0.057	0.079	0.077	0.058	0.077	0.12	0.080

XB T=8

Vol	Cmt	OD@570nm								Mean
200	4000	0.957	0.767	0.953	0.871	0.779	0.855	0.819	0.833	0.854
100	2000	0.405	0.435	0.422	0.409	0.373	0.34	0.397	0.315	0.387
75	1500	0.299	0.329	0.333	0.302	0.274	0.274	0.294	0.22	0.291
50	1000	0.216	0.222	0.25	0.199	0.21	0.182	0.204	0.17	0.207
25	500	0.155	0.145	0.164	0.135	0.134	0.127	0.148	0.105	0.139
12.5	250	0.119	0.093	0.1	0.102	0.107	0.12	0.155	0.074	0.109
6.25	125	0.08	0.076	0.076	0.07	0.073	0.075	0.089	0.072	0.076

MTT Assay Raw XA Data @ T=12

XA T=12

Vol	Cmt	OD@570nm								Mean
200	4000	0.986	0.895	0.995	1.057	0.977	1.263	1.307	1.213	1.080
100	2000	0.549	0.556	0.604	0.617	0.549	0.555	0.657	0.553	0.588
75	1500	0.427	0.468	0.425	0.463	0.484	0.489	0.507	0.527	0.473
50	1000	0.384	0.35	0.357	0.375	0.362	0.384	0.38	0.419	0.378
25	500	0.211	0.184	0.216	0.199	0.211	0.198	0.212	0.159	0.198
12.5	250	0.145	0.162	0.151	0.145	0.147	0.153	0.153	0.153	0.151
6.25	125	0.039	0.046	0.039	0.039	0.047	0.036	0.048	0.038	0.040

XB T=12

Vol	Cmt	OD@570nm								Mean
200	4000	0.113	0.122	0.129	0.112	0.116	0.126	0.111	0.127	0.120
100	2000	0.113	0.122	0.129	0.112	0.116	0.126	0.111	0.172	0.125
75	1500	0.211	0.22	0.246	0.215	0.164	0.166	0.173	0.195	0.199
50	1000	0.449	0.436	0.43	0.415	0.414	0.444	0.475	0.366	0.431
25	500	0.657	0.731	0.601	0.506	0.517	0.464	0.612	0.463	0.573
12.5	250	0.713	0.486	0.543	0.573	0.602	0.607	0.565	0.522	0.576
6.25	125	0.81	0.824	0.855	0.847	0.998	0.832	0.939	0.702	0.850

MTT Assay Raw XC Data @ T=2

XC T=4

Vol	C/ml	OD @ 570nm								Mean
200	40000	0.941	0.805	0.785	0.813	0.735	0.805	0.788	0.719	0.799
100	20000	0.399	0.372	0.394	0.386	0.415	0.419	0.452	0.441	0.410
50	10000	0.215	0.248	0.23	0.257	0.368	0.298	0.413	0.324	0.294
25	5000	0.141	0.148	0.166	0.171	0.17	0.153	0.171	0.147	0.158
12.5	2500	0.075	0.091	0.092	0.051	0.098	0.097	0.157	0.106	0.096
6.25	1250	0.049	0.075	0.056	0.07	0.059	0.059	0.059	0.074	0.063

XC8 T=4

Vol	C/ml	OD @ 570nm								Mean
200	40000	0.797	0.873	1.161	1.178	0.918	0.9	0.863	0.899	0.949
100	20000	0.491	0.443	0.577	0.567	0.563	0.575	0.554	0.69	0.558
50	10000	0.288	0.367	0.328	0.401	0.295	0.298	0.27	0.299	0.318
25	5000	0.108	0.147	0.139	0.155	0.144	0.183	0.133	0.136	0.143
12.5	2500	0.095	0.119	0.089	0.096	0.109	0.112	0.123	0.102	0.106
6.25	1250	0.078	0.045	0.086	0.154	0.078	0.064	0.072	0.088	0.083

MTT Assay Raw XC Data @ T=4

XC T=4

Vol	C/ml	OD @ 570nm								Mean
200	40000	1.374	1.356	1.636	1.219	1.4	1.407	1.171	1.132	0.799
100	20000	0.672	0.653	0.677	0.67	0.749	0.687	0.664	0.599	0.410
50	10000	0.363	0.403	0.372	0.369	0.376	0.378	0.357	0.408	0.294
25	5000	0.087	0.078	0.072	0.083	0.079	0.06	0.071	0.109	0.158
12.5	2500	0.053	0.055	0.057	0.051	0.056	0.054	0.05	0.057	0.096
6.25	1250	0.051	0.065	0.044	0.049	0.064	0.046	0.056	0.048	0.063

XC8 T=4

Vol	C/ml	OD @ 570nm								Mean
200	40000	0.147	0.126	0.148	0.158	0.142	0.135	0.11	0.13	0.949
100	20000	0.085	0.09	0.09	0.083	0.085	0.086	0.073	0.077	0.558
50	10000	0.061	0.057	0.062	0.061	0.063	0.065	0.055	0.06	0.318
25	5000	0.037	0.037	0.038	0.034	0.036	0.032	0.034	0.041	0.143
12.5	2500	0.033	0.037	0.034	0.032	0.047	0.037	0.040	0.04	0.106
6.25	1250	0.032	0.032	0.032	0.033	0.035	0.034	0.039	0.038	0.083

MTT Assay Raw OD Data @ T=48

OD T=48

Vol	Cells	OD@570nm								Mean
200	40000	0.665	0.655	0.758	0.869	0.798	0.77	0.83	0.813	0.772
100	20000	0.419	0.407	0.504	0.449	0.471	0.499	0.492	0.518	0.460
75	15000	0.353	0.346	0.421	0.429	0.411	0.397	0.395	0.41	0.395
50	10000	0.286	0.276	0.309	0.357	0.338	0.339	0.299	0.331	0.319
25	5000	0.182	0.166	0.183	0.192	0.152	0.157	0.149	0.154	0.159
12.5	2500	0.098	0.086	0.119	0.14	0.119	0.112	0.092	0.093	0.107
6.25	1250	0.081	0.092	0.057	0.079	0.077	0.068	0.077	0.12	0.080

OD T=48

Vol	Cells	OD@570nm								Mean
200	40000	0.957	0.767	0.953	0.871	0.779	0.855	0.819	0.833	0.854
100	20000	0.405	0.435	0.422	0.409	0.373	0.34	0.337	0.315	0.357
75	15000	0.299	0.339	0.333	0.302	0.274	0.274	0.294	0.22	0.291
50	10000	0.216	0.222	0.25	0.199	0.21	0.182	0.204	0.17	0.207
25	5000	0.155	0.145	0.164	0.135	0.134	0.127	0.148	0.103	0.139
12.5	2500	0.119	0.098	0.1	0.102	0.107	0.12	0.155	0.074	0.109
6.25	1250	0.08	0.076	0.076	0.07	0.073	0.075	0.089	0.072	0.076

MTT Assay Raw OD Data @ T=72

OD T=72

Vol	Cells	OD@570nm								Mean
200	40000	1.166	1.102	1.206	1.577	1.144	1.347	0.884	0.737	1.144
100	20000	0.562	0.565	0.634	0.552	0.504	0.462	0.441	0.397	0.517
75	15000	1.109	0.743	0.483	0.391	0.335	0.314	0.37	0.213	0.495
50	10000	0.553	0.4	0.374	0.363	0.268	0.324	0.237	0.135	0.394
25	5000	0.194	0.225	0.234	0.223	0.159	0.168	0.166	0.121	0.166
12.5	2500	0.081	0.076	0.092	0.086	0.106	0.086	0.085	0.095	0.088
6.25	1250	0.069	0.081	0.084	0.12	0.083	0.08	0.13	0.107	0.094

OD T=72

Vol	Cells	OD@570nm								Mean
200	40000	0.964	0.841	1.283	1.473	1.879	1.239	0.997	0.465	1.146
100	20000	0.756	0.764	0.682	0.605	0.581	0.592	0.559	0.661	0.651
75	15000	0.636	0.461	0.459	0.466	0.479	0.466	0.511	0.542	0.498
50	10000	0.559	0.34	0.362	0.365	0.309	0.283	0.348	0.363	0.370
25	5000	0.205	0.179	0.184	0.178	0.2	0.163	0.157	0.144	0.176
12.5	2500	0.128	0.115	0.101	0.108	0.119	0.111	0.097	0.108	0.111
6.25	1250	0.102	0.092	0.084	0.075	2.2 [illegible]	0.092	0.079	0.093	0.105	0.094

SURVIVAL ANALYSIS

HTori-3 Survival @ T=0

16.07.97

Control & Code	Dose Gy	Number of Cells Seeded per Flask	Numbers of Colonies Scored per Plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
ctl / ctl	0	125	63	56	86	56	65	75	62	68	77	74	68.20	9.70	10	68.20 ± 9.70	54.56 ± 2.46	1.000 ± 0.0636
a1 / a1	2	167	71	63	73	61	68	57	64	66	62		65.00	5.05	9	91.12 ± 5.05	38.92 ± 1.01	0.713 ± 0.0370
a2 / a2	4	250	72	64	70	72	64	73	66	69	61	60	67.10	4.75	10	136.40 ± 4.75	26.84 ± 0.60	0.492 ± 0.0247
a3 / a3	6	500	64	71	66	64	70	64	73	81	69	66	68.80	5.35	10	272.80 ± 5.35	13.76 ± 0.34	0.252 ± 0.0129
a4 / a4	8	2500	134	138	165	156	133	120	131	131	132	134	137.40	13.18	10	1364.00 ± 13.18	5.50 ± 0.17	0.101 ± 0.0055
ctl / ctl	0	125	60	45	51	56	52	45	50	46	48		50.33	5.12	9	50.33 ± 5.12	40.27 ± 1.37	1.000 ± 0.0480
a1 / a1	2	167	45	51	50	49	55	48	51	51	51	57	50.80	3.36	10	67.25 ± 3.36	30.42 ± 0.64	0.755 ± 0.0301
a2 / a2	4	250	44	40	35	38	41	34	39	54	52	40	41.70	6.62	10	100.67 ± 6.62	16.68 ± 0.84	0.414 ± 0.0251
a3 / a3	6	500	54	47	30	35	37	42	33	40	45	36	39.90	7.25	10	201.33 ± 7.25	7.98 ± 0.46	0.198 ± 0.0132
a4 / a4	8	2500	28	30	28	31	38	41	33	30	40		33.22	5.12	9	1006.67 ± 5.12	1.33 ± 0.07	0.033 ± 0.0020
ctl / ctl	0	125	69	71	72	68	76						71.20	3.11	5	71.20 ± 3.11	56.96 ± 1.11	1.000 ± 0.0277
a1 / a1	2	167	78	79	80	89	72						79.60	6.11	5	95.12 ± 6.11	47.66 ± 1.64	0.837 ± 0.0331
a2 / a2	4	250	84	80	82	79	91						83.20	4.76	5	142.40 ± 4.76	33.28 ± 0.85	0.584 ± 0.0188
a3 / a3	6	500	99	86	116	96	102						99.80	10.87	5	284.80 ± 10.87	19.96 ± 0.97	0.350 ± 0.0184
a4 / a4	8	2500	185	200	221	181	184						194.20	16.69	5	1424.00 ± 16.69	7.77 ± 0.30	0.136 ± 0.0059

