NOTE TO USERS

Page(s) not included in the original manuscript and are unavailable from the author or university. The manuscript was scanned as received.

20

This reproduction is the best copy available.

UMI®

DISSERTATION

Transgenerational Radiation Genetics: The Use of the Japanese Medaka (*Oryzias latipes*) to Investigate Adaptive Response and Genomic Instability

Submitted by

Wendy Wells Kuhne, M.S.

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2006

COLORADO STATE UNIVERSITY

April 26, 2006

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY WENDY WELLS KUHNE ENTITLED "TRANSGENERATIONAL RADIATION GENETICS: THE USE OF THE JAPANESE MEDAKA (*ORYZIAS LATIPES*) TO INVESTIGATE ADAPTIVE RESPONSE AND GENOMIC INSTABILITY" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

(Thomas G. Hinton)

Adviser

Co-Adviser

Department Head

ABSTRACT OF DISSERTATION

TRANSGENERATIONAL RADIATION GENETICS: THE USE OF THE JAPANESE MEDAKA (*ORYZIAS LATIPES*) TO INVESTIGATE ADAPTIVE RESPONSE AND GENOMIC INSTABILITY

Over the past decade or so, two new areas of concern that may have an important bearing on the issue of genetic hazard assessment are the so-called adaptive response (AR), meaning a reduction in radiation sensitivity caused by previous low doses of radiation, and genomic instability (GI), meaning an increase in the rate of damage occurring several generations after the generation in which the damage was inflicted. The intent of this project was to initiate research to address both of these issues using an *in vivo* fish model, the Japanese Medaka (*Oryzias latipes*). The information generated would be useful to human risk assessment as well as risk assessment for non-human species by challenging the recommended daily dose limits for aquatic species of 1 cGy/day by the International Atomic Energy Agency and the U.S. Department of Energy.

To investigate GI an F0 generation was established and starting at 6-hours post-fertilization received either no irradiation or chronic gamma irradiation from Cesium-137 (12 cGy/day) for ~ 3 months. During this time the F0 generation underwent critical stages of development such as germ cell formation, and developed from embryo to juvenile and finally to adult stages. At 2 months of age, adult medaka were randomly paired for breeding. Endpoints such as survival at 60-days post-fertilization, wet weight

(mg), sex-ratios, fertility and fecundity were measured to determine the effect of the chronic low dose-rate irradiation.

Additionally, unexposed F1 embryos generated from these pairs were analyzed by a G2 Chromosome Assay to determine if a GI, caused by damage to parental germ cells and transmitted to subsequent F1 offspring, could be measured. This was done by analysis of cytogenetic damage in F1 offspring, from unirradiated or chronically irradiated F0 parents, receiving no irradiation or a 500 cGy acute challenge dose of gamma rays.

Chronic irradiation of F0 embryos from an early embryonic stage to adulthood at 60-days post-fertilization resulted in a 38% greater mortality than the unirradiated F0 fish (P=0.02). There was no indication of an effect on sex-ratios or wet-weight between males and females (P=0.08). Fecundity was marginally affected with the chronically irradiated pairs producing 16% fewer eggs (mean±STD) per female per day than unirradiated females, 16±7 and 19±9 respectively (P=0.05). A statistically significant difference was measured among the two groups (P=0.02) for the end point of fertilization.

Cytogenetic analysis of background chromatid-type aberration frequencies in F1 offspring from unirradiated and irradiated F0 parents indicated no statistical differences. The means and standard errors were 0.01±0.01 and 0.02±0.01 respectively (P=0.87). Analysis of F1 embryos receiving the 500 cGy challenge dose showed means and standard errors of 0.18±0.03 and 0.20±0.06 for F1 offspring from unirradiated and irradiated F0 parents. These means when compared to the respective background aberration frequencies were statistically significant indicating the presence of radiation

induced aberrations (P=0.003). However, the means when compared against each other were not statistically different (P=0.97). The lack of a measurable statistical difference in both the background and radiation induced aberration frequencies in offspring from unirradiated and irradiated parents indicates that a cytogenetic GI could not be demonstrated in this study.

To investigate the adaptive response, chromatid-type aberration frequencies produced from adapting doses of 2 or 21 cGy followed by a 500 cGy challenge dose of Cesium-137 gamma rays were compared with aberration frequencies produced by the 500 cGy challenge dose alone. The adapting doses were delivered *in vivo* and chronically over periods of 4 and 42 hours, respectively, at a dose-rate of 12 cGy/day The challenge dose was delivered *in vivo* and acutely at a dose-rate of 390 cGy/min and within 30 minutes of the termination of the adapting dose. To determine if a transgenerational effect was present, F1 offspring from unirradiated and chronically irradiated parents were utilized in the experiment.

Results from the G2 chromosome assay, as analyzed by a linear comparison of means, indicated that there were no statistically significant differences in the total aberration frequencies produced with either the 2 or 21 cGy adapting dose followed by the 500 cGy challenge dose versus the 500 cGy challenge dose alone, P=0.93 and P=0.83 respectively, for offspring originating from either unirradiated or irradiated F0 parents. A high degree of variability in the mitotic indices was observed in the medaka embryos which complicated the statistical analyses. More research is obviously needed to clearly understand the total adapting dose required, the timing required for the delivery of the

challenge dose after the adapting dose, and the appropriate stage, embryo, juvenile or adult, required to successfully demonstrate any *in vivo* AR in medaka fish.

Wendy Wells Kuhne
Department of Environmental and Radiological Health Sciences
Colorado State University
Fort Collins, Colorado 80523
Summer 2006

ACKNOWLEDGEMENTS

There are many people I need to thank for helping me complete this journey. Pursuing a Ph.D. is something that not very many people get the chance to do and I am truly humbled that Drs. Ward Whicker and Joel Bedford allowed me this great opportunity. It has been a true honor to be a student under both of these professors and to learn from their experiences in the field of Radioecology and Radiation Biology. I hope that they will always be proud of the person that I am and the scientist that I have become. I wish to thank the other members of my committee; Dr. Wil Clements, Dr. Tom Hinton and Dr. John Zimbrick. Their encouragement, support and tutelage during this journey have been invaluable.

A special thank you goes to Dr. John Pinder for his assistance with statistical analyses and to Dr. Jim Durham for his assistance with dosimetry. A special thank you goes to Anna Williams for her assistance with the chromosomal aberration scoring.

I would like to thank the faculty, staff and students of Radiological Health Sciences. During my stay in the Department I have met some truly great individuals. Having the opportunity to learn from professors such as Dr. Shawki Ibrahim, Dr. John Pinder, Dr. Tom Borak and Dr. Jim Durham has been tremendous. I can never repay the support and encouragement I have received from people like Julie Asmus, Chuck Sampier, Sandy Wiggin, Mary Pridgen, Wendy Seay, and Marion Dahlgren.

I definitely would like to thank all of the members of the facilities crew, Plumbing and HVAC, which helped to maintain the greenhouse where the medaka fish were housed. These men were called into service during the week, after hours and on

weekends to address and fix problems. Their hard work and attention to detail ensured that my medaka were happy and healthy. I appreciate all of their efforts and they were a pleasure to work with.

I would like to say thank you to all of my friends here in Fort Collins and around the United States who have supported me during this journey. Thanks for helping me to stay grounded, stay focused and reminding me to always enjoy each day.

And finally I have to say a huge thank you to my family. I think this journey has been harder on them than it has been for me. They have always been supportive, both emotionally and financially, and have never questioned that I would be successful in this quest. I am so very thankful for my mother, father and brother, Ramona, Kurt and Jason.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1 – The Overall Study: Scientific Rationale and the Japanese Medaka Model

Rationale and Objectives of the Overall Study

The U.S. Department of Energy's (DOE) Low Dose Radiation Research Program focuses funding efforts in part, on projects using whole organisms to explore the possibility of transgenerational effects arising from exposure of ionizing radiation. Two priority areas of research identified by the DOE were genomic instability and adaptive response. The adaptive response (AR) was reported in human lymphocytes by Olivieri et al. in 1984 (1) when they documented that fewer chromatid type aberrations occurred from a high dose exposure (*in vitro*) if cells were first "primed" with a low dose. The possibilities of inducing resistance by exposure to a low priming dose are relevant to guidelines for those working in hazardous high-level exposure conditions (2). Indeed, if low doses of ionizing radiation stimulate an adaptive response (perhaps by stimulating cellular repair mechanisms) then radiation risks from low doses could be lower than predicted by the linear, non-threshold model (3).

The other biological response of interest, genomic instability (GI), is defined as a phenomenon whereby radiation-induced effects become evident in the progeny of irradiated cells several generations after the generation in which the radiation damage was inflicted (4). This response is characterized by an increased rate of the effect, that remains elevated at the subsequent generations, as compared to rates measured in generations prior to irradiation or the first few generations immediately following the exposure to the radiation (5-6). The concept of a delayed response challenges basic paradigms under which the biological effects of ionizing radiation are conventionally interpreted, and increasing documentation of its occurrence exists in the literature for somatic cells *in vitro* (7-9). The biological significance of a GI in somatic cells raises

the issue of germ cells and the possibility of transgenerational effects. Could there be damage relating to normal genome maintenance function that occurs in parental germ cells that is passed to subsequent unirradiated offspring so a genome instability could be inherited? And if so, could the damage present in the offspring be linked to radiation induced carcinogenesis? Evidence for an *in vivo* transgenerational effect has appeared in the literature for animal models (10-15) however, there is some debate over whether GI is a universal phenomenon, and whether it could be initiated from ionizing radiation through low dose-rate environmental exposures.

In 1992 a grant was submitted to the DOE by Dr. Joel Bedford and myself to the Glue Grant Program. The Glue Grant program is designed to support post- doctoral or graduate-student research that will enable laboratories with complementary expertise to develop and apply innovative or collaborative approaches to low dose research. In our case this grant would be a collaborative effort between Dr. Bedford and myself to conduct low dose research that would span the fields of radiation biology and radioecology. In the grant we proposed to use the Japanese Medaka fish as an *in vivo* model to examine the research areas of GI and AR. While both research areas are of particular interest due to their implications of risks to humans, there is additional concern for the effect of chronic, low-level radiation on non-human species.

The International Atomic Energy Agency (IAEA) conducted an extensive literature review on the effects of radiation on plants and animals over a decade ago and determined that reproduction was a more sensitive endpoint than mortality (16). A general recommendation was made that the maximum daily dose to individuals be limited to 1 cGy/day for aquatic species. This guideline was accepted by the U.S. Department of

Energy (DOE) as a criterion in their technical standard for Radiation Protection of the Public and the Environment (17). However, there is limited information available in the literature to challenge this recommendation and to explore whether there could be the potential for introducing conditions such as GI and AR at this level of chronic irradiation.

Much of the information available on these two research areas focus on cells in culture or *in vivo* models receiving acute doses of radiation to either the male or the female and generally to the adult stages. This project would be novel in its approach at investigating these two areas by using an *in vivo* model where chronic irradiation would be delivered to embryo stages throughout adulthood to examine effects on the F0 and F1 generations. This type of approach would simulate an environmental exposure where both F0 parents would receive the chronic irradiation and this type of radiation schedule would allow for measurement of basic biological endpoints such as survival, sex-ratios, weight, fecundity and fertility as well as cytogenetic endpoints for measurement of possible damage done to parental germ cells that are passed to the F1 offspring. The following chapters will describe the objectives, materials and methods, results and discussion of work conducted under the guidelines of the grant.

In the Glue Grant we proposed to investigate GI by assessing the possible transmission of damage in germ cells of irradiated males to their unirradiated F1 offspring thru the presence of symmetrical translocations. In order to measure the symmetrical translocations it was necessary to prepare FISH whole chromosome-specific painting probes prepared by microdissection and PCR. A key to microdissection is to have a source of cells with a high mitotic index and a "normal" karyotype. A considerable amount of time and effort was given to exploring three sources of cells to

determine which would meet these conditions and be the best for use in microdissection. The three sources of cells were; 1) a pre-existing immortalized medaka fibroblast cell line; 2) whole medaka embryos; and 3) adult male testes.

Through some basic assays it was determined that the immortalized cell line, while having a high mitotic index did not display a normal karyotype. The presence of large scale rearrangements within the chromosomes rendered these cells not useful for microdissection. I was successful at obtaining normal chromosomes from whole embryos but found this process to be very time consuming and the mitotic index was often low and highly variable between individuals. The best source of chromosomes for microdissection was from the testes of adult male medaka, ~ 6 months to 1 year old.

Attempts at preparing the FISH whole chromosome-specific painting probes through microdissection and PCR showed that hybridization of the probe was possible, achieving ~80% coverage on chromosome #1, however full coverage of the chromosome was never achieved. The PCR product produced during the labeling or specific steps of the PCR protocol produced DNA products ~300 bp in length, and the desired product size was ~500-800 bp in length. Ultimately the signal produced during hybridization was often weak and did not achieve full coverage on the chromosome. I explored the possibility of changing concentrations of primers, and changing annealing and extension times and temperatures within the PCR protocol, but was unsuccessful at producing PCR products in the desired range. The protocol achieving the strongest signal and best results for the preparation of the whole chromosome-specific painting probes can be found in Appendix 1.

Because I was unable to prepare a successful whole chromosome-specific probe I made the decision to continue on with the project and to measure GI with an already established method by examining chromatid-type aberrations in the F1 offspring with classical Giemsa staining. In this project the frequency of background and radiation-induced chromatid-type aberrations occurring in F1 offspring originating from unirradiated F0 parents would be compared to F1 offspring originating from chronically irradiated F0 parents. The intent here was to determine whether or not there was some contribution of the chronically irradiated germ cells contributing to the presence of a GI, expressed as cytogenetic damage in unirradiated F1 offspring. Chapter two describes the work conducted to explore the GI in the F1 offspring and is written as a stand alone paper ready for publication.

Chapter three describes the work done to explore the possibility of a radiation induced AR. In this chapter the objectives were to determine whether or not a radiation induced AR could be measured through frequencies of chromatid-type aberrations occurring in F1 offspring receiving different adapting doses, either 2 or 21 cGy, prior to a large 500 cGy challenge dose of radiation. This project would be novel in its approach because it would deliver the adapting doses chronically and *in vivo* to whole embryos. Additionally, the presence of a transgenerational AR would be explored by measuring chromatid-type aberration frequencies in F1 offspring originating from chronically irradiated F0 parents. The intent here would be to explore whether or not there was some contribution of the chronically irradiated germ cells contributing to the presence of an AR in the F1 offspring. This chapter is written as a stand alone paper ready for publication.

Important Characteristics of the Medaka Fish

The Medaka, translated in Japanese as tiny fish with large eyes (18), is a small oviparous teleost that is historically found in both fresh and brackish water in Northeast Asia, primarily in Japan, Korea, and China. Commonly referred to as the killfish or ricefish, the medaka was first described as *Poecilia latipes* by Temminck and Schlegel in 1846 (19). However, after an extensive taxonomic review, the medaka has been re-classified and its' current systematics is Class *Osteichthyes*, Subclass *Actinopterygii* (ray-finned fishes), Order *Beloniformes*, Family *Adrianichthyidae*, and Genus *Oryzias*. There are fourteen species of the Genus *Oryzias* and the species used in this research is *latipes*, named by Jordan and Snyder in 1906 (20) to reflect their preferred habitat, the rice (*Oryza sativa*) field.

Adult medaka range in size from 2-4 cm in length and weigh approximately 300-600 mg fully grown. Adult male and female fish can be distinguished from one another by examining external morphology, specifically the dorsal and anal fins. In the adult male the rays of the dorsal and anal fins are longer and much thicker than in the female and possesses a saw-toothed distal edge. The adult male also possesses a distinct notch on the posterior margin of the dorsal fin, where as the adult female fins, both dorsal and anal, are smaller and more rounded and absent of the notch on the dorsal fin (21). The life cycle of the medaka is short, with sexual maturation being reached in 6-8 weeks after the egg is fertilized. Once sexually mature, females can produce eggs that are fertilized and develop externally on their abdomen. Brood sizes typically average 10–30 eggs per female per day. The chorions of the eggs are clear which allows observation of the developing embryo with a dissecting microscope. Medaka are hardy individuals that can

withstand temperature fluctuations between 4-40°C and a wide range of salinities. All of these attributes, in addition to a small genome size, have led the medaka to become a popular model for research (22-25).

Biologists have used this species for experimental purposes for over 80 years. The genetics, developmental biology, embryology, and specific developmental stages have been extensively characterized and researched (22). Medaka is a leading fish species being used to identify and predict human health effects following toxicant exposure. Medaka is used to study aspects of various diseases in which large numbers of experimental organisms are required, such as in low-dose risk assessment, as well as to examine factors that only slightly increase hazard exposure risk (26). The use of medaka in biomedical and environmental research, especially as a carcinogenesis model related to identifying and predicting human health effects from toxicant exposure, has received considerable attention (27-30). The sensitivity of medaka, as indicated by its low frequency of spontaneous formation of neoplasia, the availability of specimens, and the degree of control that can be maintained over extraneous life history factors, contribute to this being one of the most extensively used species in comparative toxicology (31), biology of hepatic neoplasia (31), oncogene activation (32), DNA repair (33) and mutagenesis (34-36).

One particular area in which the medaka provides an important model is in evaluating the effect of ionizing radiation on the potential for transmission of DNA damage or mutations to future generations through germ cells (34, 37-39). Germ cell development in the medaka has been reported to begin around Stage 26 (~2 days, 6 hours post-fertilization) when cleaved cells in the embryo differentiate and migrate to the

References

1. G. Olivieri, Y. Bodycote and S. Wolff, Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. *Science.* **223**, 594-597 (1984).

2. D. Billen, Spontaneous DNA Damage and its significance for the 'Negligible Dose' controversy in radiation protection. *Radiat. Res.* **124**, 242-245 (1990).

3. A. Wojcik and J. Shadley, The current status of the adaptive response to ionizing radiation in mammalian cells. *Hum. Ecol. Risk Assess.* **6**, 281-300 (2000).

4. K. Baverstock, Radiation-induced genomic instability: A paradigm-breaking phenomenon and its relevance to environmentally induced cancer. *Mutat. Res.* **454**, 89-109 (2000).

5. W.F. Morgan, J.P. Day, M.I. Kaplan, E.M. McGhee, and C.L. Limoli, Genomic instability induced by ionizing radiation. *Radiat. Res.* **146**, 247-258 (1996).

6. W.F. Morgan, Is there a common mechanism underlying genomic instability, bystander effects and other nontargeted effects of exposure to ionizing radiation? *Oncogene.* **22**, 7094-7099 (2003).

7. M.A. Kadhim, D.A. Macdonald, D.T. Goodhead, S.A. Lorimore, S.J. Marsden and E.G. Wright, Transmission of chromosomal instability after plutonium alpha-particle irradiation. *Nature.* **355**, 738-740 (1992).

8. S.A. Lorimore, M.A. Kadhim, D.A. Pocock, D.L. Papworth, D.T. Stevens, S.A. Goodhead and E.G. Wright, Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. *Proc. Natl. Acad. Sci.* **95**, 5730-5733 (1998).

9. J.B. Little, Induction of genetic instability by ionizing radiation. *CR Acad. Sci.* III **322**, 127-134 (1999).

10. L.M. Wiley, J.E. Baulch, O.G. Raabe, and T. Straume, Impaired cell proliferation in mice that persists across at least two generations after paternal irradiation. *Radiat. Res.* **148**, 145-151 (1997).

11. Y.E. Dubrova, M. Plumb, J. Brown, and A.J. Jeffreys, Radiation-induced germline instability at minisatellite loci. *Int. J. Radiat. Biol.* **74**, 689-696 (1998).

12. M.M. Vance, J.E. Baulch, O.G. Raabe, L.W. Wiley and J.W. Overstreet, Cellular reprogramming in the F_3 mouse with paternal F_0 radiation history. *Int. J. Radiat. Biol.* **78**, 513-526 (2002).

13. Y.E. Dubrova, M. Plumb, B. Gutierrez, E. Boulton, A.J. Jeffreys, Transgenerational mutation by radiation. *Nature.* **405**, 37 (2000).

14. K.P. Hoyes, B.I. Lord, C. McCann, J.H. Hendry and I.D. Morris, Transgenerational effects of preconception paternal contamination with ^{55}Fe. *Radiat. Res.* **156**, 488-494 (2001).

15. K.O. Niwa and R. Kominami, Untargeted mutation of the maternally derived mouse hypervariable minisatelline allele in F1 mice born to irradiated spermatozoa. *Proc. Natl. Acad. Sci.* **98**, 1705-1710 (2001).

16. IAEA, International Atomic Energy Agency. Effects of ionizing radiation on plants and animals at levels implied by current radiation protection standards. Technical Reports Series #332. IAE, Vienna. (1992).

17. DOE, Department of Energy. A graded approach for evaluating radiation doses to aquatic and terrestrial biota. DOE-STD-1153-2002. (2002).

18. J. Karakawa and T. Shibata, Local names (dialects) of the Medaka – 5000 Variations and their distribution (in Japenese). Miousha, Tokyo. May (2003).

19. C.J. Temminck and H. Schlegel, *Fauna Japonica.* Ed. Philipp Franz von Siebold. Pp. 224, Pl. 102, Fig. 5. (1846).

20. D.S. Jordan and J.O. Snyder, A review of the poecilidae or killfishes of Japan. *Proc. US Natl. Mus.* **31**, 287-290 (1906).

21. N. Egami, Secondary Sexual Characters. In: Medaka (Killifish): Biology and Strains. Ed. T. Yamamoto, Keigaku Publishing Company, Tokyo. Pp. 109-125. (1975).

22. T. Yamamoto, Medaka (Killifish): Biology and Strains. Keigaku Publishing Company, Tokyo. Pp. 365. (1975).

23. K. Naruse, M. Sakaizumi, and A. Shima, Medaka as a model organism for research in experimental biology. *Fish Biol. J. Medaka.* **6,** 47-52. (1994).

24. Y. Ishikawa, Medakafish as a model system for vertebrate developmental genetics. *BioEssays.* **22**, 487-495 (2000).

25. J. Wittbrodt, A. Shima and M. Schartl, Medaka – A Model organism from the far east. *Nature Reviews Genetics.* **3**, 53-64 (2002).

26. W.W. Walker, W.E. Hawkins, R.M. Overstreet and M.A. Friedman, A small fish model for assessing cancer risk at low carcinogen concentrations. *Toxicologist* **302** (1992).

27. J.D. Hendricks, Chemical carcinogenesis in fish. Aquatic Toxicology. Ed. L.J. Weber, Raven Press, New York. 149-211 (1982).

28. J.J. Black, Aquatic animal neoplasia as an indicator for carcinogenic hazards in man. IN: H. Saxen, Ed. Hazard Assessment of Chemicals: Current Developments, Vol. 3 Academic Press, New York. 181-232 (1984).

29. C.D. Metcalfe, Tests for predicting carcinogenicity in fish. *CRC Rev. Aquat. Sci.* **1**, 111-129 (1989).

30. W.E. Hawkins, W.W. Walker and R.M. Overstreet, Carcinogenicity tests using aquarium fish. Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Ed. G.M. Rand, Taylor and Francis, 421-446 (1995).

31. D.E. Hinton, J.A. Couch, S.J. Teh, and L.A. Courtney. Cytological changes during progression of neoplasia in selected fish species. *Aquat. Toxicol.* **11**, 77-112 (1988).

32. R.J. VanBeneden, K.W. Henderson, D.G. Blair, T.S. Papas and H.S. Gardner, Oncogenes in hematopoetic and hepatic fish neoplasms. *Cancer Res.* **50**, 5671s-5674s (1990).

33. T. Ishikawa, P. Masahito and S. Takayama, Usefulness of the Medaka, *Oryzias latipes* as a test animal: DNA repair process in Medaka exposed to carcinogens. *Natl. Cancer Inst. Monograph* **65**, 35-43 (1984).

34. A. Shimada and A Shima, Combination of genomic DNA fingerprinting into the medaka specific-locus test system for studying environmental germ-line mutagenesis. *Mutat. Res.* **399**, 149-165 (1998).

35. R.N. Winn, R.J. Van Beneden and J.G. Burkhart, Transfer, methylation and spontaneous mutation frequency of ΦX174am3cs70 sequences in Medaka (*Oryzias latipes*) and Mummichog (*Fundulus heteroclitus*): implications for gene transfer and environmental mutagenesis in aquatic species. *Mar. Environ. Res.* **40**, 247-265 (1995).

36. R.N. Winn, M.B. Norris, K.J. Bryaer, C. Torres and S.L. Muller, Detection of mutations in transgenic fish carrying a bacteriophage lambda *cII* transgene target. *Proc. Natl. Acad. Sci. USA*, **97(23)**, 12655-12660 (2000).

37. A. Shima and A. Shimada, Development of a possible nonmammalian test system for radiation-induced germ-cell mutagenesis using a fish, the Japanese medaka (Oryzias latipes). *Proc. Natl. Acad. Sci.* **88,** 2545-2549 (1991).

38. Y. Kubota, A. Shimada and A. Shima, DNA alterations detected in the progeny of paternally irradiated Japanese medaka fish (*Oryzias latipes*). *Proc Natl Acad Sci.* **92**, 330-334 (1995).

39. A. Shima and A. Shimada, The Medaka as a model for studying germ-cell mutagenesis and genomic instability. *Mar. Biotechnol.* **3**, S162-S167 (2001).

40. N. Satoh and N. Egami, Sex differentiation of germ cells in the teleost, Oryzias latipes, during normal embryonic development. *J. Embryol. Exp. Morphol.* **28**, 385-395 (1972).

41. N. Egami and K.I. Ijiru, Effects of irradiation on germ cells and embryonic development in teleosts. *Int. Rev. Cytol.* **59**, 195-248 (1979).

42. Y. Shimada and N. Egami, The unique responses of the primordial germ cells in the fish Oryzias latipes to gamma rays. *Int. J. Radiat. Biol.* **45**, 227-235 (1984).

43. Y. Shimada, A. Shima and N. Egami, Effects of heat, release of hypoxia, cadmium and arsenite on radiation sensitivity of primordial germ cells in the fish Oryzias latipes, *J. Radiat. Res.* **26**, 411-417 (1985).

Chapter 2 -Low Dose-Rate Effects of Chronic Gamma Irradiation on Japanese Medaka (*Oryzias latipes*)

Abstract

A general recommendation was made, by the IAEA, that the maximum daily dose to individuals be limited to 1 cGy/day for aquatic species. There is limited information available in the literature to challenge this recommendation and to explore whether there could be the potential for introducing conditions such as genomic instability at this level of chronic irradiation. The objectives of this project were to expand on the current knowledge base of genomic instability (GI) and to challenge the current recommendation of a maximum daily dose of 1 cGy for aquatic species by examining radiation effects on F0 medaka that have been chronically irradiated. Because of the lack of information in the literature on *chronic* radiation effects in medaka a maximum daily dose an order of magnitude higher than 1 cGy/day was delivered to establish a baseline for population level responses when chronic irradiation was delivered at an embryonic stage, prior to germ cell formation, and continued through sexual maturation and breeding.

To investigate GI an F0 generation was established and starting at 6-hours post-fertilization received either no irradiation or chronic gamma irradiation from Cesium-137 (12 cGy/day) for ~ 3 months. During this time the F0 generation underwent critical stages of development such as germ cell formation, and developed from embryo to juvenile and finally to adult stages. As 2 month old adults the medaka were paired up for breeding. Endpoints such as survival at 60-days post-fertilization, wet weight (mg), sex-ratios, fertility and fecundity were measured to determine the effect of the chronic low dose-rate irradiation. Additionally, F1 embryos generated from these pairs were analyzed by a G2 Chromosome Assay to determine if a GI, caused by damage to parental germ cells and transmitted to subsequent F1 offspring, could be measured. This was done by

analysis of cytogenetic damage in F1 offspring, from unirradiated or chronically irradiated F0 parents, receiving no irradiation or a 500 cGy acute challenge dose of gamma rays. Chronic irradiation of F0 embryos from an early embryonic stage to adulthood at 60-days post-fertilization resulted in a 38% greater mortality than the unirradiated F0 fish (P=0.02). There was no indication of an effect on sex-ratios or wet-weight between males and females (P=0.08). Fecundity was marginally affected with the chronically irradiated pairs producing 16% fewer eggs (16±7) per female per day than unirradiated females (19±9) (P=0.05). A statistically significant difference was measured among the two groups (P=0.02) for the end point of fertilization. Cytogenetic analysis of mean±SEM background chromatid-type aberration frequencies in F1 offspring from unirradiated and irradiated F0 parents indicated no statistical differences, 0.01±0.01 and 0.02±0.01 respectively (P=0.87). Analysis of F1 embryos receiving the 500 cGy challenge dose showed means±SEM of 0.18±0.03 and 0.20±0.06 for F1 offspring from unirradiated and irradiated F0 parents. These means when compared to the respective background aberration frequencies were statistically significant indicating the presence of radiation induced aberrations (P=0.003). However, the means when compared against each other were not statistically different (P=0.97). The lack of a measurable statistical difference in both the background and radiation induced aberration frequencies in offspring from unirradiated and irradiated parents indicates that a cytogenetic GI could not be demonstrated in this study.