SURVIVAL ANALYSIS

HTori-3 Survival @ T=2 weeks

01.08.97

Control & Code	Dose Gy	Number of Cells Seeded per Flask	Numbers of Colonies S [1]	[2]	[3]	[4]	[5]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
ctl / ctl	0	125	63	60	62	65	62	62.40	1.82	5	62.40 ± 1.82	49.92 ± 0.65	1.000 ± 0.0184
a1 / a1	2	125	69	60	61	66	60	63.20	4.09	5	62.40 ± 4.09	50.56 ± 1.46	1.013 ± 0.0321
a2 / a2	4	125	59	73	51	57	52	58.40	8.82	5	62.40 ± 8.82	46.72 ± 3.16	0.936 ± 0.0644
a3 / a3	6	125	53	61	45	62	56	55.40	6.88	5	62.40 ± 6.88	44.32 ± 2.46	0.888 ± 0.0506
a4 / a4	8	125	63	63	60	63	67	63.20	2.49	5	62.40 ± 2.49	50.56 ± 0.89	1.013 ± 0.0222
ctl / ctl	0	125	66	67	57	69	66	65.00	4.64	5	65.00 ± 4.64	52.00 ± 1.66	1.000 ± 0.0451
a1 / a1	2	125	62	64	68	73	59	65.20	5.45	5	65.00 ± 5.45	52.16 ± 1.95	1.003 ± 0.0493
a2 / a2	4	125	62	55	61	59	56	58.60	3.05	5	65.00 ± 3.05	46.88 ± 1.09	0.902 ± 0.0356
a3 / a3	6	125	64	56	55	54	65	58.80	5.26	5	65.00 ± 5.26	47.04 ± 1.88	0.905 ± 0.0463
a4 / a4	8	125	68	70	69	60	65	66.40	4.04	5	65.00 ± 4.04	53.12 ± 1.44	1.022 ± 0.0428
ctl / ctl	0	125	80	84	72	82	86	80.80	5.40	5	80.80 ± 5.40	64.64 ± 1.93	1.000 ± 0.0423
a1 / a1	2	125	68	77	73	72	73	72.60	3.21	5	80.80 ± 3.21	58.08 ± 1.15	0.899 ± 0.0322
a2 / a2	4	125	74	85	90			83.00	8.19	3	80.80 ± 8.19	66.40 ± 3.78	1.027 ± 0.0661
a3 / a3	6	125	80	99	86	83	85	86.60	7.30	5	80.80 ± 7.30	69.28 ± 2.61	1.072 ± 0.0516
a4 / a4	8	125	88	94	89	92	87	90.00	2.92	5	80.80 ± 2.92	72.00 ± 1.04	1.114 ± 0.0370

SURVIVAL ANALYSIS

Htori-3 Survival @ T=4 weeks

15.08.97

Number & Control	Dose Gy	Number of Cells Seeded per Flask	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
ctl / 0	0	125	51	56	56	53	61	55.40	3.78	5	55.40 ± 3.78	44.32 ± 1.35	1.000 ± 0.043
a1 / 2	0	125	54	52	56	66	74	60.40	9.32	5	55.40 ± 9.32	48.32 ± 3.33	1.090 ± 0.082
a2 / 4	0	125	61	53	50	61	56	56.20	4.87	5	55.40 ± 4.87	44.96 ± 1.74	1.014 ± 0.050
a3 / 6	0	125	54	41	48	45	44	46.40	4.93	5	55.40 ± 4.93	37.12 ± 1.76	0.838 ± 0.047
a4 / 8	0	125	54	50	56	46	54	52.00	4.00	5	55.40 ± 4.00	41.60 ± 1.43	0.939 ± 0.043
ctl / 0	0	125	86	85	80	74		81.25	5.50	4	81.25 ± 5.50	65.00 ± 2.20	1.000 ± 0.072
a1 / 2	0	125	35	31				33.00	2.83	2	81.25 ± 2.83	26.40 ± 1.60	0.406 ± 0.036
a2 / 4	0	125	45	60	72	61		59.50	11.09	4	81.25 ± 11.09	47.60 ± 4.44	0.732 ± 0.082
a3 / 6	0	125	32	27	34	38	34	33.00	4.00	5	81.25 ± 4.00	26.40 ± 1.43	0.406 ± 0.034
a4 / 8	0	125	59	59	59	65	50	58.40	5.37	5	81.25 ± 5.37	46.72 ± 1.92	0.719 ± 0.054
ctl / 0	0	125	52	60	76	69	66	64.60	9.10	5	64.60 ± 9.10	51.68 ± 3.26	1.000 ± 0.089
a1 / 2	0	125	87	73	79	76	76	78.20	5.36	5	64.60 ± 5.36	62.56 ± 1.92	1.211 ± 0.085
a2 / 4	0	125	78	78	79	50	50	67.00	15.52	5	64.60 ± 15.52	53.60 ± 5.55	1.037 ± 0.126
a3 / 6	0	125	14	19	11	11	14	13.80	3.27	5	64.60 ± 3.27	11.04 ± 1.17	0.214 ± 0.026
a4 / 8	0	125	7	8	9	14	14	10.40	3.36	5	64.60 ± 3.36	8.32 ± 1.20	0.161 ± 0.025
ctl / 0	0	125	61	44	42	47	54	49.60	7.83	5	49.60 ± 7.83	39.68 ± 2.80	1.000 ± 0.095
a1 / 2	0	125	50	53	55	53		52.75	2.06	4	49.60 ± 2.06	42.20 ± 0.82	1.064 ± 0.070
a2 / 4	0	125	45	34	44	46	41	42.00	4.85	5	49.60 ± 4.85	33.60 ± 1.73	0.847 ± 0.069
a3 / 6	0	125	41	35	44	44	31	39.00	5.79	5	49.60 ± 5.79	31.20 ± 2.07	0.786 ± 0.072
a4 / 8	0	125	29	32	39	32	40	34.40	4.83	5	49.60 ± 4.83	27.52 ± 1.73	0.694 ± 0.062

SURVIVAL ANALYSIS

Htori-3 Survival @ T=8 weeks

16.09.97

Number & Control	Dose Gy	Number of Cells Seeded per Flask	Numbers of Colonies S [1]	[2]	[3]	[4]	[5]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
ctl / ctl	0	150	63	64	59	62	65	62.60	2.30	5	62.60 ± 2.30	41.73 ± 0.69	1.000 ± 0.0233
a1 / a1	2	150	50	65	66	51	52	56.80	7.98	5	62.60 ± 7.98	37.87 ± 2.38	0.907 ± 0.0589
a2 / a2	4	150	52	51	61	59	59	56.40	4.56	5	62.60 ± 4.56	37.60 ± 1.36	0.901 ± 0.0358
a3 / a3	6	150	58	54	49	51	56	53.60	3.65	5	62.60 ± 3.65	35.73 ± 1.09	0.856 ± 0.0296
a4 / a4	8	150	54	57	61	61	51	56.80	4.38	5	62.60 ± 4.38	37.87 ± 1.31	0.907 ± 0.0347
ctl / ctl	0	150	98	84	100	89	101	94.40	7.50	5	94.40 ± 52.88	62.93 ± 2.24	1.000 ± 0.0503
a1 / a1	2	150	88	89	93	91	85	89.20	3.03	5	94.40 ± 48.01	59.47 ± 0.90	0.945 ± 0.0365
a2 / a2	4	150	83	84	83	84	78	82.40	2.51	5	94.40 ± 44.18	54.93 ± 0.75	0.873 ± 0.0332
a3 / a3	6	150	81	82	88	89	88	85.60	3.78	5	94.40 ± 47.59	57.07 ± 1.13	0.907 ± 0.0369
a4 / a4	8	150	86	98	84	97	103	93.60	8.20	5	94.40 ± 53.10	62.40 ± 2.45	0.992 ± 0.0525
ctl / ctl	0	150	65	63	68	69	71	67.20	3.19	5	67.20 ± 37.57	44.80 ± 1.43	1.000 ± 1.43
a1 / a1	2	150	55	65	62	69	55	61.20	6.18	5	67.20 ± 30.43	40.80 ± 2.76	0.911 ± 2.76
a2 / a2	4	150	60	59	58	55	55	57.40	2.30	5	67.20 ± 30.37	38.27 ± 1.03	0.854 ± 1.03
a3 / a3	6	150	62	69	71	75	63	68.00	5.48	5	67.20 ± 34.85	45.33 ± 2.45	1.012 ± 2.45
a4 / a4	8	150	71	55	52	75	68	64.20	10.13	5	67.20 ± 33.89	42.80 ± 4.53	0.955 ± 4.53

SURVIVAL ANALYSIS

HT Tumour A2

20.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1] [2] [3] [4] [5]	Number of Colonies Mean	Number of Colonies Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	65 71 58 59 61	62.80	5.31	5	62.80 ± 32.77	50.24 ± 1.90	1.000 ± 0.0535
T=0 / a1	2	175	66 62 68 62 81	67.80	7.82	5	87.92 ± 39.64	38.74 ± 2.00	0.771 ± 0.0493
T=0 / a2	4	250	60 63 65 64 67	63.80	2.59	5	125.60 ± 2.16	25.52 ± 0.46	0.508 ± 0.0213
T=0 / a3	6	500	62 54 45 50 51	52.40	6.27	5	251.20 ± 6.27	10.48 ± 0.56	0.209 ± 0.0137
T=0 / a4	8	1000	28 30 32 39 30	31.80	4.27	5	502.40 ± 4.27	3.18 ± 0.19	0.063 ± 0.0045
T=0 / a5	16	10000	4 1 1 0 0	1.20	1.64	5	5024.0 ± 1.64	0.01 ± 0.01	0.000 ± 0.0001
T=4 / ctl	0	125	55 65 67 45 50	56.40	9.48	5	56.4 ± 9.48	45.12 ± 3.39	1.000 0.0841
T=4 / a1	2	125	50 53 55 61 54	54.60	4.04	5	56.4 ± 4.04	43.68 ± 1.44	0.968 ± 0.0486
T=4 / a2	4	125	44 46 52 54 45	48.20	4.49	5	56.4 ± 4.49	38.56 ± 1.61	0.855 ± 0.0481
T=4 / a3	6	125	34 35 31 39 40	35.80	3.70	5	56.4 ± 3.70	28.64 ± 1.32	0.635 ± 0.0379
T=4 / a4	8	125	22 29 32 30 19	26.40	5.59	5	56.4 ± 5.59	21.12 ± 2.00	0.468 ± 0.0478
T=4 / a5	16	125	10 5 5 10 7	7.40	2.51	5	56.4 ± 2.51	5.92 ± 0.90	0.131 ± 0.0205

SURVIVAL ANALYSIS

HT Tumour A3

20.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	100	60	64	67	54	62	61.40	4.88	5	61.40 ± 4.88	61.40 ± 2.18	1.000 ± 0.0503
T=0 / a1	2	133	61	64	66	60	63	62.80	2.39	5	81.66 ± 2.39	47.22 ± 0.80	0.769 ± 0.0303
T=0 / a2	4	200	74	74	78	67	79	74.40	4.72	5	122.80 ± 4.72	37.20 ± 1.06	0.606 ± 0.0276
T=0 / a3	6	400	81	92	81	82	82	83.60	4.72	5	245.60 ± 4.72	20.90 ± 0.53	0.340 ± 0.0148
T=0 / a4	8	1335	89	82	92	96	103	92.40	7.83	5	819.69 ± 7.83	6.92 ± 0.26	0.113 ± 0.0059
T=4 / ctl	0	250	19	19	18	15		17.75	1.89	4	17.75 ± 1.89	7.10 ± 0.38	1.000 ± 0.0754
T=4 / a1	2	250											
T=4 / a2	4	250											
T=4 / a3	6	250	28	25	25	24	24	25.20	1.64	5	17.75 ± 1.64	10.08 ± 0.29	1.420 ± 0.0653
T=4 / a4	8	250	48	32	35	37	32	36.80	6.61	5	17.75 ± 6.61	14.72 ± 1.18	2.073 ± 0.1821

SURVIVAL ANALYSIS

HT Tumour A4

31.6.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	52	53	49	50	54	51.60	2.07	5	51.6 ± 2.07	41.28 ± 0.93	1.000 ± 0.03
T=0 / a1	2	167	42	40	48	43	44	43.40	2.97	5	68.9 ± 2.97	25.99 ± 1.33	0.630 ± 0.04
T=0 / a2	4	250	23	29	30	30	22	26.80	3.96	5	103.2 ± 3.96	10.72 ± 1.77	0.260 ± 0.04
T=0 / a3	6	500	15	10	12	10	10	11.40	2.19	5	206.4 ± 2.19	2.28 ± 0.98	0.055 ± 0.02
T=0 / a4	8	1250	6	7	7	7	8	7.00	0.71	5	516.0 ± 0.71	0.56 ± 0.32	0.014 ± 0.01
T=0 / a5	12	12500	2	2				2.00	0.00	2	5160.0 ± 0.00	0.02 ± 0.00	0.000 ± 0.00
T=0 / a6	16	125000	1	1	1			1.00	0.00	3	51600.0 ± 0.00	0.00 ± 0.00	0.000 ± 0.00
T=4 / ctl	0	125	57	76	70	60	70	66.60	7.86	5	66.60 ± 7.86	53.28 ± 2.81	1.000 ± 0.06
T=4 / a2	2	125	83	71	74	99	83	82.00	10.91	5	66.60 ± 10.91	65.60 ± 3.90	1.231 ± 0.08
T=4 / a3	4	125	72	67	76	57	56	65.60	8.91	5	66.60 ± 8.91	52.48 ± 3.19	0.985 ± 0.06
T=4 / a4	6	125											
T=4 / a5	8	125	14	19	19	19	17	17.60	2.19	5	66.60 ± 2.19	14.08 ± 0.78	0.264 ± 0.02
T=4 / a6	12	125	49	40	50	28	47	42.80	9.15	5	66.60 ± 9.15	34.24 ± 3.27	0.643 ± 0.06