Introduction

The International Atomic Energy Agency (IAEA) conducted an extensive literature review on the effects of radiation on plants and animals over a decade ago and determined that reproduction was a more sensitive endpoint than mortality (1). A general recommendation was made that the maximum daily dose to individuals be limited to 1 cGy/day for aquatic species. This guideline was accepted by the U.S. Department of Energy (DOE) as a criterion in their technical standard for Radiation Protection of the Public and the Environment (2). However, there is limited information available in the literature to challenge this recommendation and to explore whether there could be the potential for introducing conditions such as genomic instability at this level of chronic irradiation.

Genomic instability (GI), is defined as a phenomenon whereby radiation-related effects arise several generations after the generation in which the radiation damage was inflicted (3). It is generally characterized by an increased rate of damages, such as chromosomal aberrations, micronuclei formation, gene mutation and amplification, cellular transformation and delayed reproductive death, in the progeny of irradiated cells (4-5). The concept of a delayed response challenges some basic paradigms under which the biological effects of ionizing radiation are conventionally interpreted. Results from experiments studying this phenomenon have been met with great controversy. Documentation of its occurrence exists in the literature primarily through studies of *in vitro* exposure of cultured somatic cells (4, 6-9). However, there are other reports, indicating that GI is not a universal phenomenon and may occur in cells that are not normal to begin with (10-13).

Evidence of heritable effects from *in vivo* parental exposure to ionizing radiation have appeared in the literature, and these studies report increased germ line mutations in subsequent unirradiated offspring (14-19). Dubrova et al. (17) speculates that, "*these types of findings have potential implications for risk evaluation in humans. The persistence of instability into the germ line of unexposed offspring of irradiated mice raises the issue of delayed genetic risk.*" While there is evidence of increased germ line mutations occurring in children born in heavily contaminated areas after the Chernobyl accident (15), there is no evidence that it occurs in children of atomic bomb survivors (20) or Chernobyl cleanup workers (21-23) or in children from patients receiving chemotherapy or radiotherapy treatments for cancer (24-26). Attempts are being made to sort out the discrepancies between the human and animal data (27) and to determine the consequences of a GI in the germline (28-30). These data not only have importance for understanding human risks but also for the development of protection criteria for non-human species. Such concerns continue to grow, in part, because of the need for developing credible cleanup criteria for contaminated sites, disposal of nuclear materials, and the increased threat of radioactive releases by terrorist activities or accidents (31).

One vertebrate model that has been shown to be useful for addressing human risk and environmental exposures is the Japanese Medaka fish. The medaka is a small oviparous teleost that is historically found in both fresh and brackish water in Northeast Asia, primarily in Japan, Korea, and China. The medaka has been extensively studied since the early 1920's, yielding a tremendous amount of information on their basic biology, genetics and developmental embryology (32-33). Adult medaka range in size from 2-4 cm in length and weigh approximately 300-600 mg when fully grown. The life

cycle of the medaka is short, with sexual maturation being reached in only 6-8 weeks after the egg is fertilized. Once sexually mature, females can produce eggs that are fertilized and develop externally on their abdomen. Brood sizes typically average 10 –30 eggs per female per day and the clear chorion of these eggs allows for observation of the embryo during critical developmental stages.

The medaka provides an important model for evaluating the effect of ionizing radiation on the potential for transmission of DNA damage or mutations to future generations through germ cells (34-38). Germ cell development in the medaka has been reported to begin around Stage 26 (~2 days, 6 hours post-fertilization) when cleaved cells in the embryo differentiate and migrate to the gonadal area. Germ cells remain mitotically dormant for a period of time (Stages 26-31), while the numbers of cells increase due to continuous movement of cells into the gonad. Sex differentiation of germ cells occurs after hatching. The cells cease to divide in male fry, while they remain active in female fry (39). This unique cycle leads to a variation in radiosensitivity of germ cells during the development of the embryo and has triggered a tremendous amount of work to evaluate the effects of ionizing radiation on germ cells during the developmental stages (40-41) and to evaluate its effects when administered in combination with varying temperatures, hypoxic conditions and chemical exposures (42).

Previous work with the medaka has shown a transgenerational genomic instability occurring in F1 offspring originating from acute paternal irradiation (34-38, 43). In these studies, only one parent was irradiated to determine sex-specific effects and the mutation frequencies reported in these experiments showed frequencies similar to what was

reported by Russell and Kelley (44) in their work with mice, thus suggesting the medaka as a useful model for developing human risk factors.

The objectives of this project were to expand on the current knowledge base and to challenge the current recommendation of a maximum daily dose of 1 cGy for aquatic species by examining radiation effects on F0 medaka that have been chronically irradiated. Because of the lack of information in the literature on *chronic* radiation effects in medaka I decided to deliver a maximum daily dose an order of magnitude higher than 1 cGy/day to establish a baseline for population level responses when chronic irradiation was delivered at an embryonic stage, prior to germ cell formation, and continued through sexual maturation and breeding. This type of radiation schedule would allow for the endpoints of survival, adult weight, sex-ratios, fertility and fecundity to be measured. Additionally, genomic instability in F1 offspring would be evaluated by analysis of chromatid-type aberrations occurring in subsequent unirradiated F1 offspring and offspring receiving an acute challenge dose.

Materials and Methods

Model Organism Japanese Medaka (CAB Strain)

The animal model used in this experiment was the Japanese Medaka fish, referred to as the CAB strain. It is an inbred strain of *Oryzias latipes* from the Southern Population of Japan. It has an orange-red phenotype and is the same species that can be commercially obtained from Carolina Biological Supply, North Carolina, and thus is referred to as the "CAB" strain.

Animal Care Use and Guidelines

Animal care procedures followed in this study are in accordance with Colorado State University Animal Care and Use Committee guidelines (Protocol Nos. 01-189A-01 and 03-181A-02).

Establishment and Care of F0 Generation; From Egg to Adult Stage

Eggs, representing the F0 generation, were collected gently by hand from the abdomen of 6-month old adult females which had bred with 6-month old adult males. Eggs were collected each morning for 5 days. The eggs were inspected under a dissecting microscope 6-h after being collected to determine if fertilization was successful. Fertilized eggs were distinguished from unfertilized eggs by the presence of the blastula cap of cells seen at Stage 10 as described by Iwamatsu (45). Fertilized eggs were randomly selected for either the unirradiated or irradiated treatment and placed into 250 ml glass jars containing 125 ml of the parental tank water and 125 ml of aerated tap water. The eggs were maintained at a density of 60 eggs per jar and at 22±2°C under 12 h light:12 h dark light cycles for 10-15 days until hatch out.

One week after hatchout, medaka were moved to 2.5-L glass bowls at a density of 6 fish per bowl. These fish were maintained at 22±2°C under 12 h light:12 h dark light cycles and fed 2X daily Aquatox flake food (Aquatic Ecosystems, Inc., Apopka, Florida) and 1X daily live *Artemia napulii* (Great Salt Lake Brine Shrimp, Parker International, Inc., Salt Lake City, UT).

Individual males and females, approximately 2-months old, were randomly selected and paired up for breeding. Pairs were maintained in 2.5 L glass bowls at 26-28°C and under a light cycle of 16 h light: 8 h dark. Adult medaka were fed 3X daily a diet of Aquatox flake food (Aquatic Ecosystems, Inc., Apopka, Florida) and 1X daily live

Artemia napulii (Great Salt Lake Brine Shrimp, Parker International, Inc., Salt Lake City, UT). Individual females began producing eggs ~5-10 days after the initiation of breeding conditions.

Water Quality Control and Assurance

Water renewal for this experiment was conducted as a static renewal with water changes occurring every 2-3 days. Water quality parameters of pH and total dissolved solids (mg/L) were monitored during the course of this experiment using a hand held meter from Thermo Orion Corporation, Beverly, MA (Meter 635, Probe 617500). Temperature (°C) and dissolved oxygen (%) were measured using a hand held dissolved oxygen meter from Thermo Orion Corporation, Beverly, MA (Meter 835A, Probe 083010A).

Chronic Irradiation of F0 Generation

Chronic gamma irradiation, administered using a 370 Ci Cesium-137 source (J.L. Shepherd and Associates), was delivered to F0 medaka eggs starting at 6-h post-fertilization (Stage 10) as described in Iwamatsu (45). Dosimetry was conducted using a calibrated Rad/Cal Ion Chamber Survey Meter (Model 2025, SN 3843) and Probe (SN 7357) in combination with calibrated Lithium Fluoride thermoluminescent dosimeters (TLD 100s). TLDs were read using a Harshaw QS 3500, Serial No. 9305053 (Bicron, OH) TLD reader. The dose was delivered continuously at an average dose-rate of 12 cGy/day except for when the source was shielded for feeding and maintenance of the tanks. The average daily dose to the fish was ~ 10±1 cGy/day.

F0 Generation Survival, Sex Ratios, Wet Weight, Fertility and Fecundity Under Unirradiated and Chronic Irradiation Conditions

Medaka, ~2-months of age, were visually inspected to determine survival at 60-days post-fertilization and to determine sex ratios of males to females produced under unirradiated and chronic irradiation conditions. Gender was determined by inspection of the anal and dorsal fins as described by Egami (46). Wet weights of adult males and females were taken at 6-months of age by placing the adult fish in 250 ml beakers containing a known amount and weight of water (Mettler Toldeo, PB602-SRR, 118362467). Fertility and fecundity of the F0 medaka was assessed by the collection and observation of F1 embryos collected from F0 breeding pairs. Collections were made at 14 days after the initiation of breeding conditions to ensure that females were producing the maximum number of eggs per day. Fecundity and breeding success of individual pairs were measured by counting the number of eggs produced per female per day and the number of eggs fertilized. Eggs were observed using a dissecting microscope and fertilization was determined to be successful if at 6-h post-fertilization (Stage 10) there was the presence of the blastula cap of cells as described by Iwamatsu (45).

Chromatid-Type Aberrations in F1 Offspring

Metaphase cells were collected from whole embryos to determine the presence of genomic instability using the G2 chromosomal assay. Whole embryos, ~48-hours post-fertilization (Stage 25), were rinsed in a 0.5% bleach solution and 2X in Phosphate Buffer Solution (Dulbecco's PBS) to remove debris from the outer surface of the egg. The embryos were placed in 1X Ringers solution and the chorion and yolk removed by poking a hole in the chorion and allowing the yolk and embryo to be manually removed.

The embryos were placed in 1 ml of PBS and the cells dissociated by liberal pipeting. The cells were centrifuged at 1000 RPM for 10 minutes. The supernatant was removed and 1 ml of 0.05% trypsin and 0.02% EDTA-Na in PBS was added for 5 minutes at 33°C. The trypsinization process was stopped by adding 4 ml of fresh L-15 media (Leibovitz's L-15 (Sigma, St. Louis, MO) cell media supplemented with 20% fetal bovine serum, streptomycin (50 $\mu g\ ml^{-1}$) and penicillin (50 U ml^{-1}). Mitotic cells were collected by adding colcemid (0.1 $\mu g\ ml^{-1}$) (GIBCO, Grand Island, NY) to the cell suspension for 3 hours. The cells were centrifuged at 1000 RPM for 10 minutes. The supernatant was removed and the cells resuspended in 10 ml of hypotonic solution (75 mM KCl) for 20 minutes. To fix the cells, fresh methanol:acetic acid (3:1 vol/vol) was added drop-wise (~1 ml) to the hypotonic solution and allowed to remain at room temperature for 5 minutes. The cell suspension was centrifuged at 1000 RPM for 10 minutes. The cells were fixed an additional 2X with methanol:acetic acid (3:1 vol/vol) for 5 minutes and centrifuged at 1000 RPM for 4 minutes. The fixed cells were dropped onto cold wet slides and allowed to air dry. Once dry, the slides were stained in freshly prepared 10% Giemsa in pH 6.8 buffer for 10 minutes.

Acute Challenge Dose to F1 Embryos

A 500 cGy acute challenge dose was delivered *in vivo* to Stage 25 whole embryos using a 3000 Ci Cesium-137 gamma source (J.L. Shepherd and Associates, Mark I 68-A (SS-056) to measure radiation induced chromatid-type aberrations. Dosimetry was conducted using calibrated TLD 100s. TLDs were read using a Harshaw QS 3500, Serial No. 9305053 (Bicron, OH) TLD reader and the dose-rate was determined to be 390 cGy/min.

Aberration Scoring

Slides were coded by a second party and scored on a Zeiss Axioskop 2 Plus (Carl Zeiss Microimaging, Thornwood, NY, 10594) with 100X objectives and a 2X optivar. Images were taken using a Nikon Digital Eclipse Camera (DXM 1200) and edited using the Automatic Camera Tamer or ACT 1 Software program (Version 2.20). The aberration scoring criteria suggested by Savage was used for determining chromatid-type aberrations (47).

Statistical Analysis

Analysis of data for survival, wet weight, fecundity and fertility was performed by use of a one-way Analysis of Variance test (ANOVA). Genomic instability was assessed by measuring mean chromatid-type aberration frequencies in F1 offspring from unirradiated and chronically irradiated F0 parents using a G2 chromosome assay. F1 offspring used in the G2 assay received either no irradiation to determine mean background aberration frequencies or received a 500 cGy challenge dose of gamma radiation to determine mean radiation-induced aberration frequencies. The F1 embryos collected for the G2 chromosome assay on average had a low and highly variable mitotic index. The statistical analysis of the data took into account this high degree of variability by only including embryos having a minimum of 5 scoreable cells, making the experimental unit the embryo and not the breeding pair. Because evaluating the effects of different levels of the interactions of F0 and F1 irradiations are central to testing the objectives of the study, each specific objective was tested using a linear comparison of means using an F-test for analysis of variance of the means (48) (Appendix 2). All

analyses were conducted using Statistical Analysis Software (SAS, Version 9.1, Raleigh, NC) (49).