SURVIVAL ANALYSIS

HT Tumour A5

28.05.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Numt Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	39	56	52	50	43	49	57	51	56	48	50.10	5.78	10	50.10 ± 19.31	40.08 ± 1.46	1.000 ± 0.0516
T=0 / a1	2	192	50	41	48	39	41	38	45	42	55	50	44.90	5.63	10	76.95 ± 17.23	23.39 ± 0.93	0.583 ± 0.0314
T=0 / a2	4	313	24	29	27	33	31	29	36	35	39	33	31.60	4.50	10	125.45 ± 3.77	10.10 ± 0.45	0.252 ± 0.0146
T=0 / a3	8	1250	12	13	9	16	12	11	12	18	18	14	13.50	2.99	10	501.00 ± 3.17	1.08 ± 0.08	0.027 ± 0.0021
T=0 / a4	12	2500	1	1	1	1	1	1	1	2	4		1.44	1.01	9	1002.00 ± 1.01	0.06 ± 0.01	0.001 ± 0.0003
T=4 / ctl	0	125	53	50									51.50	2.12	2	51.50 ± 2.12	41.20 ± 1.20	1.000 ± 0.0412
T=4 / a1	2	250	82										82.00	#DIV/0!	1	103.00 ± #DIV/0!	32.80 ± #DIV/0!	0.796 ± #DIV/0!
T=4 / a2	4	625	89	86	93	103	86						91.40	7.09	5	257.50 ± 7.09	14.62 ± 0.51	0.355 ± 0.0161
T=4 / a3	8	1250	165	170									167.50	3.54	2	515.00 ± 3.54	13.40 ± 0.20	0.325 ± 0.0106
T=4 / a4	12	2500	560	580									570.00	14.14	2	1030.00 ± 14.14	22.80 ± 0.40	0.553 ± 0.0188

SURVIVAL ANALYSIS

HT Tumour A6

20.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Numbe Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	25	18	23	25	24	23.00	2.92	5	23.00 ± 11.32	18.40 ± 1.04	1.000 ± 0.0802
T=0 / a1	2	133	19	18	15	14	16	16.40	2.07	5	24.47 ± 7.41	12.33 ± 0.70	0.670 ± 0.0537
T=0 / a2	4	200	9	10	8	6	8	8.20	1.48	5	36.80 ± 1.71	4.10 ± 0.33	0.223 ± 0.0220
T=0 / a3	6	400	2	1	1	3	7	2.80	2.49	5	73.60 ± 2.49	0.70 ± 0.28	0.038 ± 0.0153
T=0 / a4	8	2000	1	4	1	3	0	1.80	1.64	5	368.00 ± 1.64	0.09 ± 0.04	0.005 ± 0.0020
T=4 / ctl	0	250	69	80	92	80	73	78.80	8.76	5	78.80 ± 8.76	31.52 ± 1.57	1.000 ± 0.0703
T=4 / a1	2	250						#DIV/0!	#DIV/0!	0	78.80 #DIV/0!	#DIV/0! #DIV/0!	#DIV/0! #DIV/0!
T=4 / a2	4	250						#DIV/0!	#DIV/0!	0	78.80 #DIV/0!	#DIV/0! #DIV/0!	#DIV/0! #DIV/0!
T=4 / a3	6	250						#DIV/0!	#DIV/0!	0	78.80 #DIV/0!	#DIV/0! #DIV/0!	#DIV/0! #DIV/0!
T=4 / a4	8	250	62	70	72	62	70	67.20	4.82	5	78.80 ± 4.82	26.88 ± 0.86	0.853 ± 0.0555

SURVIVAL ANALYSIS

HT Tumour A7

21.6.98

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	71	69	59	54	60	62.60	7.16	5	62.60 ± 31.91	50.08 ± 2.56	1.000 ± 0.0724
T=0 / a1	2	192						#DIV/0!	#DIV/0!	0	96.15 ± #DIV/0!	#DIV/0! ± #DIV/0!	#DIV/0! ± #DIV/0!
T=0 / a2	4	313						#DIV/0!	#DIV/0!	0	156.75 ± #DIV/0!	#DIV/0! ± #DIV/0!	#DIV/0! ± #DIV/0!
T=0 / a3	8	2500	147	145	151	155	148	149.20	3.90	5	1252.00 ± 3.90	5.97 ± 0.07	0.119 ± 0.0063
T=4 / ctl	0	125	89	96	92	81	96	90.80	6.22	5	90.80 ± 6.22	72.64 ± 2.23	1.000 ± 0.0433
T=4 / a1	2	125						#DIV/0!	#DIV/0!	0	90.80 ± #DIV/0!	#DIV/0! ± #DIV/0!	#DIV/0! ± #DIV/0!
T=4 / a2	4	125						#DIV/0!	#DIV/0!	0	90.80 ± #DIV/0!	#DIV/0! ± #DIV/0!	#DIV/0! ± #DIV/0!
T=4 / a3	8	125	53	49	38	42	60	48.40	8.73	5	90.80 ± 8.73	38.72 ± 3.13	0.533 ± 0.0460

SURVIVAL ANALYSIS

HT Tumour A8

20.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1] [2] [3] [4] [5]	Number of Colonies Mean	Number of Colonies Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	27 25 24 31 35	28.40	4.56	5	28.40 ± 15.77	22.72 ± 1.63	1.000 ± 0.1016
T=0 / a1	2	167	34 45 45 35 34	38.60	5.86	5	37.94 ± 17.93	23.11 ± 1.57	1.017 ± 0.1005
T=0 / a2	4	250	18 14 16 18 22	17.60	2.97	5	56.80 ± 1.91	7.04 ± 0.53	0.310 ± 0.0323
T=0 / a3	6	500	21 21 25 25 22	22.80	2.05	5	113.60 ± 2.05	4.56 ± 0.18	0.201 ± 0.0165
T=0 / a4	8	1250	20 22 21 22 18	20.60	1.67	5	284.00 ± 1.67	1.65 ± 0.06	0.073 ± 0.0058
T=4 / ctl	0	250	41 37 45 35 35	38.60	4.34	5	38.60 ± 4.34	15.44 ± 0.78	1.000 ± 0.0710
T=4 / a2	4	250	33 30 35 29 34	32.20	2.59	5	38.60 ± 2.59	12.88 ± 0.46	0.834 ± 0.0670
T=4 / a3	6	250	38 44 36 35 36	37.80	3.63	5	38.60 ± 3.63	15.12 ± 0.65	0.979 ± 0.0820
T=4 / a4	8	250	30 29 31 34 33	31.40	2.07	5	38.60 ± 2.07	12.56 ± 0.37	0.813 ± 0.0632

SURVIVAL ANALYSIS

HT Tumour A9

20.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1] [2] [3] [4] [5]	Number of Colonies Mean	Number of Colonies Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	25 28 25 24 21	24.60	2.51	5	24.60 ± 11.14	19.68 ± 0.90	1.000 ± 0.0645
T=0 / a1	2	167	22 22 15 14 16	17.80	3.90	5	32.87 ± 7.24	10.66 ± 1.04	0.542 ± 0.0585
T=0 / a2	4	250	20 15 12 17 14	15.60	3.05	5	49.20 ± 3.37	6.24 ± 0.55	0.317 ± 0.0313
T=0 / a3	6	500	8 7 7 11 14	9.40	3.05	5	98.40 ± 3.05	1.88 ± 0.27	0.096 ± 0.0145
T=0 / a4	8	1250	8 17 11 7 5	9.60	4.67	5	246.00 ± 4.67	0.77 ± 0.17	0.039 ± 0.0087
T=0 / ctl	0	250	21 24 23 26 26	24.00	2.12	5	24.00 ± 2.12	9.60 ± 0.38	1.000 ± 0.0559
T=0 / a1	2	625	25 25 26 25 22	24.60	1.52	5	60.00 ± 1.52	3.94 ± 0.11	0.410 ± 0.0219
T=0 / a2	4	1250	18 15 7 20	15.00	5.72	4	120.00 ± 5.72	1.20 ± 0.23	0.125 ± 0.0245
T=0 / a3	6	2500	6 6 7 13	8.00	3.37	4	240.00 ± 3.37	0.32 ± 0.07	0.033 ± 0.0072
T=0 / a4	8	5000	6 8 4 12 2	6.40	3.85	5	480.00 ± 3.85	0.13 ± 0.03	0.013 ± 0.0036
T=0 / ctl	0	250	25 26 28 26 31	27.20	2.39	5	27.20 ± 2.39	10.88 ± 0.43	1.000 ± 0.06
T=0 / a1	8	250	13 12 12 17 18	14.40	2.88	5	27.20 ± 2.88	5.76 ± 0.52	0.529 ± 0.05

SURVIVAL ANALYSIS

HT Tumour G1

13.07.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	52	37	46	43	46	47	50	41	58	51	47.10	6.01	10	47.10 ± 6.01	37.68 ± 1.90	1.000 ± 0.0713
T=0 / a1	2	167	44	36	41	38	40	44	45	37	40	37	40.20	3.26	10	62.93 ± 3.26	24.07 ± 1.03	0.639 ± 0.0423
T=0 / a2	4	250	25	34	25	36	28	30	31	28	26	26	28.90	3.81	10	94.20 ± 3.81	11.56 ± 1.21	0.307 ± 0.0355
T=0 / a3	6	500	27	19	15	17	21	17	16	20	15	22	18.90	3.75	10	188.40 ± 3.75	3.78 ± 1.19	0.100 ± 0.0319
T=0 / a4	8	2500	17	20	25	18	21	24	29	21			21.88	3.94	8	942.00 ± 3.94	0.88 ± 1.39	0.023 ± 0.0370
T=4 / ctl	0	125	55	42	52	58	56						52.60	6.31	5	52.60 ± 6.31	42.08 ± 2.82	1.000 ± 0.0948
T=4 / a1	2	125	61	61	73	53	65						62.60	7.27	5	52.60 ± 7.27	50.08 ± 3.25	1.190 ± 0.1110
T=4 / a2	4	125	53	58	49	58	62						56.00	5.05	5	52.60 ± 5.05	44.80 ± 2.26	1.065 ± 0.0893
T=4 / a3	6	125	33	29	30	35	40						33.40	4.39	5	52.60 ± 4.39	26.72 ± 1.96	0.635 ± 0.0632
T=4 / a4	8	125	46	49	46	43	60						48.80	6.61	5	52.60 ± 6.61	39.04 ± 2.96	0.928 ± 0.0938
T=0 / ctl	0	125	49	44	56								49.67	6.03	3	49.67 ± 6.03	39.73 ± 3.48	1.000 ± 0.1239
T=0 / a1	2	125	47	47	44	45	50						46.60	2.30	5	49.67 ± 2.30	37.28 ± 1.03	0.938 ± 0.0862
T=4 / a3	0	150	70	75	78	83	99						81.00	11.11	5	81.00 ± 11.11	54.00 ± 4.97	1.000 ± 0.1302
T=4 / a4	8	150	38	35	46	38	33						38.00	4.95	5	81.00 ± 4.95	25.33 ± 2.21	0.469 ± 0.0580

SURVIVAL ANALYSIS

HT Tumour G2

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	55	63	64	53	61	65	54	63	58	67	60.30	4.97	10	60.30 ± 4.97	48.24 ± 1.57	1.000 ± 0.0461
T=0 / a1	2	167	57	45	53	54	57	63	63	55			55.88	5.79	8	80.56 ± 5.79	33.46 ± 2.05	0.694 ± 0.0481
T=0 / a2	4	250	77	72	80	100							82.25	12.28	4	120.60 ± 12.28	32.90 ± 6.14	0.682 ± 0.1293
T=0 / a3	6	500	39	53	45	38	33	40	37	38	37		40.00	5.81	9	241.20 ± 5.81	8.00 ± 1.94	0.166 ± 0.0405
T=0 / a4	8	2500	15	22	25	26	25	42	38	27	27	27	27.40	7.62	10	1206.00 ± 7.62	1.10 ± 2.41	0.023 ± 0.0499
T=4 / ctl	0	125	59	71	58	61	51						60.00	7.21	5	60.00 ± 7.21	48.00 ± 3.22	1.000 ± 0.0950
T=4 / a1	8	2500	20	21	21	27	25						22.80	3.03	5	1200.00 ± 3.03	0.91 ± 1.36	0.019 ± 0.0283
T=0 / ctl	0	125	51	53	64	46	53						53.40	6.58	5	53.40 ± 6.58	42.72 ± 2.94	1.000 ± 0.0974
T=0 / a1	8	2500	43	50	48	49	54						48.80	3.96	5	1068.00 ± 3.96	1.95 ± 1.77	0.046 ± 0.0416
T=4 / a3	0	150	98	96	97	112	107						102.00	7.11	5	102.00 ± 7.11	68.00 ± 3.18	1.000 ± 0.0661
T=4 / a4	8	150	49	50	37	45	48						45.80	5.26	5	102.00 ± 5.26	30.53 ± 2.35	0.449 ± 0.0405

SURVIVAL ANALYSIS

HT Tumour G3

04.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	40	33	42	33	30	32	28	39			34.63	5.07	8	34.63 ± 13.28	27.70 ± 1.79	1.000 ± 0.0915
T=0 / a1	2	167	37	35	41	42	46	42	40	45	31	48	40.70	5.21	10	46.26 ± 15.89	24.37 ± 1.65	0.880 ± 0.0823
T=0 / a2	4	250	31	36	38	30	41	33	21	36	32	34	33.20	5.43	10	69.25 ± 3.86	13.28 ± 1.72	0.479 ± 0.0693
T=0 / a3	6	625	29	21	23	30	30	23	29	25	20	28	25.80	3.85	10	173.13 ± 4.00	4.13 ± 1.22	0.149 ± 0.0450
T=0 / a4	8	1250	28	26	24	30	29	24	29	33	25	22	27.00	3.37	10	346.25 ± 3.37	2.16 ± 1.06	0.078 ± 0.0388
T=4 / ctl	0	125	69	70	80	81	67	75	78	73	66		73.22	5.61	9	73.22 ± 5.61	58.58 ± 1.87	1.000 ± 0.0451
T=4 / a1	2	125	81	81	70	77	80	71	63	76	71	75	74.50	5.78	10	73.22 ± 5.78	59.60 ± 1.83	1.017 ± 0.0450
T=4 / a2	4	156	88	90	95	88	94	101	81	89			90.75	5.95	8	91.38 ± 5.95	58.17 ± 2.10	0.993 ± 0.0479
T=4 / a4	8	400	156	138	170	157	154	179	146	165	155		157.78	12.29	9	234.31 ± 12.29	39.44 ± 4.10	0.673 ± 0.0731
T=0 / ctl	0	133	86	89	84	72							82.75	7.46	4	82.75 ± 7.46	62.22 ± 3.73	1.000 ± 0.0847
T=0 / a1	8	1333	130	148	141	161							145.00	12.99	4	829.37 ± 12.99	10.88 ± 6.49	0.175 ± 0.1049

SURVIVAL ANALYSIS

HT Tumour G4

11.07.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	87	86	76	64	68	80	73	74	63		74.56	8.72	9	74.56 ± 8.72	59.64 ± 2.91	1.000 ± 0.0689
T=0 / a1	2	156	51	66	60	57	58	64	72	53	57	51	58.90	6.81	10	93.05 ± 6.81	37.76 ± 2.15	0.633 ± 0.0475
T=0 / a2	4	156	36	38	33	35	41	34	27	31	34		34.33	4.00	9	93.05 ± 4.00	22.01 ± 1.33	0.369 ± 0.0287
T=0 / a3	6	625	39	43	30	34	30	32	38	36	36		35.33	4.33	9	372.78 ± 4.33	5.65 ± 1.44	0.095 ± 0.0246
T=0 / a4	8	1250	21	19	16	11	12	13	18				15.71	3.82	7	745.56 ± 3.82	1.26 ± 1.44	0.021 ± 0.0242
T=4 / ctl	0	125	93	90	82	81	95						88.20	6.38	5	88.20 ± 6.38	70.56 ± 2.85	1.000 ± 0.0633
T=4 / a1	2	125	90	94	86	98	91						91.80	4.49	5	88.20 ± 4.49	73.44 ± 2.01	1.041 ± 0.0582
T=4 / a2	4	125	55	56	58	60	49						55.60	4.16	5	88.20 ± 4.16	44.48 ± 1.86	0.630 ± 0.0405
T=4 / a3	6	125	73	72	71	66	68						70.00	2.92	5	88.20 ± 2.92	56.00 ± 1.30	0.794 ± 0.0429
T=4 / a4	8	125	63	54	63	56	52						57.60	5.13	5	88.20 ± 5.13	46.08 ± 2.29	0.653 ± 0.0455

SURVIVAL ANALYSIS

HT Tumour G5

30.07.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	37	38	30	36	51	50	41	45	48	45	42.10	6.84	10	42.10 ± 6.84	33.68 ± 2.16	1.000 ± 0.0908
T=0 / a1	2	156	39	27	23	25	26	26	34	28	25	35	28.80	5.29	10	52.54 ± 5.29	18.46 ± 1.67	0.548 ± 0.0609
T=0 / a2	4	208	19	18	21	10	18	20	12	19	23	16	17.60	3.98	10	70.05 ± 3.98	8.46 ± 1.26	0.251 ± 0.0407
T=0 / a3	6	625	18	24	18	17	17	14	11	24	18	24	18.50	4.38	10	210.50 ± 4.38	2.96 ± 1.38	0.088 ± 0.0415
T=0 / a4	8	1250	10	11	11	11	8	12	14	16	13	8	11.40	2.50	10	421.00 ± 2.50	0.91 ± 0.79	0.027 ± 0.0236
T=4 / ctl	0	125	72	72	74	82	82						76.40	5.18	5	76.40 ± 5.18	61.12 ± 2.32	1.000 ± 0.0536
T=4 / a1	2	125											#DIV/0!	#DIV/0!	0	76.40 ± #DIV/0!	#DIV/0! ± #DIV/0!	#DIV/0! ± #DIV/0!
T=4 / a2	4	125											#DIV/0!	#DIV/0!	0	76.40 ± #DIV/0!	#DIV/0! ± #DIV/0!	#DIV/0! ± #DIV/0!
T=4 / a3	6	125	44										44.00	#DIV/0!	1	76.40 ± #DIV/0!	35.20 ± #DIV/0!	0.576 ± #DIV/0!
T=4 / a4	8	125	18	16	18	31	23						21.20	6.06	5	76.40 ± 6.06	16.96 ± 2.71	0.277 ± 0.0456

SURVIVAL ANALYSIS

HT Tumour G6

28.05.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1] [2] [3] [4] [5] [6] [7] [8] [9] [10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	83	2 4 2 0 1 2 7 4 4 4	3.00	2.00	10	3.00 ± 2.00	3.61 ± 0.63	1.000 ± 0.2475
T=0 / a1	2	138	3 4 4 4 4 2 6 9 8	4.89	2.32	9	4.99 ± 2.32	3.54 ± 0.77	0.980 ± 0.2739
T=0 / a2	4	208	4 8 9 2 7 8 4 4 7 8	6.10	2.38	10	7.52 ± 2.38	2.93 ± 0.75	0.811 ± 0.2519
T=0 / a3	8	416	1 1 1 3 1 1	1.33	0.82	6	15.04 ± 0.82	0.32 ± 0.33	0.089 ± 0.0935
T=0 / a4	12	1667	1 1 1	1.00	0.00	3	60.25 ± 0.00	0.06 ± 0.00	0.017 ± 0.0029
T=4 / ctl	0	125	62 57 60 52 51	56.40	4.83	5	56.40 ± 4.83	45.12 ± 2.16	1.000 ± 0.0677
T=4 / a1	2	138	77 65 62 65 67 80 71 73 75 76	71.10	6.06	10	62.27 ± 6.06	51.52 ± 1.92	1.142 ± 0.0692
T=4 / a2	4	166	81 80 71 62 60 55 665 68 62 71	127.50	189.05	10	74.90 ± 189.05	76.81 ± 59.78	1.702 ± 1.3274
T=4 / a3	8	250	86 91 96 96 95 78 76 83 98	88.78	8.32	9	112.80 ± 8.32	35.51 ± 2.77	0.787 ± 0.0721
T=4 / a4	12	500	7 5 7 4 4 8 7	6.00	1.63	7	225.60 ± 1.63	1.20 ± 0.62	0.027 ± 0.0137
T=0 / ctl	0	125	14 10 10 9 14	11.40	2.41	5	11.40 ± 2.41	9.12 ± 1 08	1.000 ± 0.1670
T=0 / a1	2	2500	20 18 20 22 20	20.00	1.41	5	228.00 ± 1.41	0.80 ± 0.63	0.088 ± 0.0701

SURVIVAL ANALYSIS

HT Tumour G7

30.07.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	(10)	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	56	53	70	61	75	53	58	63	56	62		60.70	7.21	10	60.70 ± 7.21	48.56 ± 2.28	1.000 ± 0.066
T=0 / a1	2	192	63	69	52	55	50	61	60	51	65	51		57.70	6.78	10	93.24 ± 6.78	30.05 ± 2.15	0.619 ± 0.053
T=0 / a2	4	313	40	43	35	39	39	40	40	44	58	72		45.00	11.30	10	151.99 ± 11.30	14.38 ± 3.57	0.296 ± 0.075
T=0 / a3	6	1250	22	20	28	24	26	31	24	30	30	37		27.20	5.03	10	607.00 ± 5.03	2.18 ± 1.59	0.045 ± 0.033
T=0 / a4	8	2500	16	15	16	12	11	15	12	13	14	15		13.90	1.79	10	1214.00 ± 1.79	0.56 ± 0.57	0.011 ± 0.012
T=4 / ctl	0	125	40	23	24	22	36							29.00	8.37	5	29.00 ± 8.37	23.20 ± 3.74	1.000 ± 0.228
T=4 / a1	2	125	31	28	29	29	30							29.40	1.14	5	29.00 ± 1.14	23.52 ± 0.51	1.014 ± 0.052
T=4 / a2	4	167	23	27	21	23	32							25.20	4.38	5	38.74 ± 4.38	15.09 ± 1.96	0.650 ± 0.090
T=4 / a3	6	250	19	24	18	22	21							20.80	2.39	5	58.00 ± 2.39	8.32 ± 1.07	0.359 ± 0.049
T=4 / a4	8	666	15	18	17	19	19							17.60	1.67	5	154.51 ± 1.67	2.64 ± 0.75	0.114 ± 0.033

SURVIVAL ANALYSIS

HT Tumour G8

17.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	83	55	73	74	72	71	67	75	66	71	70.70	7.23	10	70.70 ± 7.23	56.56 ± 2.29	1.000 ± 0.0571
T=0 / a1	2	192	89	73	68	63	72	79	89	77	76	69	75.50	8.51	10	108.60 ± 8.51	39.32 ± 2.69	0.695 ± 0.0553
T=0 / a2	4	313	73	62	55	54	53	58	57	54	66	69	60.10	7.06	10	177.03 ± 7.06	19.20 ± 2.23	0.339 ± 0.0418
T=0 / a3	6	1250	55	40	47	42	62	56	66	56	63	65	55.20	9.39	10	707.00 ± 9.39	4.42 ± 2.97	0.078 ± 0.0526
T=0 / a4	8	2500	18	22	24	19	20	24	26	23	25	20	22.10	2.73	10	1414.00 ± 2.73	0.88 ± 0.86	0.016 ± 0.0153
T=4 / ctl	0	125	81	87	70	75	63	83	81	75	74	81	77.00	7.04	10	77.00 ± 7.04	61.60 ± 2.23	1.000 ± 0.0511
T=4 / a1	2	125	68	63	64	56	69	60	62	72	80	66	66.00	6.75	10	77.00 ± 6.75	52.80 ± 2.13	0.857 ± 0.0490
T=4 / a2	4	167	68	70	76	84	89	79	69				76.43	8.06	7	102.87 ± 8.06	45.77 ± 3.05	0.743 ± 0.0578
T=4 / a3	6	250	99	94	100	94	116	104	105	106	105	87	101.00	8.10	10	154.00 ± 8.10	40.40 ± 2.56	0.656 ± 0.0493
T=4 / a4	8	666	161	178	143	167	174	175	170	167	176	180	169.10	10.86	10	410.26 ± 10.86	25.39 ± 3.43	0.412 ± 0.0582