Results

F0 Generation

Fish chronically irradiated with an average of 10 cGy/day starting at 6-h post-fertilization until 60-days post-fertilization showed a statistically significant difference (P=0.02) in survival rates versus unirradiated fish of the same age. The unirradiated fish displayed a mean survival rate of 48% while chronically irradiated fish displayed a mean survival rate of 28% (Table 2-1). One problem encountered during the 60-day exposure centered on the variation in the water quality in terms of dissolved oxygen and pH. The maintenance of a high level of dissolved oxygen is an important factor to overall fish health. Analysis of water quality indicated a statistically significant difference in pH and dissolved oxygen (%) levels in the unirradiated versus irradiated medaka groups ($P < 0.05$ respectively) (Table 2-2). Typically for life cycle assays it is recommended that dissolved oxygen (%) levels remain at 60% for flow through systems (50); however, there is no recommended level for static renewal experiments. During the course of this static renewal experiment the average for the unirradiated and irradiated medaka was ~40% and 30% respectively. The chronic irradiation could have contributed to the lower pH and dissolved oxygen levels through the process of radiolysis of the water molecules. The lower dissolved oxygen levels may have contributed to a physiological stress that was added to the chronic irradiation insult. Visual inspection of the surviving individuals showed no significant differences in sex-ratios or mean wet weights compared between males and females among the groups (P=0.08) (Table 2-3).

Statistically significant differences in fertility and fecundity were measured. Unirradiated F0 females demonstrated 100% fecundity where all females were successful in producing eggs, whereas chronically irradiated F0 females demonstrated a decrease in fecundity with only a 94% success rate for the production of eggs. Comparison of the average number of eggs produced per female per day between unirradiated and irradiated pairs indicated a marginally statistically significant difference, with the unirradiated females averaging 19±9 eggs per day versus 16±7 eggs per day in the chronically irradiated F0 fish (P=0.05) (Table 2-3). A statistically significant difference (P=0.02) was measured among the two groups for the end point of fertilization.

F1 Embryos Chromatid-Type Aberrations (Genomic Instability)

The results of the chromatid-type aberration analysis indicated F1 embryos from unirradiated F0 parents displayed a total background aberration frequency of 0.01±0.01 and F1 embryos from chronically irradiated F0 parents displayed a total background aberration frequency of 0.02±0.01 (Table 2-4) (Figure 2-1). Total aberration frequencies were not statistically different from one another (P=0.87). Analysis of specific types and combinations of aberrations such as gaps, breaks, total exchanges, gaps+breaks, total exchanges-(gaps+breaks), and total aberrations indicated no statistically significant differences (Table 2-5).

The total radiation induced aberration frequencies from the 500 cGy challenge dose, measured in F1 offspring from unirradiated F0 parents, was 0.18±0.03 compared with 0.20±0.06 for F1 offspring from chronically irradiated F0 parents (Table 2-6) (Figure 2-1). These aberration frequencies are not statistically different from one another (P=0.97). Analysis of specific types and combinations of aberrations such as gaps,

breaks, total exchanges, gaps+breaks, total exchanges-(gaps+breaks), and total aberrations again indicated no statistically significant differences (Table 2-7). However, the aberration frequencies produced by the 500 cGy challenge dose, when compared with background aberration frequencies, were found be statistically significant (P=0.003) indicating a measurable radiation-induced aberration frequency (Table 2-8). The lack of a measurable statistical difference in both the background aberration frequencies and the radiation induced frequencies in F1 offspring from unirradiated and irradiated F0 parents indicates that a cytogenetic GI could not be demonstrated.

Discussion

The Japanese Medaka fish has proven to be a useful vertebrate model for assessing risk from damage inflicted to germ cells and the possible transmission of genomic instability to future generations from exposure to ionizing radiation. While previous work has focused on effects when exposures are given acutely and to only one parent, primarily males, this project was designed to mimic an environmental exposure in which both parents are chronically irradiated from embryonic stages, prior to germ cell formation, through breeding. The dose-rate chosen for the present experiment exceeded by an order of magnitude the recommended 1 cGy per day limit recommended by the IAEA and accepted by the DOE, and was purposely selected in an effort to establish population level responses to the chronic irradiation. There is limited information available on the cytogenetic responses of medaka embryos to chronic gamma radiation. The information generated in this experiment will be helpful in assessing whether the use of cytogenetic endpoints would be appropriate for determining the effects of chronic low

dose-rate radiation and the potential of inducing GI at dose-rates of 1 cGy per day and lower.

Chronic irradiation of F0 embryos from an early embryonic stage to adulthood at 60-days post-fertilization resulted in a 38% greater mortality than the unirradiated F0 fish. The variation in the water quality in terms of dissolved oxygen and pH could have contributed to a physiological stress that was added to the chronic irradiation insult. The total whole body dose received by the fish during the 60-day exposure from embryo to adulthood was ~600 cGy. Chronic radiation effects to aquatic species have shown that fish are the most sensitive to radiation (51-52) and that whether freshwater or marine, tropical or temperature, all species have similar radiosensitivities (53-55). In regards to chronic irradiation with gamma rays no excess mortality was observed when chronic irradiation was delivered to embryo and juvenile stages of chinoook salmon, *Onchorhynchus tschawytscha*, for 60-80 days at dose-rates of 0.5-9.5 cGy/day (56-59). However, the increase in mortality observed in my experiment at 60-days post-fertilization at a dose-rate of 10±1 cGy/day is consistent with those previously reported by Hyodo-Taguchi and Etoh (51) when the gamma rays were delivered to early stage embryos. In their experiment Stage 9 embryos were exposed to chronic gamma irradiation (~10 rads/day for 10 days) until hatchout. The dose received by these individuals during embryonic development to hatchout resulted in 50% mortality at 30-days post-hatching and 80% mortality at 401 days, thus showing a marked increase in mortality during the juvenile to adult stages.

Chronic radiation of zebrafish, *Danio rerio*, from hatchout fish to adult and continuing for 1 year showed no effect on mortality at dose-rates ranging from 0.72, 2.4

and 17 cGy/day (60). However, effects on reproduction were observed at a dose-rate of 17 cGy/day. Histological examination of adult zebrafish showed damage to testes but no apparent damage to ovaries. Chronically irradiated breeding pairs showed decreased numbers of viable eggs produced per laying opportunity as compared to unirradiated breeding pairs. This result is consistent with the findings of a statistically significant decrease in the fertility of chronically irradiated breeding pairs in my experiment.

An increased rate of chromosomal aberrations is a hallmark of genomic instability and has been reported in studies where acute parental irradiation of adult medaka has resulted in increased mutation frequencies in subsequent unirradiated F1 offspring (38). The intent of my project was to collect early stage embryos to determine if a GI could be measured as chromatid-type aberrations in F1 offspring from unirradiated and chronically irradiated F0 parents. The background total aberration frequencies and radiation induced total aberration frequencies in F1 offspring from unirradiated and chronically irradiated F0 parents were compared for statistical differences. The mean total background aberration frequencies from F1 offspring from unirradiated F0 parents and chronically irradiated F0 parents showed no demonstrable GI. Additionally, F1 offspring receiving a 500 cGy *in vivo* challenge dose of gamma rays showed no demonstrable GI if they originated from either unirradiated or chronically irradiated F0 parents.

While statistical analysis of the whole embryos indicated the lack of a demonstrable GI in F1 embryos originating from chronically irradiated F0 parents, there was a noticeable increase in mortality of embryos prior to Stage 25. This increase in mortality indicates the possibility of instability occurring prior to Stage 25 or an increased sensitivity of the embryos to the chronic gamma radiation. Hyodo-Taguchi and

Etoh (51) reported that eggs produced from females chronically irradiated (~10 cGy/day) were capable of being fertilized by unirradiated males, however, the embryos were unable to develop into later stages of embryogenesis, thus showing that the ova may have carried some genetic damage from the chronic irradiation that was passed to the offspring.

Potential explanations for the lack of a demonstrable GI using chromatid-type aberrations in this experiment could lie in the use of an *in vivo* embryo model and the technique used for measuring the chromatid-type aberrations. Previous research on GI has primarily focused on the use of lymphocyte cells in culture despite the fact that they are fundamentally different than cells that would compose a solid organ. While the use of lymphocyte cells has shown that GI can be initiated *in vitro*, perpetuated *in vivo* when the process was experimentally initiated *in vitro*, and initiated in apparently normal human hematopoietic cells *in vitro* (6) the work of Whitehouse and Tawn (12) showed no apparent increase *in vivo* in humans chronically exposed to radiation indicating the possibility of a much higher threshold for the induction of GI as compared to isolated cells in culture. Potentially the experimental design of this project by using whole embryos, which are composed of already differentiated organs and tissues and the fact that only one generation was evaluated could have been contributing to the lack of the a measurable GI. Additionally, the use of whole chromosome staining by traditional Giemsa methods has been found to be less sensitive than other methods, such as G-banding, for measuring chromatid-type aberrations (12).

Future work is planned to continue the on-going development of whole chromosome specific painting probes to explore the possible transmission of a GI via

more stable cytogenetic endpoints such as a symmetrical translocations. The high degree of observed mitotic variability between individual medaka embryos contributed to the difficulty in measuring a cytogenetic GI. Therefore additional molecular endpoints and assays will be explored to determine if there is a more sensitive tool for measuring possible GI at the early stages of embryogenesis in the medaka and at dose-rates achieving the recommended maximum daily dose of 1 cGy/day.

Acknowledgements

This work was supported by grants provided by the U.S. Department of Energy (DE-FG02-03ER63645) and the National Institute of Health, National Cancer Institute NRSA Training Grant (CA09236). A special thank you to Dr. John Pinder for his help with statistical analyses and Dr. Jim Durham for his help with dosimetry. Also, thank you to Anna Williams for her assistance with chromosome aberration scoring.

References

1. IAEA, International Atomic Energy Agency. Effects of ionizing radiation on plants and animals at levels implied by current radiation protection standards. Technical Reports Series #332. IAE, Vienna. (1992).

2. DOE, Department of Energy. A graded approach for evaluating radiation doses to aquatic and terrestrial biota. DOE-STD-1153-2002. (2002).

3. K. Baverstock, Radiation-induced genomic instability: A paradigm-breaking phenomenon and its relevance to environmentally induced cancer. *Mutat. Res.* **454**, 89-109 (2000).

4. W. F. Morgan, J.P. Day, M.I. Kaplan, E.M. McGhee, and C.L. Limoli, Genomic instability induced by ionizing radiation. *Radiat. Res.* **146**, 247-258 (1996).

5. W.F. Morgan, Is there a common mechanisms underlying genomic instability, bystander effects and other nontargeted effects of exposure to ionizing radiation? *Oncogene.* **22**, 7094-7099 (2003).

6. M. A. Kadhim, D.A. Macdonald, D.T. Goodhead, S.A. Lorimore, S.J. Marsden and E.G. Wright, Transmission of chromosomal instability after plutonium alpha-particle irradiation. *Nature.* **355**, 738-740 (1992).

7. S.A. Lorimore, M.A. Kadhim, D.A. Pocock, D.L. Papworth, D.T. Stevens, S.A. Goodhead and E.G. Wright, Chromosomal instability in the descendants of unirradiated surviving cells after alpha-particle irradiation. *Proc. Natl. Acad. Sci.* **95**, 5730-5733 (1998).

8. J.B. Little, H. Nagasawa, T. Pfenning and H. Vetrovs, Radiation-induced genomic instability: Delayed mutagenic and cytogenetic effects of X Rays and Alpha Particles. *Radiat. Res.* **149**, 299-307 (1997).

9. J.B. Little, Induction of genetic instability by ionizing radiation. *CR Acad. Sci.* III **322**, 127-134 (1999).

10. L. Abramsson-Zetterberg, G. Zetterberg, S. Sundell-Bertman, and J. Grawe, Absence of genomic instability in mice following prenatal low dose-rate gamma-irradiation. *Int. J. Radiat. Biol.* **76**, 971-977 (2000).

11. S.D. Bouffler, J.W. Haines, A.A. Edwards, J.D. Harrison, and R. Cox, Lack of detectable transmissible chromosomal instability after *in vivo* or *in vitro* exposure of mouse bone marrow cells to ^{224}Ra alpha particles. *Radiat. Res.* **155**, 345-352 (2001).

12. C.A. Whitehouse and E. J. Tawn, No evidence for chromosomal instability in radiation workers with *in vivo* exposure to plutonium. *Radiat. Res.* **156**, 467-475 (2001).

13. L.C. Dugan and J.S. Bedford, Are chromosomal instabilities induced by exposure of cultured normal human cells to low- or high-LET radiation? *Radiat. Res.* **159**, 301-311 (2003).

14. L.M. Wiley, J.E. Baulch, O.G. Raabe, and T. Straume, Impaired cell proliferation in mice that persists across at least two generations after paternal irradiation. *Radiat. Res.* **148**, 145-151 (1997).

15. Y.E. Dubrova, M. Plumb, J. Brown, and A.J. Jeffreys, Radiation-induced germline instability at minisatellite loci. *Int. J. Radiat. Biol.* **74**, 689-696 (1998).

16. M.M. Vance, J.E. Baulch, O.G. Raabe, L.W. Wiley and J.W. Overstreet, Cellular reprogramming in the F_3 mouse with paternal F_0 radiation history. *Int. J. Radiat. Biol.* **78**, 513-526 (2002).

17. Y.E. Dubrova, M. Plumb, B. Gutierrez, E. Boulton, A.J. Jeffreys, Transgenerational mutation by radiation. *Nature.* **405**, 37 (2000).

18. K.P. Hoyes, B.I. Lord, C. McCann, J.H. Hendry and I.D. Morris, Transgenerational effects of preconception paternal contamination with ^{55}Fe. *Radiat. Res.* **156**, 488-494 (2001).

19. K.O. Niwa and R. Kominami, Untargeted mutation of the maternally derived mouse hypervariable minisatelline allele in F1 mice born to irradiated spermatozoa. *Proc. Natl. Acad. Sci.* **98**, 1705-1710 (2001).

20. BEIR VII: Health effects from exposure to low levels of ionizing radiation, Phase 2. National Research Council of the National Academies. The National Academies Press. Washington, DC (2006).

21. L.A. Livshits, S.G. Malyarchuk, S.A. Kravchenko, G.H. Matsuka, E.M. Lukyanova, Y.G. Antipkin, L.P. Arabskaya, E. Petit, F. Giraudeau and B. Le Guen, Children of Chernobyl cleanup workers do not show elevated rates of mutations in minisatellite alleles. *Radiat. Res.* **155**, 74-80 (2001).

22. R.J.C. Slebos, R.E. Little, D.M. Umbach, Y. Antipkin, T.D. Zadaorzhnaja, N.A. Mendel, C.A. Sommer, K. Conway, E. Parrish and J.A. Taylor, Mini- and microsatellite muttaions in children from Chernobyl accident clean-up workers. *Mutat. Res.* **559**, 143-151 (2004).

23. A. Kiuru, A. Auvinen, M. Luokkamaki, K. Makkonen, T. Veidebaum, M. Tekkel, M. Rahu, T. Hakulinen, K. Servomaa and R. Mustonen, Hereditary minisatellite

mutations among the offspring of Estonian Chernobyl cleanup workers. *Radiat. Res.* **159**, 651-655 (2003).

24. J.A. Armour, M.H. Brinkworth and A. Kamischke, Direct analysis by small-pool PCR of MS205 minisatellite mutation rates in sperm after mutagenic therapies. *Mutat. Res.* **445**, 73-80 (1999).

25. N. Zheng, D.G. Monckton, G. Wilson, F. Hagemeister, R. Chakraborty, T.H. Connor, M.J. Siciliano and M.L. Meistrich, Frequency of minisatellite repeat number changes at the MS205 locus in human sperm before and after cancer chemotherapy. *Environ. Mol. Mutagen.* **36**, 134-145 (2000).

26. C.A. May, K. Tamaki, R. Neumann, G. Wilson, G. Zagars, A. Pollack, Y.E. Dubrova, A.J. Jeffreys and M.L. Meistrich, Minisatellite mutation frequency in human sperm following radiotherapy. *Mutat. Res.* **453**, 67-75 (2000).

27. O. Niwa, Induced genomic instability in irradiated germ cells and in the offspring; reconciling discrepancies among the human and animal studies. *Oncogene.* **22**, 7078-7086 (2003).

28. Y.E. Dubrova, Long-term genetic effects of radiation exposure. *Mutat. Res.* **544**, 433-439 (2003).

29. S.D. Bouffler, B.A. Bridges, D.N. Cooper, Y. Dubrova, T.J. McMillan, J. Thacker, E.G. Wright and R. Waters, Assessing radiation-associated mutational risk to the germline: Repetitive DNA sequences as mutational targets and biomarkers. *Radiat. Res.* **165**, 249-268 (2006).

30. R.C. Barber and Y.E. Dubrova, The offspring of irradiated parents, are they stable? *Mutat. Res.* (In Press) (2006).

31. ICRP, A framework for assessing the impact of ionizing radiation on non-human species. Publication 91. International Commission on Radiation Protection, Pergamon Press, Oxford (2003).

32. T. Yamamoto, Medaka (Killfish): Biology and Strains. Keigaku Publishing Company, Tokyo. Pp. 365. (1975).

33. J. Wittbrodt, A. Shima and M. Schartl, Medaka – A Model organism from the far east. *Nature Reviews Genetics.* **3**, 53-64 (2002).

34. A. Shima and A. Shimada, Development of a possible nonmammalian test system for radiation-induced germ-cell mutagenesis using the fish, the Japanese medaka. *Proc. Natl. Acad. Sci.* **88,** 2545-2549 (1991).

35. Y. Kubota, A. Shimada and A. Shima, DNA alterations detected in the progeny of paternally irradiated Japanese medaka fish, (Oryzias latipes), *Proc. Natl. Acad. Sci.* **92**, 330-334 (1995).

36. A. Shimada and A. Shima, Combination of genomic DNA fingerprinting and the medaka specific-locus test system for studying environmental germ-line mutagenesis. *Mutat. Res.* **399**, 149-165 (1998).

37. A. Shima and A. Shimada, The Medaka as a model for studying germ-cell mutagenesis and genomic instability. *Mar. Biotechnol.* **3**, S162-S167 (2001).

38. A. Shimada and A. Shima, Transgenerational genomic instability as revealed by a somatic mutation assay using the medaka fish. *Mutat. Res.* **552**, 119-124 (2004).

39. N. Satoh and N. Egami, Sex differentiation of germ cells in the teleost, Oryzias latipes, during normal embryonic development. *J. Embryol. Exp. Morphol.* **28**, 385-395 (1972).

40. K.I. Ijiri and N. Egami, Effects of γ-Ray irradiation on primordial germ cells in embryos of Oryzias latipes. *Radiat. Res.* **72**, 164-173 (1977).

41. Y. Shimada and N. Egami, The unique responses of the primordial germ cells in the fish Oryzias latipes to gamma-rays. *Int. J. Radiat. Biol.* **45**, 227-235 (1984).

42. Y. Shimada, A. Shima and N. Egami, Effects of heat, release from hypoxia, cadmium and arsenite on radiation sensitivity of primordial germ cells in the fish *Oryzias latipes*. *J. Radiat. Res.* **26**, 411-417 (1985).

43. A. Shimada, H. Eguchi, S. Yoshinaga and A. Shima, Dose-rate effect on transgenerational mutation frequencies in spermatogonial stem cells of the medaka fish. *Radiat. Res.* **163**, 112-114 (2005).

44. W.L. Russell and E.M. Kelly, Mutation frequencies in male mice and the estimation or genetic hazards of radiation in men. *Proc. Natl. Acad. Sci.* USA **79**, 542-544 (1982).

45. T. Iwamatsu, Stages of normal development in the Medaka Oryzias latipes. *Mech. Of Dev.* **121**, 605-618 (2004).

46. N. Egami, Secondary Sexual Characters. In: Medaka (Killifish): Biology and Strains. Ed. Yamamoto, T. Keigaku Publishing Company, Tokyo. Pp. 109-125 (1975).

47. J.R.K. Savage, Classification and relationships of induced chromosomal structural changes. *J. Med. Gen.* **12**, 103-122 (1975).

48. R.C. Littell, G.A. Milliken, W.W. Stroup, and R.D. Wolfinger, SAS system for fixed models. SAS Inst., Cary, NC (1996).

49. G. Der and B. Everett, Handbook of statistical analyses using SAS. CRC Press, Boca Raton, Fl, USA. (2001).

50. Ministry of the Environment, Japan. Medaka, Oryzias latipes, Development of test methods and suitability of medaka as test organism for detection of endocrine disrupting chemicals. Chemicals and Evaluation Research Institute. Japan. February (2003).

51. Y. Hyodo-Taguchi and N. Etoh, The fecundity and fertility of medaka exposed to chronic γ-radiation in their embryonic stages. *J. Radiat. Res.* **24**, 270-277 (1983).

52. D.S. Woodhead, The impact of radioactive discharges on native British wild-life and the implications for environmental protection. R&D Technical Report P135, Environment Agency, Bristol. (1998).

53. UNSCEAR. Sources and effects of ionizing radiation. United Nations, New York (1996).

54. D.S. Woodhead, The effects of chronic irradiation on the breeding performance of the guppy, (Poecilia reticulate) (Osteichthyes: Teleosti), *Int. J. Radiat. Biol.* **32**, 1-22 (1977).

55. Y. Hyodo-Taguchi, Effects of chronic gamma-irradiation on spermatogenesis in the fish Oryzias latipes with special reference to regeneration of testicular stem cells. In Radiation Effects on Aquatic Organisms. (ed. N. Egami), pp 91-104. Japan Scientific Societies Press, Tokyo/University Park Press, Baltimore. (1980).

56. J.F. Knowles, Long-term irradiation of a marine fish, the plaice Pleuronectes platessa: an assessment of the effects on size and composition of the testes and of possible genotoxic changes in peripheral erythrocytes. *Int. J. Radiat. Biol.* **75**, 773-782 (1999).

57. L.R. Donaldson and K. Bonham, Effects of low-level chronic irradiation on Chinook and Coho salmon eggs and alevins. *Trans. Am. Fish. Soc.* **93**, 333-341 (1964).

58. L.R. Donaldson and K. Bonham, Effects of chronic exposure of Chinook salmon eggs and alevins. *Trans. Am. Fish. Soc.* **99**, 112-119 (1970).

59. D.S. Woodhead, Contamination due to radioactive materials. In Marine Ecology, Pollution and Protection of the Seas-Radioactive Materials, Heavy Metals, and Oil. (ed. O. Kinne), John Wiley and Sons, New York, NY. 1111-1287 (1984).

60. SNIFFER, Scotland and Northern Ireland Forum for Environmental Research. An investigation into the effects of chronic radiation on fish. R&D Technical Report P3-053/TR, (2002).

Table 2-1
Survival of Unirradiated and Chronically Irradiated F0 Adults at 60-days post-fertilization.

	F0 Embryos at 6-hours post-fertilization (n)	F0 Fish at 60-Days post-fertilization (n)	Survival (%)
Unirradiated	306	148	48*
Irradiated (10 cGy/day)	323	90	28*

*Statistically significant (P=0.02) using a one-way ANOVA

Table 2-2
Water Chemistry Measurements (Mean±SEM) of pH, Temperature (°C), Dissolved Oxygen (%) and Total Dissolved Solids (mg/L) for Unirradiated and Chronically Irradiated F0 Medaka.

	F0 Unirradiated	F0 Irradiated	P-value
pH (n=10)	7.8±0.06	7.5±0.05	<0.05*
Temperature (°C) (n=10)	27.1±0.25	26.6±0.1	0.15
Dissolved Oxygen (%) (n=10)	39±1.2	29±1.2	<0.05*
Total Dissolved Solids (mg/L) (n=10)	462±50	543±61	0.30

*Water chemistry parameters of pH and Dissolved Oxygen (%) are statistically different at the α=0.05 level in the unirradiated and irradiated F0 medaka using a one-way ANOVA.

Table 2-3
Wet Weight (Mean±STD) of F0 Adults at 60-days post-fertilization and Number of Eggs produced per Female Per Day (Mean±STD) and Percentage of Eggs Fertilized (%) at 90-days post-fertilization.

	F0 Unirradiated (n=17)	F0 Irradiated (10 cGy/day) (n=24)	P-Value
Wet Weight (mg)	Females = 660±330 Males = 590±260	Females= 660±230 Males=510±150	0.08
Average Number of Eggs/ Female/ Day	19±9	16±7	0.05
Percentage of Fertilized Eggs (%)	100	94	0.02*

*Percentage of eggs fertilized per adult female per day was statistically different at the α=0.05 level in the irradiated versus unirradiated F0 medaka using a one-way ANOVA.

Table 2-4
Background Chromatid-Type Aberration Frequency in F1 Offspring Originating from Unirradiated and Chronically Irradiated F0 Parents (Mean± SEM).

	F1 Dose (cGy)	F1 Offspring (n)	Number of Mitotic Cells Scored	Number of Aberrations per Cell			Total Aberrations per Cell
				Total Gaps	Total Breaks	Total Exchanges	
F0 Unirradiated	0	11	145	0.003±0.04	0.01±0.007	0±0	0.01±0.01
F0 Irradiated (10 cGy/day)	0	13	147	0±0	0.01±0.01	0.01±0.01	0.02±0.01

*No statistical difference measured in the total aberration frequencies (P=0.87)

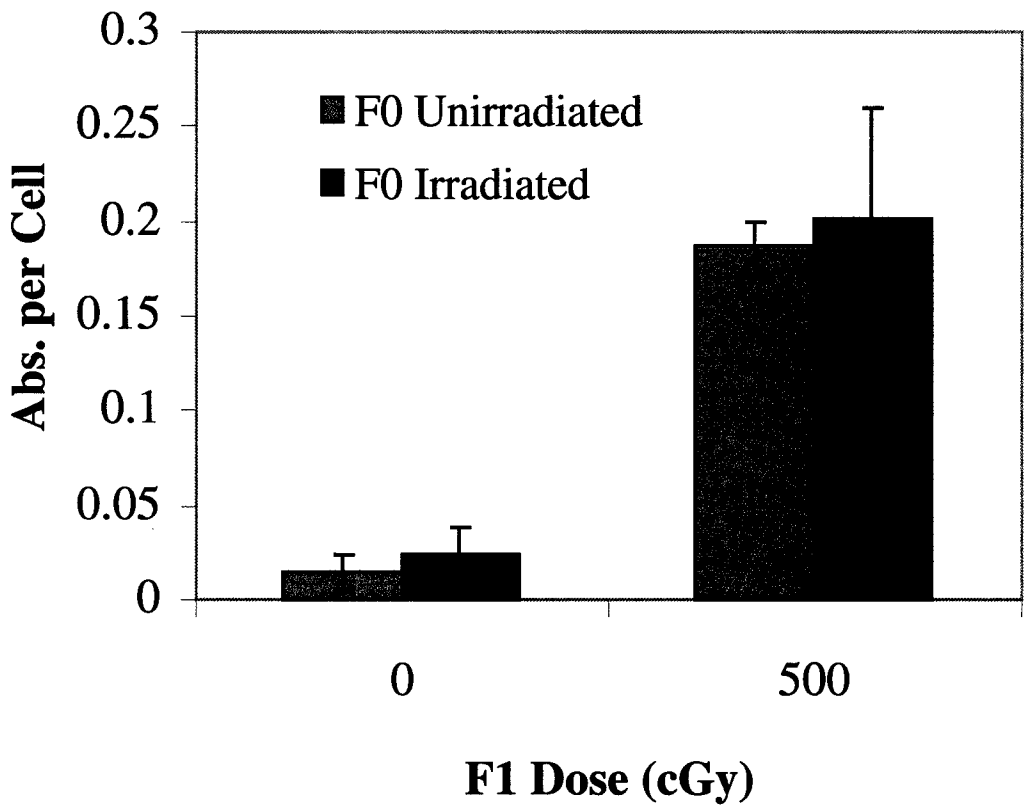

Figure 2-1 Mean total aberration frequencies in F1 offspring from unirradiated and chronically irradiated F0 Parents. Error bars represent ± 1 standard error.

Table 2-5
Linear comparison of the mean aberration frequencies for individual and combinations of aberrations in F1 offspring originating from irradiated and unirradiated F0 parents.