SURVIVAL ANALYSIS

HT Tumour G9

07.08.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	56	52	56	44	47	51.00	5.39	5	51.00 ± 5.39	40.80 ± 2.41	1.000 ± 0.0835
T=0 / a3	6	625	90	99	97	92	100	95.60	4.39	5	255.00 ± 4.39	15.30 ± 1.96	0.375 ± 0.0530
T=0 / a4	8	1250	13	16	9	10	8	11.20	3.27	5	510.00 ± 3.27	0.90 ± 1.46	0.022 ± 0.0359
T=4 / ctl	0	125	70	59	62	54	61	61.20	5.81	5	61.20 ± 5.81	48.96 ± 2.60	1.000 ± 0.0750
T=4 / a4	8	125	9	3	8	6	7	6.60	2.30	5	61.20 ± 2.30	5.28 ± 1.03	0.108 ± 0.0218

SURVIVAL ANALYSIS

HT Tumour G10

07.08.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	77	65	77	69		72.00	6.00	4	72.00 ± 6.00	57.60 ± 3.00	1.000 ± 0.0737
T=0 / a3	6	625	61	70	55	70	59	63.00	6.75	5	360.00 ± 6.75	10.08 ± 3.02	0.175 ± 0.0532
T=0 / a4	8	1250	45	45	41	51	55	47.40	5.55	5	720.00 ± 5.55	3.79 ± 2.48	0.066 ± 0.0432
T=4 / ctl	0	125	66	65	59	65		63.75	3.20	4	63.75 ± 3.20	51.00 ± 1.60	1.000 ± 0.0444
T=4 / a3	6	125	58	61	55	65	51	58.00	5.39	5	63.75 ± 5.39	46.40 ± 2.41	0.910 ± 0.0552
T=4 / a4	8	125	31	49	46	43	62	46.20	11.17	5	63.75 ± 11.17	36.96 ± 4.99	0.725 ± 0.1005

SURVIVAL ANALYSIS

HT Tumour G11

28.08.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1] [2] [3] [4] [5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	35 28 28 27 31	29.80	3.27	5	29.80 ± 3.27	23.84 ± 1.46	1.000 ± 0.0868
T=0 / a4	8	2500	20 17 17 19 17	18.00	1.41	5	596.00 ± 1.41	0.72 ± 0.63	0.030 ± 0.0266
T=4 / ctl	0	125	47 41 40 48 41	43.40	3.78	5	43.40 ± 3.78	34.72 ± 1.69	1.000 ± 0.0861
T=4 / a4	8	125	35 35 31 37 35	34.60	2.19	5	43.40 ± 2.19	27.68 ± 0.98	0.797 ± 0.0632

SURVIVAL ANALYSIS

HT Tumour G12

27.08.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	19	16	19	25	18	19.40	3.36	5	19.40 ± 3.36	15.52 ± 1.50	1.000 ± 0.1370
T=0 / a4	8	2500	34	29	36	46	23	33.60	8.56	5	388.00 ± 8.56	1.34 ± 3.83	0.087 ± 0.2468
T=4 / ctl	0	125	35	38	38	35	40	37.20	2.17	5	37.20 ± 2.17	29.76 ± 0.97	1.000 ± 0.0461
T=4 / a4	8	125	22	23	21	23	18	21.40	2.07	5	37.20 ± 2.07	17.12 ± 0.93	0.575 ± 0.0364

SURVIVAL ANALYSIS

HT Tumour G13

11.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	125	55	40	48	49	30	24	24	21	22		34.78	13.33	9	34.78 ± 13.33	27.82 ± 3.55	1.000 ± 0.1807
T=0 / a1	2	156	38	61	50	63	60	37	45	53	52	49	50.80	9.04	10	43.40 ± 9.04	32.56 ± 1.83	1.170 ± 0.1634
T=0 / a2	4	208	36	40	42	46	45	35	40	40	42	49	41.50	4.33	10	57.87 ± 4.33	19.95 ± 0.66	0.717 ± 0.0946
T=0 / a3	6	312	52	33	33	31	37	32	35	30	31	26	34.00	6.98	10	86.81 ± 6.98	10.90 ± 0.71	0.392 ± 0.0561
T=0 / a4	8	625	32	30	29	30	34	31	26	22	32	35	30.10	3.81	10	173.89 ± 3.81	4.82 ± 0.19	0.173 ± 0.0232
T=4 / ctl	0	125	104	111	117	115	115	105	119	106	110		111.33	5.50	9	111.33 ± 5.50	89.07 ± 1.47	1.000 ± 0.0233
T=4 / a1	2	125	116	107	103	109	99	94	99	102	110	100	103.90	6.54	10	111.33 ± 6.54	83.12 ± 1.65	0.933 ± 0.0241
T=4 / a3	6	125	113	123	118	143	156	118	124	118	116		125.44	14.41	9	111.33 ± 14.41	100.36 ± 3.84	1.127 ± 0.0470
T=4 / a4	8	125	226	225	225	244	230	227	232	260	256	264	238.90	15.69	10	111.33 ± 15.69	191.12 ± 3.97	2.146 ± 0.0569

SURVIVAL ANALYSIS

HT Strontium @ T=0

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1] [2] [3] [4] [5] [6] [7] [8] [9] [10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	160	85 68 83 75 62 76 71 73	74.13	7.53	8	74.13 ± 7.53	46.33 ± 2.66	1.000 ± 0.0813
T=0 / a1	0.25	160	79 69 88 76	78.00	7.87	4	74.13 ± 7.87	48.75 ± 3.94	1.052 ± 0.1043
T=0 / a2	0.5	160	73 86 82 91	83.00	7.62	4	74.13 ± 7.62	51.88 ± 3.81	1.120 ± 0.1044
T=0 / a3	0.75	160	91 87 86 80	86.00	4.55	4	74.13 ± 4.55	53.75 ± 2.27	1.160 ± 0.0828
T=0 / a4	1	177	80 81 85 82	82.00	2.16	4	82.00 ± 2.16	46.33 ± 1.08	1.000 ± 0.0620
T=0 / a5	2	200	72 83 72 74	75.25	5.25	4	92.66 ± 5.25	37.63 ± 2.63	0.812 ± 0.0734
T=0 / a6	3	267	55 35 65 44	49.75	13.05	4	123.70 ± 13.05	18.63 ± 6.52	0.402 ± 0.1427
T=0 / a7	4	400	73 69 77 77	74.00	3.83	4	185.31 ± 3.83	18.50 ± 1.91	0.399 ± 0.0473
T=0 / a8	5	800	137 153 143 125	139.50	11.70	4	370.63 ± 11.70	17.44 ± 5.85	0.376 ± 0.1282
T=0 / a9	6	1600	104 112 98 123	109.25	10.81	4	298.13 ± 10.81	6.83 ± 5.41	0.366 ± 0.2902
T=0 / a10	7	3200	163 171 172 170	169.00	4.08	4	596.25 ± 4.08	5.28 ± 2.04	0.283 ± 0.1096
T=0 / a11	8	6400	318 329 336 355	334.50	15.55	4	1192.51 ± 15.55	5.23 ± 7.77	0.281 ± 0.4172

SURVIVAL ANALYSIS

HT Strontium @ T=4

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=4 / ctl	0	125	23	22	24	22	25	23.20	1.30	5	23.20 ± 1.30	18.56 ± 0.58	1.000 ± 0.0444
T=4 / a1	2	125	30	28	22	24	26	26.00	3.16	5	23.20 ± 3.16	20.80 ± 1.41	1.121 ± 0.0839
T=4 / a2	4	125	22	19	20	20	21	20.40	1.14	5	23.20 ± 1.14	16.32 ± 0.51	0.879 ± 0.0390
T=4 / a3	8	125	18	14	12	16	10	14.00	3.16	5	23.20 ± 3.16	11.20 ± 1.41	0.603 ± 0.0785
T=4 / ctl	0	125	25	28	30	22	25	26.00	3.08	5	26.00 ± 3.08	20.80 ± 1.38	1.000 0.0733
T=4 / a1	2	125	30	22	21	24	27	24.80	3.70	5	26.00 ± 3.70	19.84 ± 1.66	0.954 ± 0.0850
T=4 / a2	4	125	22	28	25	24	27	25.20	2.39	5	26.00 ± 2.39	20.16 ± 1.07	0.969 ± 0.0597
T=4 / a3	8	125	19	12	18	22	11	16.40	4.72	5	26.00 ± 4.72	13.12 ± 2.11	0.631 ± 0.1034
T=4 / ctl	0	500	91	91	101	99	88	94.00	5.66	5	94.00 ± 5.66	18.80 ± 2.53	1.000 0.1382
T=4 / a1	2	500	118	108	108	115	93	108.40	9.66	5	94.00 ± 9.66	21.68 ± 4.32	1.153 ± 0.2326
T=4 / a2	4	500	90	109	92	101	83	95.00	10.12	5	94.00 ± 10.12	19.00 ± 4.53	1.011 ± 0.2408
T=4 / a3	8	500	134	112	122	132	134	126.80	9.65	5	94.00 ± 9.65	25.36 ± 4.32	1.349 ± 0.2296

SURVIVAL ANALYSIS

HT Point Source

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	[1]	[2]	[3]	[4]	Number of Colonies Mean	Number of Colonies Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	266	80	82	86	88	84.00	3.65	4	84.00 ± 3.65	31.58 ± 1.83	1.000 ± 0.0818
T=0 / a1	1	311	35	37	41	35	37.00	2.83	4	98.21 ± 2.83	11.90 ± 1.41	0.377 ± 0.0498
T=0 / a2	3	280	47	50	44	47	47.00	2.45	4	88.42 ± 2.45	16.79 ± 1.22	0.532 ± 0.0495
T=0 / a3	5	272	61	66	68	65	65.00	2.94	4	85.89 ± 2.94	23.90 ± 1.47	0.757 ± 0.0639
T=4 / ctl	0	267	69	70	75	66	70.00	3.74	4	70.00 ± 3.74	26.22 ± 1.87	1.000 ± 0.1009
T=4 / a1	1	272	74	79	75	68	74.00	4.55	4	71.31 ± 4.55	27.21 ± 2.27	1.038 ± 0.1140
T=4 / a2	3	335	84	79	81	76	80.00	3.37	4	87.83 ± 3.37	23.88 ± 1.68	0.911 ± 0.0914
T=4 / a3	6	403	109	104	101	98	103.00	4.69	4	105.66 ± 4.69	25.56 ± 2.35	0.975 ± 0.1133

SURVIVAL ANALYSIS

Tumour A7 Feeder

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=4 / ctl	0	125	55	57	55	47	57						54.20	4.15	5	54.20 ± 4.15	43.36 ± 1.85	1.000 ± 0.0605
T=4 / HTF	8	125	47	33	37	43	43						40.60	5.55	5	54.20 ± 5.55	32.48 ± 2.48	0.749 ± 0.0656
T=4 / ctl	0	125	48	58	52	59	45						52.40	6.11	5	52.40 ± 6.11	41.92 ± 2.73	1.000 ± 0.0921
T=4 / A7F	8	125	34	39	40	38	40						38.20	2.49	5	52.40 ± 2.49	30.56 ± 1.11	0.729 ± 0.0544
T=4 / ctl	0	125	54	43	49	35	51						46.40	7.54	5	46.40 ± 7.54	37.12 ± 3.37	1.000 ± 0.1284
T=4 / NF	8	125	32	31	50	37	40						38.00	7.65	5	46.40 ± 7.65	30.40 ± 3.42	0.819 ± 0.1184