Aberration Type	Observed Mean Difference[2]	Standard Error	P-Value[1]
Gaps	0.01	0.02	0.39
Breaks	0.01	0.02	0.54
Total Exchanges	0.001	0.01	0.91
Gaps+Breaks	0.03	0.02	0.31
Total Exchanges – (Gaps+Breaks)	-0.02	0.02	0.20
Total Aberrations	0.005	0.03	0.86

*[1] For an F-test of the linear comparison.
*[2]Observed mean difference is the observed mean for the irradiated minus the observed mean for the unirradiated F0 parents.

Table 2-6

Radiation induced aberration frequencies (Mean± SEM) in F1 Offspring originating from unirradiated and irradiated F0 Parents. Radiation induced aberrations were produced by exposure to a 500 cGy challenge dose of gamma rays.

	F1 Dose (cGy)	F1 Offspring (n)	Number of Mitotic Cells Scored	Number of Aberrations per Cell			Total Aberrations per Cell
				Total Gaps	Total Breaks	Total Exchanges	
F0 Unirradiated	500	20	208	0.08±0.02	0.07±0.02	0.03±0.01	0.18±0.03
F0 Irradiated	500	14	132	0.09±0.03	0.08±0.03	0.03±0.02	0.20±0.06

*No statistical difference measured in the aberration frequencies (P=0.97)

Table 2-7
Linear comparison of mean aberration frequencies for individual and combinations of specific aberrations in F1 offspring, receiving a 500 cGy gamma dose, originating from irradiated and unirradiated F0 parents.

Aberration Type	Observed Mean Difference[2]	Standard Error	P-Value[1]
Gaps	0.02	0.05	0.82
Breaks	0.01	0.06	0.86
Total Exchanges	-0.02	0.01	0.53
Gaps+Breaks	0.02	0.08	0.77
Total Exchanges – (Gaps+Breaks)	-0.02	0.05	0.71
Total Aberrations	0.003	0.09	0.97

*[1] For an F-test of the linear comparison.
*[2]Observed mean difference is the observed mean for the irradiated minus the observed mean for the for unirradiated F0 parents.

Table 2-8
Linear comparison of mean aberration frequencies for individual and combinations of specific aberrations in F1 offspring receiving a 500 cGy gamma dose versus unirradiated F1 offspring.

Aberration Type	Observed Mean Difference[2]	Standard Error	P-Value[1]
Gaps	0.08	0.02	0.0005**
Breaks	0.06	0.03	0.048**
Total Exchanges	0.02	0.01	0.12
Gaps+Breaks	0.15	0.04	0.002**
Total Exchanges – (Gaps+Breaks)	0.02	0.02	0.35
Total Aberrations	0.17	0.05	0.0003**

*[1] For an F-test of the linear comparison.
*[2]Observed mean difference is the observed mean for the F1 offspring receiving the 500 cGy dose minus the observed mean for the unirradiated F1 offspring.
**Statistically significant at the α=0.05 level.

Chapter 3 - *In Vivo* Adaptive Response in Japanese Medaka (*Oryzias latipes*)

Abstract

The concept of adaptive response (AR) is that if cells are exposed to a small dose of radiation prior to a larger dose, then the biological response may be less than if the cells were exposed to the larger dose alone. An adaptive response to radiation has been observed in several *in vitro* cell lines and occasionally in *in vivo* model systems with both high and low LET radiation when the adapting doses have been delivered as acute exposures. The objectives of this experiment were to assess whether or not an AR could be measured when the adapting dose was applied *in vivo* and chronically to the freshwater fish Japanese Medaka, and to determine whether there was a transgenerational effect. The AR would be assessed by comparing chromatid-type aberration frequencies produced from adapting doses of 2 or 21 cGy followed by a 500 cGy challenge dose of Cesium-137 gamma rays with aberration frequencies produced by the 500 cGy challenge dose alone. The adapting doses were delivered *in vivo* and chronically over periods of 4 and 42 hours, respectively, at a dose-rate of 12 cGy/day. The challenge dose was delivered *in vivo* and acutely at a dose-rate of 390 cGy/min and within 30 minutes of the termination of the adapting dose. To determine if a transgenerational effect was present, F1 offspring from unirradiated and chronically irradiated parents were utilized in the experiment. Results from the G2 chromosome assay, as analyzed by a linear comparison of mean aberration frequencies, indicated that there were no statically significant differences in the total aberration frequencies produced with either the 2 or 21 cGy adapting dose followed by the 500 cGy challenge dose versus the 500 cGy challenge dose alone, P=0.93 and P=0.83 respectively, for offspring originating from either unirradiated or irradiated F0 parents. A high degree of variability in the mitotic indices was observed

in the medaka embryos which complicated the statistical analyses. More research is obviously needed to clearly understand the total adapting dose required, the timing required for the delivery of the challenge dose after the adapting dose, and the appropriate stage, embryo, juvenile or adult, required to successfully demonstrate any *in vivo* AR in medaka fish.

Introduction

The concept of adaptive response (AR) is that if cells are exposed to a small dose of radiation prior to a larger dose, then the biological response may be less than if the cells were exposed to the larger dose alone. An adaptive response was reported by Olivieri et al. in 1984 (1) when he exposed human lymphocyte cell cultures to low-doses of tritiated thymidine and followed that exposure with an acute X-ray dose of 1.5 Gy. These authors reported a 50% reduction in the number of chromatid-type aberrations in the cultures that received the priming dose from the tritiated thymidine followed by the 1.5 Gy of X-rays versus cultures receiving the 1.5 Gy dose alone. Since this initial report, the AR has been reported using low LET radiation and a variety endpoints such as micronucleus formation, DNA repair, mutation frequency and neoplastic transformation using a variety of *in vitro* systems such as human lymphoblasts and fibroblasts (2-5), mammalian cell lines such C3H 10T1/2 mouse embryo cells, (6) A_L human hamster cells (7), and gold-fish cells CAF-31 (8). Additionally, the AR has been measured in human cell lines when the adapting radiation was high LET neutrons and alpha particles (9-10). The commonality in all of these experiments was that the total adapting doses were in the range of 1-2 cGy, given as acute doses with dose-rates averaging ~20 cGy/min followed by an acute challenge dose. However, adapting doses as high as 50 cGy have been shown to induce a AR when given at the considerably lower dose-rates of 0.5-1 cGy/min (11).

Recent studies have focused on applying the adapting dose to *in vivo* model systems using lower dose-rates. When both the adapting and challenge doses were applied to *in vivo* mouse models, Trp53 heterozygous mice (12-13) and BalbC mice (14),

using dose-rates ranging from 0.5-2.8 cGy/min and keeping the total exposure time for the adapting dose to <20 minutes , the results were again positive for a AR.

While the possibility of an induced resistance to damage by exposure to low priming doses of ionizing radiation seems favorable, the long term implications and possible transgenerational effects are still not well known. The presence of a AR at low-doses and low dose-rates challenges the linear, no-threshold model upon which radiation protection practices are based, where the risk is directly proportional to dose, and every dose, no matter how low, increases cancer risk (15). One group has reported that the adaptive response can affect the kinds of mutations induced, principally by reducing the number of deletion-type mutations in comparison to point mutations (3). Studies by Ueno et al. (7) also indicated an increased number of "complex" mutations with a reduction in proportion of simple mutations, prompting them to conclude that: *"...radioadaptation may be two-faced, reducing mutant yields on the one hand, while increasing the proportion of mutants with dangerous characteristics on the other hand". This result, if verifiable, would have important implications for the assessment of biological hazards following exposures to low levels of ionizing radiation and perhaps other agents as well."* Observations that might argue against the likely operation of a transgenerational adaptive response are reports indicating that the effect of the adapting dose, independent of cell type and delivery either *in vitro* or *in vivo,* disappeared in about one day (11, 14).

The objectives of this experiment were to assess whether or not an AR could be measured when the adapting dose was applied *in vivo* and chronically to the freshwater fish Japanese Medaka and to determine whether there was a transgenerational effect. The

AR would be assessed by measuring chromatid-type aberrations in the first post-irradiation mitosis of F1 embryos. F1 embryos would receive adapting doses of either 2 or 21 cGy, delivered over 4 and 42 hours, prior to a 500 cGy challenge dose, or receive the 500 cGy challenge dose alone. This radiation scheme would be delivered to F1 embryos originating from unirradiated F0 parents and from chronically irradiated F0 parents.

Materials and Methods

Model Organism Japanese Medaka (CAB Strain)

The animal model used in this experiment was the Japanese Medaka fish. The fish were from an inbred strain of *Oryzias latipes* from the Southern Population of Japan. It has an orange-red phenotype and is the same species that can be commercially obtained from Carolina Biological Supply Company, North Carolina, and thus it is referred to as the "CAB" strain.

Animal Care Use and Guidelines

Animal care procedures followed in this study are in accordance with Colorado State University Animal Care and Use Committee guidelines (Protocol Nos. 01-189A-01 and 03-181A-02).

Establishment of F0 Generation; From Egg to Adult Stage

Eggs, representing the F0 generation, were collected gently by hand from the abdomen of 6-month old adult females which had bred with 6-month old adult males. Eggs were collected each morning for 5 days. The eggs were inspected under a dissecting microscope 6-h after being collected to determine if fertilization was

successful. Fertilized eggs were distinguished from unfertilized eggs by the presence of the blastula cap of cells as described by Iwamatsu (16). Fertilized eggs were randomly selected for either the unirradiated or irradiated treatment and placed into 250 ml glass jars containing 125 ml of the parental tank water and 125 ml of aerated tap water. The eggs were maintained at a density of 60 eggs per jar and at 22±2°C under 12 h light:12 h dark light cycles for 10-15 days until hatch out.

One week after hatchout medaka were moved to 2.5-L glass bowls at a density of 6 fish per bowl. These fish were maintained at 22±2°C under 12 h light:12 h dark light cycles and fed 2X daily Aquatox flake food (Aquatic Ecosystems, Inc., Apopka, Florida) and 1X daily live *Artemia napulii* (Great Salt Lake Brine Shrimp, Parker International, Inc., Salt Lake City, UT).

Individual males and females, approximately 2-months old, were randomly selected and paired up for breeding. Pairs were maintained in 2.5 L glass bowls at 26-28°C and under a light cycle of 16 h light: 8 h dark. Adult medaka were fed 3X daily a diet of Aquatox flake food (Aquatic Ecosystems, Inc., Apopka, Florida) and 1X daily live *Artemia napulii* (Great Salt Lake Brine Shrimp, Parker International, Inc., Salt Lake City, UT). Individual females began producing eggs ~5-10 days after the initiation of breeding conditions.

Water Quality Control and Assurance

Water renewal for this experiment was conducted as static renewal with water changes occurring every 2-3 days. Water quality parameters of pH and total dissolved solids (mg/L) were monitored during the course of this experiment using a hand held meter from Thermo Orion Corporation, Beverly, MA (Meter 635, Probe 617500).

Temperature (°C) and dissolved oxygen (%) were monitored using a hand held meter from Thermo Orion Corporation, Beverly, MA (Meter 835A, Probe 083010A).

Chronic Irradiation of F0 Generation

Chronic gamma irradiation from a 370 Ci J.L. Shepherd and Associates Cesium-137 beam irradiator, Model 81-14 (SS-0070), (Serial No. 7014) source was delivered to F0 medaka eggs starting at 6-h post-fertilization and continuing until breeding and collection of the F1 generation was complete, ~3 months total exposure. Dosimetry was conducted using a calibrated Rad/Cal Ion Chamber Survey Meter (Model 2025, SN 3843) and Probe (SN 7357) in combination with calibrated Lithium Fluoride thermoluminescent dosimeters (TLD 100s). TLDs were read using a Harshaw QS 3500, Serial No. 9305053 (Bicron, OH) TLD reader. The dose was delivered continuously at an average dose-rate of 12 cGy/day except for when the source was shielded for feeding and maintenance of the tanks. The average daily dose to the fish was ~ 10±1 cGy/day.

Collection of F1 Embryos

Fertilization of F1 embryos occurred generally within an hour after the lights were turned on and the medaka had been fed in the morning. Eggs were collected by hand from the abdomen of the female and placed in 125 ml glass beakers containing ½ parental tank water and ½ aerated tap water. The eggs were immediately removed from the chronic irradiation field.

Irradiation of F1 Offspring using Cesium-137 Gamma Irradiation

Adapting doses of 2 and 21 cGy were delivered chronically at a dose-rate of 0.5 cGy/h to whole embryos using the Cesium-137 irradiator used for the F0 generation.

Embryos were maintained in 250 ml of water during delivery of the adapting dose at a temperature of 22±2°C. The challenge dose of 500 cGy was delivered acutely using a 3,000 Ci J.L. Shepherd Mark I Cesium-137 irradiator at a dose rate of 390 cGy/min. Embryos were maintained in 50 ml centrifuge tubes containing aerated water. For those individuals receiving both the adapting and challenge doses, the challenge dose was delivered within 30 minutes of the end of the adapting dose. Dosimetry was conducted using a calibrated Rad/Cal Ion Chamber Survey Meter (Model 2025, SN 3843) and Probe (SN 7357) in combination with calibrated Lithium Fluoride thermoluminescent dosimeters (TLD 100s). TLDs were read using a Harshaw QS 3500, Serial No. 9305053 (Bicron, OH) TLD reader.

Metaphase Chromosome Collection and Slide Preparation for G2 Assay

Metaphase cells were collected from early stage whole embryos. Whole embryos, ~48-hours post-fertilization (Stage 25), were rinsed in a 0.5% bleach solution and 2X in Phosphate Buffer Solution (Dulbecco's PBS) to remove debris from the outer surface of the egg. The embryos were placed in 1X Ringers solution and the chorion and yolk removed by poking a hole in the chorion and manually removing the embryo and yolk from the chorion. The embryos were placed in 1 ml of PBS and the cells dissociated by liberal pipeting. The cells were centrifuged at 1000 RPM for 10 minutes. The supernatant was removed and 1 ml of 0.05% trypsin and 0.02% EDTA-Na in PBS was added for 5 minutes at 33°C. The trypsinization process was stopped by adding 4 ml of fresh L-15 media (Leibovitz's L-15 (Sigma, St. Louis, MO) cell media supplemented with 20% fetal bovine serum, streptomycin (50μg ml^{-1}) and penicillin (50 U ml^{-1}). Mitotic cells were collected by adding colcemid (0.1 μg ml^{-1}) (GIBCO, Grand Island,

NY) to each cell suspension for 3 hours. For cells receiving the adapting and challenge dose or challenge dose alone the colcemid was administered within 1 hour of the challenge dose. The cells were centrifuged at 1000 RPM for 10 minutes. The supernatant was removed and the cells resuspended in 10 ml of hypotonic solution (75 mM KCl) for 20 minutes. To fix the cells, fresh methanol:acetic acid (3:1 vol/vol) was added drop-wise (~1 ml) to the hypotonic solution and allowed to remain at room temperature for 5 minutes. The cell suspension was centrifuged at 1000 RPM for 10 minutes. The cells were fixed an additional 2X with methanol:acetic acid (3:1 vol/vol) for 5 minutes and centrifuged at 1000 RPM for 4 minutes. The fixed cells were dropped onto cold wet slides and allowed to air dry. Once dry the slides were stained in freshly prepared 10% Giemsa in pH 6.8 buffer for 10 minutes.

Aberration Scoring

Slides were coded by a second party and scored on a Zeiss Axioskop 2 Plus (Carl Zeiss Microimaginig, Thornwood, NY, 10594) with 100X objectives and a 2X optivar. Images were taken using a Nikon Digital Eclipse Camera (DXM 1200) and edited using the Automatic Camera Tamer or ACT 1 Software program (Version 2.20). The aberration scoring criteria suggested by Savage was used for determining chromatid-type aberrations (17).

Statistical Analysis

Adaptive response was assessed by measuring mean chromatid-type aberration frequencies in F1 offspring from unirradiated and chronically irradiated F0 parents using a G2 chromosome assay. F1 offspring used in the G2 assay received either no irradiation

to determine mean background aberration frequencies or received adapting doses of 2 or 21 cGy immediately followed by a 500 cGy challenge dose of gamma radiation, or the 500 cGy challenge dose alone. The F1 embryos collected for the G2 chromosome assay on average had a low and highly variable mitotic index. The statistical analysis of the data took into account this high degree of variability by only including embryos having a minimum of 5 scoreable cells, making the experimental unit the embryo and not the breeding pair. Because evaluating the effects of different levels of the interactions of F0 and F1 irradiations are central to testing the objectives of the study, each specific objective was tested using a linear comparison of means (18). The comparisons were carried out using an F-Test for an analysis of variance of the means (Appendix 2). All analyses were conducted using Statistical Analysis Software (SAS, Version 9.1, Raleigh, NC) (19).

Results

Background Aberration Frequencies

Analysis of total background aberration frequencies (mean±SEM) in the unirradiated F1 offspring showed no statistically significant differences between F1 embryos originating from unirradiated parents and F1 embryos originating from parents receiving chronic gamma irradiation, 0.01±0.01 and 0.02±0.01 respectively (P=0.86) (Table 3-1). A linear comparison of mean aberration frequencies was conducted to determine if differences were measurable for specific types and frequencies of aberrations, such as gaps, total exchanges, gaps+breaks, total exchanges – (gaps+breaks) and total aberrations. Comparisons of the mean frequencies of these types of aberrations

again indicated that no statistically significant differences could be demonstrated (P=0.86) (Table 3-2) (Figure 3-1).

Radiation-Induced Aberration Frequencies

Aberration frequencies in F1 offspring receiving the 500 cGy challenge dose alone were compared against background aberration frequencies in unirradiated F1 offspring to determine the amount of radiation induced aberrations. F1 offspring from unirradiated F0 parents showed a total aberration frequency from the 500 cGy challenge dose of 0.18±0.03 and 0.20±0.06 for F1 offspring from chronically irradiated parents. Both aberration frequencies were statistically different from the unirradiated background aberration frequencies (P=0.003) indicating the presence of radiation induced aberrations. There was no indication that the radiation induced effect was greater for F1 embryos originating from unirradiated F0 parents versus chronically irradiated F0 parents (P=0.97) (Table 3-1) (Figure 3-1).

***In Vivo* 2 cGy Adapting Dose**

In this experiment an *in vivo* adapting dose of 2 cGy of gamma rays was delivered chronically, over 4 hours, to medaka embryos prior to a 500 cGy challenge dose. The total aberration frequency for F1 offspring from unirradiated F0 parents was 0.23±0.10 versus 0.15±0.04 for F1 offspring originating from irradiated F0 parents. These total aberration frequencies, when compared with aberration frequencies measured by the 500 cGy challenge dose alone, were not statistically different (P=0.93) (Table 3-1). Analysis of specific aberration frequencies, such as rates of gaps, breaks gaps+breaks, total exchanges, total exchanges – (gaps+breaks) and total aberrations produced by the 2 + 500

cGy versus 500 cGy alone were compared by a linear comparison of means. Results did indicate a AR for gaps (P=0.04) for embryos originating from unirradiated and irradiated F0 parents, however, when more complex aberrations such as breaks and exchanges were included in the analysis the AR was no longer statistically-demonstrable (Table 3-3). Comparison of the total aberration frequencies measured in F1 embryos receiving the 2 + 500 cGy doses were not statistically different for embryos originating from unirradiated F0 parents or irradiated F0 parents (P=0.32) (Table 3-1) (Figure 3-1) indicating no transgenerational effect.

In Vivo 21 cGy Adapting Dose

In this experiment an *in vivo* adapting dose of 21 cGy of gamma rays was delivered chronically, over 42 hours, to medaka embryos prior to a 500 cGy challenge dose. The total aberration frequency for F1 offspring from unirradiated F0 parents was 0.15±0.02 versus 0.22±0.03 for F1 offspring originating from irradiated F0 parents. These aberration frequencies, when compared against those measured by the 500 cGy challenge dose alone, were not statistically different (P=0.83) (Table 3-1). Analysis of specific aberration frequencies such as rates of gaps, breaks gaps+breaks, total exchanges, total exchanges – (gaps+breaks) and total aberrations produced by the 21 + 500 cGy doses versus the 500 cGy dose alone were compared by linear comparison of mean aberration frequencies and did not indicate the presence of a AR (Table 3-4). The total aberration frequencies for F1 offspring, from both unirradiated and irradiated F0 parents, receiving the 21 + 500 cGy challenge showed no difference in mean frequencies indicating no transgenerational effect (P=0.39) (Table 3-1) (Figure 3-1).

2 cGy Adapting Dose versus 21 cGy Adapting Dose

The adapting doses used in this experiment, 2 and 21 cGy, were initiated and delivered to various stages of embryonic development in the medaka. When individual aberration frequencies were analyzed for F1 offspring receiving these adapting doses the frequency of chromosomal exchanges approached statistical significance (P=0.07); however, inclusion of all types of aberrations indicated that the difference between the total aberration frequencies produced by either adapting dose was not statistically significant (P=0.92) (Table 3-5). This indicates that the frequency of aberrations produced by the 2 cGy adapting dose prior to the 500 cGy challenge dose versus the aberrations produced by the 21 cGy adapting dose prior to the 500 cGy challenge dose showed no measurable differences (Figure 3-1).

Discussion

The work presented here was designed to explore adaptive response to radiation when the adapting dose was delivered chronically and *in vivo* to whole embryos of the Japanese Medaka fish. Additionally, the experiment was designed to explore the possibility of any transgenerational effects being passed from chronically irradiated F0 parents to subsequent F1 offspring that could express itself as a AR. A G2 Chromsome Assay was used to measure "primary" structural aberrations occurring on a single sister-chromatid as a result of exposure to gamma radiation. Results of the experiment showed no statistically significant differences in aberration frequencies in F1 embryos receiving either the 2 or 21 cGy challenge dose prior to the 500 cGy challenge dose versus the 500 cGy challenge dose alone. Additionally, no statistically significant differences in aberration frequencies were demonstrated, irrespectively of whether these embryos

originated from unirradiated or chronically irradiated F0 parents, thus indicating no detectable transgenerational effect. The lack of a statistically demonstrable *in vivo* AR in this model system could be due to several factors, either functioning singly or in combination with one another. Factors of total adapting dose, dose-rate, timing of the delivery of the challenge dose relative to that of the adapting dose and life stage of the *in vivo* model have been indicated as being crucial for a positive result for AR (20-21).

The mechanism by which adaptive response occurs is not clearly understood but it is theorized that it functions by enhancing DNA repair and antioxidant ability, and enhancing the production of proteins needed to minimize the impact from indirect damaging effects of the subsequent high challenge dose (22-27). If adapting doses are given at sufficiently high doses or dose-rates, then there is the potential of causing too much radiation induced damage for the DNA repair systems to fully operate. In essence, if the repair systems can not keep up with the rates and types of damage, no protective effect can be measured (28). There was some concern that this was the case with this experiment. The adapting doses chosen for this experiment included the traditional 2 cGy dose, which has been proven successful in a variety of *in vitro* and *in vivo* model systems, and a higher 21 cGy dose to challenge the upper limits for a measurable adaptive response. Research conducted by Shadley and Weincke (11) showed that an adaptive response could be measured in somatic cells when an adapting dose as high as 50 cGy was delivered prior to the challenge dose. The presence of the adaptive response however, was dependent upon the dose-rate being at 0.5-1 cGy/min (11), thus the adapting dose could be characterized as an acute exposure. In this experiment, a AR was not demonstrated for either the 2 or the 21 cGy adapting dose, suggesting the possibility

that the chronic nature of the adapting doses could be the limiting factor in successfully measuring a AR.

Another important factor in the observation of an adaptive response to radiation appears to be the time between the delivery of the challenge dose and the adapting dose. Typically, when AR has been shown in *in vitro* and *in vivo* models, the challenge doses were applied several hours after the adapting dose (20-21). In this experiment the challenge dose was applied in < 30 min, as close to the ending of the adapting dose as possible to simulate an environmental exposure. In the present study, it was hypothesized that the individuals were residing in an area with an elevated level of background radiation and were subsequently exposed to a high challenge dose that could have originated from an accidental or terrorist release. Based on the results of previous work (20-21) and the results in this experiment, it does not appear that an AR could be demonstrated in the environment at these levels.

Another possible explanation for the lack of an *in vivo* AR in medaka embryos was the timing of the embryonic stage at which the adapting dose was delivered. Studies using mouse models have not been able to detect an AR in very early embryonic stages of development. When C57B1/6 mice were exposed at the 2 cell to blastocyst stage of development, no AR for chromosomal aberrations could be measured (29-30). However, when adapting doses were given to later stages of development, a AR could be shown (31-35). In these experiments it was determined that the embryonic stage at which the adapting dose was given was very important, as was the endpoint measured and the dose-rate at which the adapting dose was applied. In the present experiment the adapting doses were applied to embryonic Stages 10 and 25. These embryonic stages have been

shown to be somewhat radioresistant to low LET radiation (36) as compared to other embryonic stages in regards to the endpoint of hatchability, however, these stages are still considered to be very early in the embryonic development of the medaka and are characterized by a high degree of cell cycling which could contribute to the overall radiation sensitivity of the embryo.

The induction of cytogenetic damage has been reported to be one of the most sensitive endpoints that can be used to study the effects of radiation exposure to aquatic organisms (37). However, difficulties in using aquatic species for these purposes are that they often have karyotypes that have large numbers of very small chromosomes. Additionally the mitotic indices' of adult stages of aquatic species are often low, requiring the use of embryos (38). In this experiment one problem encountered with the use of the medaka embryos was that the mitotic index was highly variable between individuals of the same age. This high degree of variability made it very difficult to address cytogenetic radiation effects due to contributions from breeding pairs and this situation limited the statistical analyses to individual level responses alone. It is possible that another technique or assay is required in order to control for this high degree of variability between individual medaka embryos and to address breeding pair effects to successfully demonstrate any AR in the early embryonic stages of development.

More research is obviously needed to clearly understand the total adapting dose required, the timing required for the delivery of the challenge dose after the adapting dose, and the appropriate stage, embryo, juvenile or adult, required to successfully demonstrate any *in vivo* AR. Additionally, more research is needed to determine if, indeed, an *in vivo* AR exists at all, and if so, whether there are any potential long term

effects on the individuals expressing the AR and to their subsequent offspring in regards to possible transmission of a transgenerational genomic instability.

Acknowledgements

This work was supported by grants provided by the U.S. Department of Energy (DE-FG02-03ER63645) and the National Institute of Health, National Cancer Institute NRSA Training Grant (CA09236). A special thank you to Dr. John Pinder for his assistance with statistical analyses and Dr. Jim Durham for his assistance with dosimetry. Also, thank you to Anna Williams for her assistance with chromosome aberration scoring.

References

1. G. Olivieri, J. Bodycote, S. Wolff, Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. *Science.* **223**, 594-597 (1984).

2. E.I. Azzam, S.M. deToledo, G.P. Raaphorst, R.E.J. Mitchel, Radiation-induced radioresistance in a normal skin fibroblast cell line. In: Low Dose Irradiation and Biological Defence Mechanisms (T. Sugahara, L.A. Sagan, T. Aoyama, Eds.) pp. 291-294. Elsivier, Amsterdam, (1992).

3. O. Rigaurd, D. Papadopoulo, and E. Moustachi, Decreased deletion mutation in radioadapted human lymphoblasts. *Radiat. Res.* **133,** 94-101 (1993).

4. J.L. Redpath and R.J. Antoniono, Induction of an adaptive response against spontaneous neoplastic transformation in vitro by low-dose gamma radiation. *Radiat. Res.* **149**, 517-520 (1998).

5. E.J. Broome, D.L. Brown and R.E.J. Mitchel, Dose responses for adaption to low doses of ^{60}Co γ and ^{3}H β-particle radiation in normal human fibroblasts. *Radiat. Res.* **158**, 181-186 (2002).

6. E. I. Azzam, G.P. Raaphorst and R.E.J. Mitchel, Radiation-induced adaptive response for protection against micronucleus formation and neoplastic transformation in C3H 10T1/2 mouse embryo cells. *Radiat. Res.* **138** (Suppl.), S28-S31 (1994).

7. A.M. Ueno, D.B. Vannais, D.L. Gustafson, J.C. Wong, C.A. Waldren, A low, adaptive dose of gamma-rays reduced the number and altered the spectrum of S1 mutants in human-hamster hybrid A_L cells. *Mutat. Res.* **358,** 161-169 (1996).

8. Y. Kurihara, M. Rienkjkarn, and H. Etoh, Cytogenetic adaptive response of cultured fish cells to low doses of x-rays. *J. Radiat. Res.* **33**, 267-274 (1992).