SURVIVAL ANALYSIS

Strontium T=0 and T=4

27.03.98

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	250	16	12	12	12	10	12.40	2.19	5	12.40 ± 2.19	4.96 ± 0.98	1.000 ± 0.2794
T=0 / a1	2	625	13	11	15	18	8	13.00	3.81	5	31.00 ± 3.81	2.08 ± 1.70	0.419 ± 0.3532
T=0 / a2	4	1250	8	12	7	9	5	8.20	2.59	5	62.00 ± 2.59	0.66 ± 1.16	0.132 ± 0.2348
T=0 / a3	8	5000	1	2	2	0	0	1.00	1.00	5	248.00 ± 1.00	0.02 ± 0.45	0.004 ± 0.0902
T=0 / a4	16	20000	0	0	0	0	0	0.00	0.00	5	992.00 ± 0.00	0.00 ± 0.00	0.000 ± #DIV/0!
T=4 / ctl	0	500	91	91	101	99	88	94.00	5.66	5	24.80 ± 5.66	18.80 ± 2.53	3.790 ± 0.9060
T=4 / a1	2	500	118	108	108	115	93	108.40	9.66	5	24.80 ± 9.66	21.68 ± 4.32	4.371 ± 1.2264
T=4 / a2	4	500	90	109	92	101	83	95.00	10.12	5	24.80 ± 10.12	19.00 ± 4.53	3.831 ± 1.1857
T=4 / a3	8	500	134	112	122	132	134	126.80	9.65	5	24.80 ± 9.65	25.36 ± 4.32	5.113 ± 1.3333

SURVIVAL ANALYSIS

Stontium Survival @ T=4

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=4 / ctl	0	500	91	91	101	99	88	94.00	5.66	5.00	94.00 ± 5.66	18.80 ± 2.53	1.00 ± 0.19
T=4 / a1	2	500	118	108	108	115	93	108.40	9.66	5.00	94.00 ± 9.66	21.68 ± 4.32	1.15 ± 0.28
T=4 / a2	4	500	90	109	92	101	83	95.00	10.12	5.00	94.00 ± 10.12	19.00 ± 4.53	1.01 ± 0.28
T=4 / a3	8	500	134	112	122	132	134	126.80	9.65	5.00	94.00 ± 9.65	25.36 ± 4.32	1.35 ± 0.29
T=4 / ctl	0	125	25	28	30	22	25	26.00	3.08	5.00	23.50 ± 3.08	20.80 ± 1.38	1.11 ± 0.17
T=4 / a5	2	125	30	22	21	24	27	24.80	3.70	5.00	23.50 ± 3.70	19.84 ± 1.66	1.06 ± 0.17
T=4 / a6	4	125	22	28	25	24	27	25.20	2.39	5.00	23.50 ± 2.39	20.16 ± 1.07	1.07 ± 0.16
T=4 / a7	8	125	19	12	18	22	11	16.40	4.72	5.00	23.50 ± 4.72	13.12 ± 2.11	0.70 ± 0.15
T=4 / ctl	0	125	23	22	24	22	25	23.20	1.30	5.00	23.50 ± 1.30	18.56 ± 0.58	0.99 ± 0.14
T=4 / a10	2	125	30	28	22	24	26	26.00	3.16	5.00	23.50 ± 3.16	20.80 ± 1.41	1.11 ± 0.17
T=4 / a11	4	125	22	19	20	20	21	20.40	1.14	5.00	23.50 ± 1.14	16.32 ± 0.51	0.87 ± 0.12
T=4 / a12	8	125	18	14	12	16	10	14.00	3.16	5.00	23.50 ± 3.16	11.20 ± 1.41	0.60 ± 0.11

SURVIVAL ANALYSIS

HT X-Ray Acute Repeat 1

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	200	130	139	120	124	114	125.40	9.58	5	125.40 ± 9.58	62.70 ± 4.28	1.000 ± 0.0966
T=0 / a1	0.25	222	139	150	130	108	132	131.80	15.43	5	139.19 ± 15.43	59.37 ± 6.90	0.947 ± 0.1277
T=0 / a2	0.5	225	125	141	145	146	127	136.80	10.06	5	141.08 ± 10.06	60.80 ± 4.50	0.970 ± 0.0977
T=0 / a3	0.75	235	120	129	108	129	126	122.40	8.85	5	147.35 ± 8.85	52.09 ± 3.96	0.831 ± 0.0849
T=0 / a4	1	250	129	128	140	134	149	136.00	8.69	5	156.75 ± 8.69	54.40 ± 3.89	0.868 ± 0.0858
T=0 / a5	2	444	167	176	146	174	172	167.00	12.21	5	278.39 ± 12.21	37.61 ± 5.46	0.600 ± 0.0962
T=0 / a7	8	666	177	158	158	158	167	163.60	8.44	5	417.58 ± 8.44	24.56 ± 3.78	0.392 ± 0.0659

SURVIVAL ANALYSIS

HT X-Ray Acute Repeat 2

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	150	98	91	80	132	100.25	22.43	4	100.25 ± 22.43	66.83 ± 11.21	1.000 ± 0.2373
T=0 / a1	0.25	168	83	95	88	110	94.00	11.75	4	112.08 ± 11.75	56.05 ± 5.87	0.839 ± 0.1659
T=0 / a2	0.5	178	106	97	107	102	103.00	4.55	4	118.90 ± 4.55	57.90 ± 2.27	0.866 ± 0.1493
T=0 / a3	0.75	188	102	101	120	120	110.75	10.69	4	125.31 ± 10.69	59.07 ± 5.34	0.884 ± 0.1685
T=0 / a4	1	213	104	99	117	106	106.50	7.59	4	142.49 ± 7.59	49.95 ± 3.80	0.747 ± 0.1377
T=0 / a5	2	355	126	140	110	106	120.50	15.61	4	237.12 ± 15.61	33.96 ± 7.80	0.508 ± 0.1446
T=0 / a6	3	426	140	130	110	125	126.25	12.50	4	284.64 ± 12.50	29.64 ± 6.25	0.444 ± 0.1195
T=0 / a7	4	530	159	179	146	146	157.50	15.59	4	354.35 ± 15.59	29.71 ± 7.79	0.444 ± 0.1384
T=0 / a8	5	800	124	121	117	116	119.50	3.70	4	534.93 ± 3.70	14.93 ± 1.85	0.223 ± 0.0466
T=0 / a9	6	1599	140	117	117	126	125.00	10.86	4	474.05 ± 10.86	7.82 ± 5.43	0.264 ± 0.1832
T=0 / a10	7	3202	139	134	138	139	137.50	2.38	4	949.08 ± 2.38	4.29 ± 1.19	0.145 ± 0.0402
T=0 / a11	8	3999	120	122	121	120	120.75	0.96	4	1185.52 ± 0.96	3.02 ± 0.48	0.102 ± 0.0161

SURVIVAL ANALYSIS

HT X-Ray Acute Repeat 3

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	200	129	111	95	116	127	113	139	130			120.00	13.95	8	120.00 ± 13.95	60.00 ± 4.93	1.00 ± 0.12
T=0 / a1	0.25	210	138	113	118	118							121.75	11.09	4	125.78 ± 11.09	58.08 ± 5.54	0.97 ± 0.12
T=0 / a2	0.5	222	124	120	133	129							126.50	5.69	4	133.09 ± 5.69	57.03 ± 2.84	0.95 ± 0.09
T=0 / a3	0.75	235	160	162	157	164							160.75	2.99	4	141.01 ± 2.99	68.40 ± 1.49	1.14 ± 0.10
T=0 / a4	1	267	174	169	145	167							163.75	12.84	4	160.05 ± 12.84	61.39 ± 6.42	1.02 ± 0.14
T=0 / a5	2	444	167	170	158	169							166.00	5.48	4	266.39 ± 5.48	37.39 ± 2.74	0.62 ± 0.07
T=0 / a6	3	530	154	169	178	159							165.00	10.68	4	317.86 ± 10.68	31.15 ± 5.34	0.52 ± 0.10
T=0 / a7	4	665	136	152	148	158							148.50	9.29	4	399.19 ± 9.29	22.32 ± 4.65	0.37 ± 0.08
T=0 / a8	5	1002	145	148	144	168							151.25	11.30	4	600.99 ± 11.30	15.10 ± 5.65	0.25 ± 0.10
T=0 / a9	6	1999	178	167	167	157							167.25	8.58	4	622.70 ± 8.58	8.37 ± 4.29	0.27 ± 0.14
T=0 / a10	7	3997	173	184	162	173							173.00	8.98	4	1244.80 ± 8.98	4.33 ± 4.49	0.14 ± 0.14
T=0 / a11	8	4997	104	98	91	87							95.00	7.53	4	1556.46 ± 7.53	1.90 ± 3.76	0.06 ± 0.12

SURVIVAL ANALYSIS

HT X-Ray Acute Repeat 4

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	267	170	146	148	165	148	155.40	11.22	5	155.40 ± 11.22	58.20 ± 5.02	1.00 ± 0.12
T=0 / a1	0.25	270	148	145	134	134	137	139.60	6.50	5	157.15 ± 6.50	51.70 ± 2.91	0.89 ± 0.09
T=0 / a2	0.5	300	141	141	156	165	168	154.20	12.83	5	174.61 ± 12.83	51.40 ± 5.74	0.88 ± 0.12
T=0 / a3	0.75	314	161	165	164	144	159	158.60	8.50	5	182.76 ± 8.50	50.51 ± 3.80	0.87 ± 0.10
T=0 / a4	1	406	176	193	201	171	209	190.00	16.19	5	236.30 ± 16.19	46.80 ± 7.24	0.80 ± 0.14
T=0 / a5	2	1026	273	273	274	263	280	272.60	6.11	5	597.16 ± 6.11	26.57 ± 2.73	0.46 ± 0.06
T=0 / a6	4	889	153	151	149	156	146	151.00	3.81	5	517.42 ± 3.81	16.99 ± 1.70	0.29 ± 0.04
T=0 / a7	8	1044	19	20	21	22	24	21.20	1.92	5	607.63 ± 1.92	2.03 ± 0.86	0.03 ± 0.02
T=0 / a8	16	34760	0	0	0	0	0	0.00	0.00	5	20231.10 ± 0.00	0.00 ± 0.00	0.00 ± #DIV/0!

SURVIVAL ANALYSIS

X-Ray Acute @ T=4

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=4 / ctl	0	500	141	156	154	185	160	159.20	16.08	5	149.60 ± 16.08	31.84 ± 7.19	1.06 ± 0.34
T=4 / a1	2	500	134	128	129	123	134	129.60	4.62	5	149.60 ± 4.62	25.92 ± 2.06	0.87 ± 0.21
T=4 / a2	4	500	140	117	123	125	123	125.60	8.59	5	149.60 ± 8.59	25.12 ± 3.84	0.84 ± 0.23
T=4 / a3	8	500	137	132	141	141	136	137.40	3.78	5	149.60 ± 3.78	27.48 ± 1.69	0.92 ± 0.22
T=4 / ctl	0	125	38	37	32	39	41	37.40	3.36	5	37.40 ± 3.36	29.92 ± 1.50	1.00 ± 0.23
T=4 / a5	2	125	38	30	35	39	40	36.40	4.04	5	37.40 ± 4.04	29.12 ± 1.81	0.97 ± 0.23
T=4 / a6	4	125	29	26	31	30	25	28.20	2.59	5	37.40 ± 2.59	22.56 ± 1.16	0.75 ± 0.17
T=4 / a7	8	125	18	25	21	22	20	21.20	2.59	5	37.40 ± 2.59	16.96 ± 1.16	0.57 ± 0.13
T=4 / ctl	0	125	41	42	46	37	32	39.60	5.32	5	39.60 ± 5.32	31.68 ± 2.38	1.00 ± 0.24
T=4 / a10	2	125	40	38	39	32	28	35.40	5.18	5	39.60 ± 5.18	28.32 ± 2.32	0.89 ± 0.21
T=4 / a11	4	125	33	37	22	25	29	29.20	6.02	5	39.60 ± 6.02	23.36 ± 2.69	0.74 ± 0.19
T=4 / a12	8	125	22	33	30	18	20	24.60	6.54	5	39.60 ± 6.54	19.68 ± 2.93	0.62 ± 0.17

SURVIVAL ANALYSIS

HT X-Ray Chronic Repeat 1

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	267	143	140	156	155	155						149.80	7.66	5	149.80 ± 7.66	56.10 ± 3.43	1.000 ± 0.0864
T=0 / a1	0.25	280	132	148	147	138	127						138.40	9.18	5	157.09 ± 9.18	49.43 ± 4.11	0.881 ± 0.0908
T=0 / a2	0.5	290	113	103	135	131	110						118.40	13.89	5	162.70 ± 13.89	40.83 ± 6.21	0.728 ± 0.1193
T=0 / a3	0.75	300	177	153	166	180	170						169.20	10.62	5	168.31 ± 10.62	56.40 ± 4.75	1.005 ± 0.1045
T=0 / a4	1	355	182	187	165	189	192						183.00	10.70	5	199.17 ± 10.70	51.55 ± 4.79	0.919 ± 0.1021
T=0 / a5	2	533	209	232	219	217	214						218.20	8.58	5	299.04 ± 8.58	40.94 ± 3.84	0.730 ± 0.0817
T=0 / ctl	0	267	132	142	140	159	141						142.80	9.88	5	142.80 ± 9.88	53.48 ± 4.42	1.000 ± 0.1028
T=0 / a1	4	761	183	186	164	171	198						180.40	13.28	5	407.01 ± 13.28	23.71 ± 5.94	0.443 ± 0.1143
T=0 / ctl	0	266	146	165	169	155	160						159.00	8.97	5	159.00 ± 8.97	59.77 ± 4.01	1.000 ± 0.0908
T=0 / a3	8	885	41	52	58	53	50						50.80	6.22	5	529.00 ± 6.22	5.74 ± 2.78	0.096 ± 0.0469

SURVIVAL ANALYSIS

HT X-Ray Chronic Repeat 2

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	133	57	60	52	60	93	91	102	84			74.88	19.61	8	74.88 ± 19.61	56.30 ± 6.93	1.000 ± 0.1742
T=0 / a1	0.25	133	75	83	76	83							79.25	4.35	4	74.88 ± 4.35	59.59 ± 2.17	1.058 ± 0.1360
T=0 / a2	0.5	133	74	81	93	75							80.75	8.73	4	74.88 ± 8.73	60.71 ± 4.37	1.078 ± 0.1538
T=0 / a3	0.75	133	94	92	83	76							86.25	8.34	4	74.88 ± 8.34	64.85 ± 4.17	1.152 ± 0.1601
T=0 / a4	1	133	76	66	61								67.67	7.64	3	74.88 ± 7.64	50.88 ± 4.41	0.904 ± 0.1361
T=0 / a5	2	177	63	69	73	69							68.50	4.12	4	99.65 ± 4.12	38.70 ± 2.06	0.687 ± 0.0923
T=0 / a6	3	205	60	61	58	66							61.25	3.40	4	115.41 ± 3.40	29.88 ± 1.70	0.531 ± 0.0720
T=0 / a7	4	266	61	54	51	45							52.75	6.65	4	149.75 ± 6.65	19.83 ± 3.33	0.352 ± 0.0733
T=0 / ctl	0	133	75	80	68	60	89	107	77	109			83.13	17.53	8	83.13 ± 17.53	62.50 ± 6.20	1.000 ± 0.1581
T=0 / a9	5	333	39	42	46	45							43.00	3.16	4	208.13 ± 3.16	12.91 ± 1.58	0.207 ± 0.0359
T=0 / a10	6	445	24	25	20	15							21.00	4.55	4	278.13 ± 4.55	4.72 ± 2.27	0.076 ± 0.0375
T=0 / a11	7	666	35	40	30	37							35.50	4.20	4	416.25 ± 4.20	5.33 ± 2.10	0.085 ± 0.0352
T=0 / a12	8	1333	44	56	41	51							48.00	6.78	4	833.13 ± 6.78	3.60 ± 3.39	0.058 ± 0.0547

SURVIVAL ANALYSIS

HT X-Ray Chronic Repeat 3

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	200	80	78	80	91	78	58	75	68			76.00	9.67	8	76.00 ± 9.67	38.00 ± 3.42	1.000 ± 0.1272
T=0 / a1	0.25	205	122	105	148	101							119.00	21.37	4	77.90 ± 21.37	58.05 ± 10.68	1.528 ± 0.3129
T=0 / a2	0.5	210	100	127	118	116							115.25	11.24	4	79.80 ± 11.24	54.88 ± 5.62	1.444 ± 0.1968
T=0 / a3	0.75	216	113	128	136	113							122.50	11.45	4	82.08 ± 11.45	56.71 ± 5.72	1.492 ± 0.2017
T=0 / a4	1	222	112	121	113	115							115.25	4.03	4	84.36 ± 4.03	51.91 ± 2.02	1.366 ± 0.1338
T=0 / a5	2	266	119	133	118	113							120.75	8.58	4	101.08 ± 8.58	45.39 ± 4.29	1.195 ± 0.1558
T=0 / a6	3	307	174	78	155	169							144.00	44.73	4	116.66 ± 44.73	46.91 ± 22.36	1.234 ± 0.5989
T=0 / a7	4	444	106	103	105	101							103.75	2.22	4	168.72 ± 2.22	23.37 ± 1.11	0.615 ± 0.0625
T=0 / ctl	0	200	111	116	96	108	91	103	100	122			105.88	10.38	8	105.88 ± 10.38	52.94 ± 3.67	1.000 ± 0.1136
T=0 / a9	5	533	74	66	65	68							68.25	4.03	4	282.16 ± 4.03	12.80 ± 2.02	0.242 ± 0.0439
T=0 / a10	6	640	102	95	94	102							98.25	4.35	4	338.80 ± 4.35	15.35 ± 2.17	0.290 ± 0.0487
T=0 / a11	7	800	114	103	131	132							120.00	14.02	4	423.50 ± 14.02	15.00 ± 7.01	0.283 ± 0.1349
T=0 / a12	8	1600	116	134	129	135							128.50	8.74	4	847.00 ± 8.74	8.03 ± 4.37	0.152 ± 0.0836

SURVIVAL ANALYSIS

HT X-Ray Chronic Repeat 4

12.06.97

Number & Control	Dose Gy	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / ctl	0	160	86	96	94	117							98.25	13.23	4	98.25 ± 13.23	61.41 ± 6.61	1.000 ± 0.1523
T=0 / a1	0.25	160	122	105	148	95							117.50	23.19	4	98.25 ± 23.19	73.44 ± 11.59	1.196 ± 0.2285
T=0 / a2	0.5	160	95	112	127	115							112.25	13.20	4	98.25 ± 13.20	70.16 ± 6.60	1.142 ± 0.1634
T=0 / a3	0.75	161	113	126	134	111							121.00	10.92	4	98.86 ± 10.92	75.16 ± 5.46	1.224 ± 0.1590
T=0 / a4	1	160	108	121	112	109							112.50	5.92	4	98.25 ± 5.92	70.31 ± 2.96	1.145 ± 0.1324
T=0 / a5	2	228	119	114	130	110							118.25	8.66	4	140.01 ± 8.66	51.86 ± 4.33	0.845 ± 0.1151
T=0 / a6	3	267	170	74	169	155							142.00	45.85	4	163.95 ± 45.85	53.18 ± 22.92	0.866 ± 0.3848
T=0 / a7	4	320	102	100	101	88							97.75	6.55	4	196.50 ± 6.55	30.55 ± 3.28	0.497 ± 0.0756
T=0 / a8	6	530	66	105	74	69							78.50	17.97	4	325.45 ± 17.97	14.81 ± 8.99	0.241 ± 0.1486
T=0 / a9	8	1600	92	80	93	80							86.25	7.23	4	982.50 ± 7.23	5.39 ± 3.61	0.088 ± 0.0596

SURVIVAL ANALYSIS

X-Ray Chronic @ T=4

12.06.97

Number & Control	Delayed Dose (Gy)	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=4 / ctl	0	125	50	51	54	55	56	53.20	2.59	5	53.20 ± 2.59	42.56 ± 1.16	1.000 ± 0.0385
T=4 / a1	2	125	48	51	52	47	59	51.40	4.72	5	53.20 ± 4.72	41.12 ± 2.11	0.966 ± 0.0561
T=4 / a2	4	125	44	42	45	50	51	46.40	3.91	5	53.20 ± 3.91	37.12 ± 1.75	0.872 ± 0.0475
T=4 / a3	8	125	33	35	36	38	39	36.20	2.39	5	53.20 ± 2.39	28.96 ± 1.07	0.680 ± 0.0312
T=4 / ctl	0	125	55	65	58	61	52	58.20	5.07	5	58.20 ± 5.07	46.56 ± 2.27	1.000 ± 0.0558
T=4 / a5	2	125	55	47	58	52	51	52.60	4.16	5	58.20 ± 4.16	42.08 ± 1.86	0.904 ± 0.0469
T=4 / a6	4	125	51	41	47	52	44	47.00	4.64	5	58.20 ± 4.64	37.60 ± 2.07	0.808 ± 0.0497
T=4 / a7	8	125	39	28	44	41	38	38.00	6.04	5	58.20 ± 6.04	30.40 ± 2.70	0.653 ± 0.0607
T=4 / ctl	0	125	44	51	50	52	54	50.20	3.77	5	50.20 ± 3.77	40.16 ± 1.69	1.000 ± 0.0500
T=4 / a10	2	125	51	41	52	55	46	49.00	5.52	5	50.20 ± 5.52	39.20 ± 2.47	0.976 ± 0.0670
T=4 / a11	4	125	44	41	42	39	38	40.80	2.39	5	50.20 ± 2.39	32.64 ± 1.07	0.813 ± 0.0346
T=4 / a12	8	125	33	32	35	38	38	35.20	2.77	5	50.20 ± 2.77	28.16 ± 1.24	0.701 ± 0.0363

SURVIVAL ANALYSIS

Iodine 131

11.08.97

Control & Code	Dose mCi	Number of Cells Seeded per plate	Numbers of Colonies					Number of Colonies		No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
			[1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.				
T=0 / 1	0.000	150	47	50	43	45	54	47.80	4.32	5	47.80 ± 4.32	31.87 ± 1.29	1.000 ± 0.0572
T=0 / 3	0.063	200	20	31	26	29	25	26.20	4.21	5	63.73 ± 4.21	13.10 ± 0.94	0.411 ± 0.0339
T=0 / 4	0.125	400	23	26	32	33	38	30.40	5.94	5	127.47 ± 5.94	7.60 ± 0.66	0.238 ± 0.0230
T=0 / 5	0.250	3340	4	4				4.00	0.00	2	1064.35 ± 0.00	0.12 ± 0.00	0.004 ± 0.0002
T=0 / 5+	0.375	1000											
T=0 / a1	0.000	300	82	98	101	105	92	95.60	8.96	5	95.60 ± 8.96	31.87 ± 1.34	1.000 ± 0.0593
T=0 / a2	0.050	500	103	109	93	96	104	101.00	6.44	5	159.33 ± 6.44	20.20 ± 0.58	0.634 ± 0.0321
T=0 / a3	0.100	500	15	18	20	16	11	16.00	3.39	5	159.33 ± 3.39	3.20 ± 0.30	0.100 ± 0.0104
T=0 / a4	0.200	4000	27	32	21	25	21	25.20	4.60	5	1274.67 ± 4.60	0.63 ± 0.05	0.020 ± 0.0018

SURVIVAL ANALYSIS

Iodine 131

11.08.97

Control & Code	Dose mCi	Number of Cells Seeded per plate	[1]	[2]	[3]	[4]	Number of Colonies Mean	Number of Colonies Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / 1	0.0000	300	143	179	161		161.00	18.00	3	161.00 ± 18.00	53.67 ± 3.46	1.000 ± 0.0913
T=0 / 2	0.0313	300	68	54	71		64.33	9.07	3	161.00 ± 9.07	21.44 ± 1.75	0.400 ± 0.0415
T=0 / 3	0.0625	300	41	42	40		41.00	1.00	3	161.00 ± 1.00	13.67 ± 0.19	0.255 ± 0.0168
T=0 / 4	0.1250	300	3	3	3		3.00	0.00	3	161.00 ± 0.00	1.00 ± 0.00	0.019 ± 0.0012
T=0 / 5	0.2500	600	0	0	0		0.00	0.00	3	322.00 ± 0.00	0.00 ± #DIV/0!	0.000 ± #DIV/0!
T=0 / 6	1.0000	8800	0	0	0		0.00	0.00	3	4722.67 ± 0.00	0.00 ± #DIV/0!	0.000 ± #DIV/0!
T=0 / 7	2.0000	8700	0	0	0		0.00	0.00	3	4669.00 ± 0.00	0.00 ± #DIV/0!	0.000 ± #DIV/0!
T=0 / 1	0.0000	255	52	55	60		55.67	4.04	3	55.67 ± 4.04	21.83 ± 0.92	1.000 ± 0.0770
T=0 / 3	0.0625	342	24	29	29		27.33	2.89	3	74.66 ± 2.89	7.99 ± 0.49	0.366 ± 0.0325
T=0 / 3+	0.0937	782	41	41	38		40.00	1.73	3	170.71 ± 1.73	5.12 ± 0.13	0.234 ± 0.0162
T=0 / 4	0.1250	593	73	60	61		64.67	7.23	3	129.45 ± 7.23	10.91 ± 0.70	0.500 ± 0.0456
T=0 / 5	0.2500	6106	16	18	22		18.67	3.06	3	1332.94 ± 3.06	0.31 ± 0.03	0.014 ± 0.0016
T=0 / 5+	0.3750	4413	1	1	0		0.67	0.58	3	963.36 ± 0.58	0.02 ± 0.01	0.001 ± 0.0003
T=0 / 7	0.0000	306	98	103	91	0	73.00	48.91	4	73.00 ± 48.91	23.86 ± 7.99	1.000 ± 0.3412
T=0 / 6	0.0125	304	80	75	88	85	82.00	5.72	4	72.52 ± 5.72	26.97 ± 0.94	1.131 ± 0.0829
T=0 / 5	0.0250	553	184	187	180	181	183.00	3.16	4	131.92 ± 3.16	33.09 ± 0.29	1.387 ± 0.0903
T=0 / 4	0.0500	553	147	150	150	0	111.75	74.51	4	131.92 ± 74.51	20.21 ± 6.74	0.847 ± 0.2877
T=0 / 3	0.1000	470	75	73	76	84	77.00	4.83	4	112.12 ± 4.83	16.38 ± 0.51	0.687 ± 0.0493
T=0 / 2	0.1500	1105	14	19	19	20	18.00	2.71	4	263.61 ± 2.71	1.63 ± 0.12	0.068 ± 0.0068
T=0 / 1	0.2000	5064	7	9	6	0	5.50	3.87	4	1208.08 ± 3.87	0.11 ± 0.04	0.005 ± 0.0016
T=0 / 7	0.0000	332	118	116	126		120.00	5.29	3	120.00 ± 5.29	36.14 ± 0.92	1.000 ± 0.0694
T=0 / 6	0.0125	282	112	117	108	103	110.00	5.94	4	101.93 ± 5.94	39.01 ± 1.05	1.079 ± 0.0755
T=0 / 5	0.0250	276	88	87			87.50	0.71	2	99.76 ± 0.71	31.70 ± 0.18	0.877 ± 0.0568
T=0 / 4	0.0500	560	132	154	136	155	144.25	11.95	4	202.41 ± 11.95	25.76 ± 1.07	0.713 ± 0.0547
T=0 / 3	0.1000	576	52	60	65	54	57.75	5.91	4	208.19 ± 5.91	10.03 ± 0.51	0.277 ± 0.0228
T=0 / 2	0.1500	1062	20	20	21	16	19.25	2.22	4	383.86 ± 2.22	1.81 ± 0.10	0.050 ± 0.0043
T=0 / 1	0.2000	5141	10	6	13	8	9.25	2.99	4	1858.19 ± 2.99	0.18 ± 0.03	0.005 ± 0.0009

SURVIVAL ANALYSIS

Iodine 131

11.08.97

Control & Code	Dose mCi	Number of Cells Seeded per plate	Numbers of Colonies Scored per plate [1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	Number of Colonies Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=0 / 1	0.0000	300	143	179	161								161.00	18.00	3	161.00 ± 18.00	53.67 ± 3.46	1.000 ± 0.0913
T=0 / 2	0.0313	300	68	54	71								64.33	9.07	3	161.00 ± 9.07	21.44 ± 1.75	0.400 ± 0.0415
T=0 / 3	0.0625	300	41	42	40								41.00	1.00	3	161.00 ± 1.00	13.67 ± 0.19	0.255 ± 0.0168
T=0 / 4	0.1250	300	3	3	3								3.00	0.00	3	161.00 ± 0.00	1.00 ± 0.00	0.019 ± 0.0012
T=0 / 5	0.2500	600	0	0	0								0.00	0.00	3	322.00 ± 0.00	0.00 ± #DIV/0!	0.000 ± #DIV/0!
T=0 / 6	0.1000	8800	0	0	0								0.00	0.00	3	4722.67 ± 0.00	0.00 ± #DIV/0!	0.000 ± #DIV/0!
T=0 / 7	0.2000	8700	0	0	0								0.00	0.00	3	4669.00 ± 0.00	0.00 ± #DIV/0!	0.000 ± #DIV/0!
T=0 / 1	0.0000	255	52	55	60								55.67	4.04	3	55.67 ± 4.04	21.83 ± 0.92	1.000 ± 0.0593
T=0 / 3	0.0625	342	24	29	29								27.33	2.89	3	74.66 ± 2.89	7.99 ± 0.49	0.366 ± 0.0475
T=0 / 3+	0.0937	782	41	41	38								40.00	1.73	3	170.71 ± 1.73	5.12 ± 0.13	0.234 ± 0.0423
T=0 / 4	0.0125	593	73	60	61								64.67	7.23	3	129.45 ± 7.23	10.91 ± 0.70	0.500 ± 0.0529
T=0 / 5	0.2500	6106	16	18	22								18.67	3.06	3	1332.94 ± 3.06	0.31 ± 0.03	0.014 ± 0.0419
T=0 / 5+	0.3750	4413	1	1									1.00	0.00	2	963.36 ± 0.00	0.02 ± 0.00	0.001 ± 0.0419
T=0 / 7	0.0000	306	98	103	91								97.33	6.03	3	97.33 ± 6.03	31.81 ± 1.14	1.000 ± 0.0506
T=0 / 6	0.1250	304	80	75	88	85							82.00	5.72	4	96.70 ± 5.72	26.97 ± 0.94	0.848 ± 0.0464
T=0 / 5	0.2500	553	184	187	180	181							183.00	3.16	4	175.90 ± 3.16	33.09 ± 0.29	1.040 ± 0.0369
T=0 / 4	0.5000	553	147	150	150								149.00	1.73	3	175.90 ± 1.73	26.94 ± 0.18	0.847 ± 0.0362
T=0 / 3	0.1000	470	75	73	76	84							77.00	4.83	4	149.50 ± 4.83	16.38 ± 0.51	0.515 ± 0.0392
T=0 / 2	0.1500	1105	14	19	19	20							18.00	2.71	4	351.48 ± 2.71	1.63 ± 0.12	0.051 ± 0.0360
T=0 / 1	0.2000	5064	7	9	6								7.33	1.53	3	1610.77 ± 1.53	0.14 ± 0.02	0.005 ± 0.0358
T=0 / 7	0.0000	332	118	116	126								120.00	5.29	3	120.00 ± 5.29	36.14 ± 0.92	1.000 ± 0.0360
T=0 / 6	0.1250	282	112	117	108	103							110.00	5.94	4	101.93 ± 5.94	39.01 ± 1.05	1.079 ± 0.0387
T=0 / 5	0.0250	276	88	87									87.50	0.71	2	99.76 ± 0.71	31.70 ± 0.18	0.877 ± 0.0259
T=0 / 4	0.0500	560	132	154	136	155							144.25	11.95	4	202.41 ± 11.95	25.76 ± 1.07	0.713 ± 0.0390
T=0 / 3	0.1000	576	52	60	65	54							57.75	5.91	4	208.19 ± 5.91	10.03 ± 0.51	0.277 ± 0.0291
T=0 / 2	0.1500	1062	20	20	21	16							19.25	2.22	4	383.86 ± 2.22	1.81 ± 0.10	0.050 ± 0.0256
T=0 / 1	0.2000	5141	10	6	13	8							9.25	2.99	4	1858.19 ± 2.99	0.18 ± 0.03	0.005 ± 0.0255
T=0 / 1	0.000	150	47	50	43	45	54						47.80	4.32	5	47.80 ± 4.32	31.87 ± 1.29	1.000 ± 0.0006
T=0 / 3	0.063	200	20	31	26	29	25						26.20	4.21	5	63.73 ± 4.21	13.10 ± 0.94	0.411 ± 0.0005
T=0 / 4	0.125	400	23	26	32	33	38						30.40	5.94	5	127.47 ± 5.94	7.60 ± 0.66	0.238 ± 0.0005
T=0 / 5	0.250	3340	4	4									4.00	0.00	2	1064.35 ± 0.00	0.12 ± 0.00	0.004 ± 0.0004
T=0 / a1	0.000	300	82	98	101	105	92						95.60	8.96	5	95.60 ± 8.96	31.87 ± 1.34	1.000 ± 0.0006
T=0 / a2	0.050	500	103	109	93	96	104						101.00	6.44	5	159.33 ± 6.44	20.20 ± 0.58	0.634 ± 0.0005
T=0 / a3	0.100	500	15	18	20	16	11						16.00	3.39	5	159.33 ± 3.39	3.20 ± 0.30	0.100 ± 0.0004
T=0 / a4	0.200	4000	27	32	21	25	21						25.20	4.60	5	1274.67 ± 4.60	0.63 ± 0.05	0.020 ± 0.0004

SURVIVAL ANALYSIS

Iodine 131 @ T=4/8 weeks

28.05.97

Number & Control	Dose mCi	Number of Cells Seeded per plate	Numbers of Colonies [1]	[2]	[3]	[4]	[5]	Mean	Std. Dev.	No. of Plates	Viable Fraction (Colonies /cell) (± Standard Deviation)	Plating Efficiency /% (± Standard Error)	Surviving Fraction (± Standard Error)
T=4 / ctl	0	125	18	11	11	14	14	13.60	2.88	5	13.60 ± 2.88	10.88 ± 1.29	1.000 ± 0.1675
T=4 / a1	0.05	125	17	20	18	22	20	19.40	1.95	5	13.60 ± 1.95	15.52 ± 0.87	1.426 ± 0.1870
T=4 / a2	0.1	125	29	22	29	28	30	27.60	3.21	5	13.60 ± 3.21	22.08 ± 1.44	2.029 ± 0.2741
T=4 / a3	0.2	125	40	28	32	31	28	31.80	4.92	5	13.60 ± 4.92	25.44 ± 2.20	2.338 ± 0.3429
T=4 / ctl	0	125	51	50	39	45	50	47.00	5.05	5	47.00 ± 5.05	37.60 ± 2.26	1.000 ± 0.0849
T=4 / a1	0.1	125	22	31	21	32	31	27.40	5.41	5	47.00 ± 5.41	21.92 ± 2.42	0.583 ± 0.0733
T=4 / a2	0.2	125	11	16	19	11	24	16.20	5.54	5	47.00 ± 5.54	12.96 ± 2.48	0.345 ± 0.0691
T=4 / a3	0.2*	125	36	32	30	25	42	33.00	6.40	5	47.00 ± 6.40	26.40 ± 2.86	0.702 ± 0.0871
T=8 / ctl	0	125	110	113	102	106	112	108.60	4.56	5	108.60 ± 4.56	86.88 ± 2.04	1.000 ± 0.0332
T=8 / a1	0.05	125	84	86	96	94		90.00	5.89	4	108.60 ± 5.89	72.00 ± 2.94	0.829 ± 0.0391
T=8 / a2	0.1	125	71	63	65	70		67.25	3.86	4	108.60 ± 3.86	53.80 ± 1.93	0.619 ± 0.0266
T=8 / a3	0.2	125	80	75	64	62	75	71.20	7.79	5	108.60 ± 7.79	56.96 ± 3.48	0.656 ± 0.0567
T=8 / ctl	0	125	53	52	63	51	60	55.80	5.36	5	55.80 ± 5.36	44.64 ± 2.40	1.000 ± 0.0759
T=8 / a1	0.05	125	52	47	41	40	48	45.60	5.03	5	55.80 ± 5.03	36.48 ± 2.25	0.817 ± 0.0668
T=8 / a2	0.1	125	28	28	40	33	32	32.20	4.92	5	55.80 ± 4.92	25.76 ± 2.20	0.577 ± 0.0582
T=8 / a3	0.2	125	37	37	34	41	53	40.40	7.47	5	55.80 ± 7.47	32.32 ± 3.34	0.724 ± 0.1058