9. N. Gajendiran, K. Tanaka, T.S. Kumaravel and N. Kamada, Neutron-induced adaptive response studied in G0 human lymphocytes using the comet assay. *J. Radiat. Res.* **42**, 91-101 (2001).

10. S.G. Sawant, G. Randers-Peherson, N.F. Metting and E.J. Hall, Adaptive response and the bystander effect induced by radiation in C3H 10T(1/2) cells in culture. *Radiat. Res.* **156**, 177-180 (2001).

11. J.D. Shadley and J.K. Wiencke, Induction of the adaptive respone by X-rays is dependent on radiation intensity. *Int. J. Radiat. Biol.* **56**, 107-118 (1989).

12. R.E.J. Mitchel, J.S. Jackson, D.P. Morrison, and S.M. Carlisle. Low doses of radiation increase the latency of spontaneous lymphomas and spinal

osteosarcomas in cancer-prone, radiation-sensitive *Trp53* heterozygous mice. *Radiat. Res.* **159**, 320-327 (2003).

13. R.E.J. Mitchel, J.S. Jackson, and S.M. Carlisle, Upper dose thresholds for radiation-induced adaptive response against cancer in high-dose-exposed, cancer-prone, radiation-sesitive *Trp53* heterozygous mice. *Radiat. Res.* **162**, 20-30 (2004).

14. P. Morales-Ramirez, and M.T. Mendiola-Cruz, Kinetics of the early adaptive response to gamma rays: Induction of a cellular radioprotective mechanisms in murine leukocytes In Vivo. *Biosci. Reports.* **24,** 609-616 (2004).

15. ICRP, Recommendations of the International Commission for Radiological Protection. Publication 60, *Annals of the ICRP*, Pergamon Press, Oxford, 1990.

16. T. Iwamatsu, Stages of normal development in the Medaka Oryzias latipes. *Mech. Of Dev.* **121**, 605-618 (2004).

17. J.R.K. Savage, Classification and relationships of induced chromosomal structural changes. *J. Med. Gen.* **12**, 103-122 (1975).

18. R.C. Littell, G.A. Milliken, W.W. Stroup, and R.D. Wolfinger, SAS system for fixed models. SAS Inst., Cary, NC (1996).

19. G. Der and B. Everett, Handbook of statistical analyses using SAS. CRC Press, Boca Raton, Fl, USA. (2001).

20. C. Streffer, Bystander effects, adaptive response and genomic instability induced by prenatal irradiation. *Mutat. Res.* **568**, 79-87 (2004).

21. UNSCEAR. Sources and effects of ionizing radiation. United Nations, New York (1996).

22. W. Wolff, Aspects of the adaptive response to very low doses of radiation and other agents. *Mutat. Res.* **358**, 135-142 (1996).

23. L. Cai, J. Jiang. Mild hyperthermia can induce adaption to cytogenetic damage caused by subsequent X irradiation. *Radiat. Res.* **143**, 26-33 (1995).

24. K. Yamaoka R. Edamatsu, T. Itoh and A. Mori, Effects of low-dose X-ray irradiation on biomembrane in brain cortex of aged rats. *Free Radic Biol.* Med **16**, 529-534. (1994).

25. L. Cai, M. Satoh, C. Tohyama and M.G. Cherian. Metallothionein in radiation exposure: Its induction and protective role. *Toxicology* **132**, 85-98 (1999).

26. J.H. Youngbloom, J.K. Wiencke and S. Wolff, Inhibition of the adaptive response in human lymphocytes to very low doses of ionizing radiation by the protein synthesis inhibitor cycloheximide. *Mutation Res.* **227**, 257-261 (1989).

27. J.H. Kim, K.H. Hahm, C.K. Cho and S.Y. Yoo, Protein biosynthesis in low dose ionizing radiation-adapted human melamona cells. *J. Radiat. Res.* **37**, 161-169 (1996).

28. L.E. Feinendegen, The role of adaptive responses following exposure to ionizing radiation. *Human and Exp. Tox.* **18**, 426-432 (1999).

29. W.U. Muller, C. Streffer, F. Niedereichholz, Adaptive response in mouse embryos? *Int. J. Radiat. Biol.* **62**, 169-175 (1992).

30. A. Wojcik, K. Bonk, W.U. Muller, C. Streffer, U. Weissenorn, and G. Obe, Absence of adaptive response to low doses of X-rays in preimplantation embryos and spleen lymphocytes of an inbred mouse strain as compared to human peripherial lymphocytes: a cytogenetic study, *Int. J. Radiat. Biol.* **62**, 177-186 (1992).

31. R.E. Mitchel, J.A. Dolling, J. Misonoh, D.R. Boreham, In-fluence of prior exposure to low-dose adapting radiation on radiation-induced teratogenic effects in fetal mice with varying Trp53 function. *Radiat. Res.* **158**, 458-463 (2002).

32. D.R. Boreham, J.A. Dolling, J. Misonoh, R.E. Mitchel, Radaiton-induced teratogenic effects in fetal mice with varying Trp53 function. *Radiat. Res.* **158**, 449-457 (2002).

33. B. Wang, H. Ohyama, T. Nose, H. Itsukaichi, T. Nakajima, O. Yukawa, T. Odaka, K. Tanaka, E. Kojima, T. Yamada, I. Hayata, Adaptive response in embryogenesis: I. Dose and timing of radiation for reduction of prenatal death and congenital malformation during the late period of organogenesis. *Radiat. Res.* **150**, 120-122 (1998).

34. B. Wang, H. Ohyama, K. Haginoya, T. Odaka, H. Itsukaichi, M. Nose, T. Nakajima, O. Yukawa, T. Yamada, I. Hayata, Adaptive response in embryogenesis II. Retardation of postnatal development of prenatally irradiated mice. *Radiat. Res.* **152**, 119-123 (1999).

35. B. Wang, H. Ohyama, Y. Shang, K. Tanaka, S. Aizawa, O. Yukawa, I. Hayata, Adaptive response in embryongenesis. V. Existence of two efficient dose-rate ranges for 0.3 Gy priming irradiation to adapt mouse fetuses. *Radiat. Res.* **161**, 264-272 (2004).

36. N. Egami and A. Hama, Dose-rate effects on the hatchability of irradiated embryos of the fish, Oryzias latipes. *Int. J. Radiat. Biol.* **28**, 273-278 (1975).

37. IAEA, International Atomic Energy Agency. Methodology for assessing impacts of radioactivity on aquatic ecosystems, Technical Report Series No. 172. Vienna (1979).

38. NCRP, National Council on Radiation Protection and Measurements, Effects of ionizing radiation on aquatic organisms. Report No. 109. Bethesda, MD (1991).

Table 3-1
Aberration frequencies per cell measured in F1 offspring originating from unirradiated and chronically irradiated F0 parents (Mean±SEM).

	F1 Dose (cGy)	F1 Offspring (n)	Number of Mitotic Cells Scored	Number of Aberrations per Cell			
				Total Gaps	Total Breaks	Total Exchanges	Total Aberrations per Cell
F0 Unirradiated	0	11	145	0.003±0.04	0.01±0.01	0±0	0.01±0.01[AB]
	2 + 500	11	93	0.04±0.02	0.09±0.05	0.10±0.01	0.23±0.10[DEH]
	21 +500	44	546	0.04±0.01	0.08±0.01	0.03±0.01	0.15±0.02[FGH]
	500	20	208	0.08±0.02	0.07±0.02	0.03±0.01	0.18±0.03[BCDF]
F0 Irradiated (10 cGy/day)	0	13	147	0±0	0.01±0.003	0.01±0.01	0.02±0.01[AB]
	2 +500	11	78	0.04±0.02	0.11±0.040	0±0	0.15±0.04[DEH]
	21 +500	41	378	0.09±0.02	0.10±0.02	0.03±0.01	0.22±0.03[FGH]
	500	14	132	0.09±0.03	0.08±0.03	0.03±0.02	0.20±0.06[BCDF]

*[A] No statistically significant difference in background total aberration frequencies (P=0.86).
*[B] Statistically significant difference in total aberration frequencies indicating the presence of radiation induced aberrations (P=0.003)
*[C] No statistically significant difference in total aberration frequencies (P=0.97)
*[D] No statistically significant difference in total aberration frequencies (P=0.93)
*[E]No statistically significant difference in total aberration frequencies (P=0.32)
*[F] No statistically significant difference in total aberration frequencies (P=0.83)
*[G]No statistically significant difference in total aberration frequencies (P=0.39)
* [H]No statistically significant difference in total aberration frequencies (P=0.92)

Table 3-2
Linear comparison of mean aberration frequencies for individual and specific combinations of aberrations in F1 offspring from irradiated and unirradiated F0 parents.

Aberration Type	Observed Mean Difference[2]	Standard Error	P-Value[1]
Gaps	0.01	0.02	0.39
Breaks	0.01	0.02	0.54
Total Exchanges	0.001	0.01	0.91
Gaps+Breaks	0.03	0.03	0.31
Total Exchanges – (Gaps+Breaks)	-0.02	0.02	0.20
Total Aberrations	0.01	0.03	0.86

*[1]For an F-test of the linear contrast within the 2X4 ANOVA.
*[2]Observed mean difference is the observed mean for the irradiated minus the observed mean for the unirradiated F0 parents.

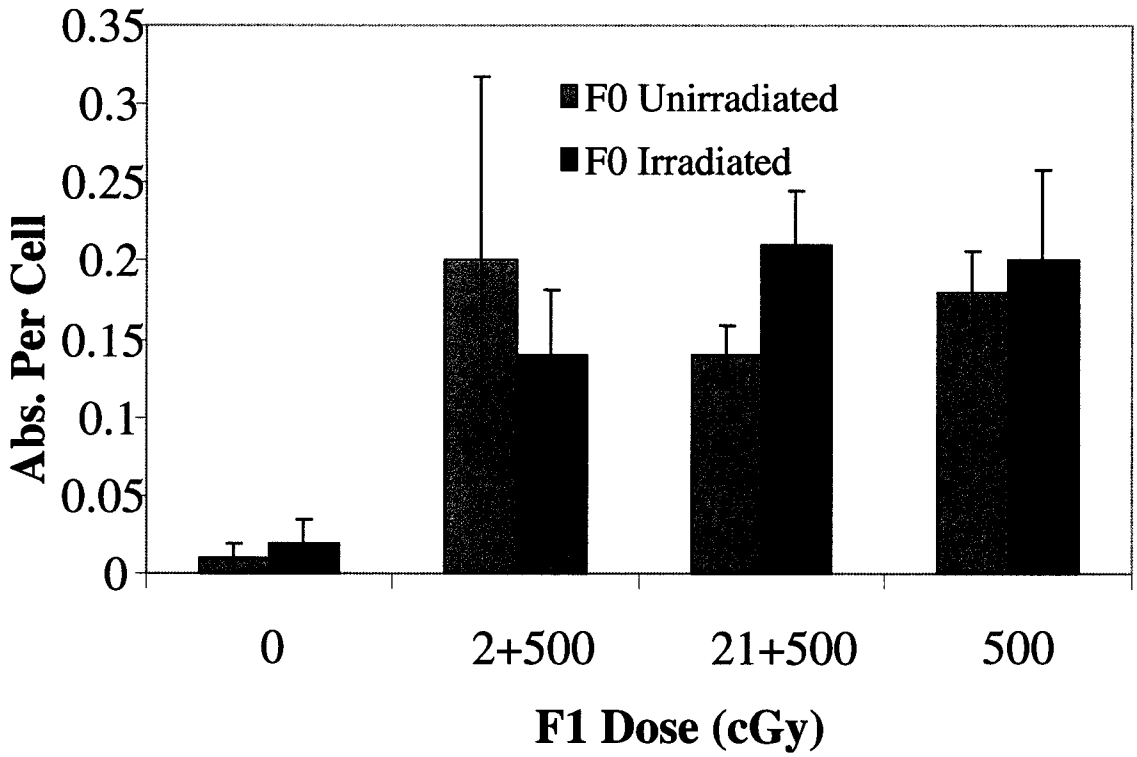

Figure 3-1 Mean total aberration frequencies in F1 offspring from unirradiated and chronically irradiated F0 parents. Error bars represent mean ± 1 standard error.

Table 3-3
Linear comparison of mean aberration frequencies for individual and specific combinations of abberations in F1 offspring receiving the 2 cGy adapting dose prior to the 500 cGy challenge dose versus those receiving the 500 cGy challenge dose alone.

Aberration Type	Observed Mean Difference[2]	Standard Error	P-Value[1]
Gaps	-0.05	0.02	0.04**
Breaks	0.03	0.03	0.43
Total Exchanges	-0.02	0.01	0.10
Gaps+Breaks	-0.02	0.04	0.56
Total Exchanges – (Gaps+Breaks)	0.03	0.02	0.44
Total Aberrations	-0.004	0.05	0.94

*[1]For an F-test of the linear contrast within the 2X4 ANOVA.
*[2]Observed mean difference is the observed mean for F1 offspring receiving the 2 + 500 cGy minus the observed mean for F1 offspring receiving the 500 cGy alone.
**Statistically significant difference measured for the formation of gaps (P=0.04) at the α=0.05 level.

Table 3-4
Linear comparison of mean aberration frequencies of individual and specific combinations of aberrations in F1 offspring receiving the 21 cGy adapting dose prior to the 500 cGy challenge dose versus the 500 cGy challenge dose alone.

Aberration Type	Observed Mean Difference[2]	Standard Error	P-Value[1]
Gaps	-0.02	0.02	0.20
Breaks	0.01	0.02	0.49
Total Exchanges	-0.001	-0.01	0.91
Gaps+Breaks	-0.01	0.03	0.83
Total Exchanges – (Gaps+Breaks)	-0.001	0.02	0.95
Total Aberrations	-0.01	0.03	0.83

*[1]For an F-test of the linear contrast within the 2X4 ANOVA.
*[2]Observed mean difference is the observed mean for F1 offspring receiving the 21 + 500 cGy minus the F1 offspring receiving the 500 cGy alone.

Table 3-5
Linear comparison of mean aberration frequencies of individual and specific combinations of aberrations in F1 offspring receiving the 21 cGy adapting dose + 500 cGy challenge dose versus F1 offspring receiving the 2 cGy adapting dose + 500 cGy challenge dose.

Aberration Type	Observed Mean Difference[2]	Standard Error	P-Value[1]
Gaps	-0.03	0.02	0.21
Breaks	0.01	0.03	0.74
Total Exchanges	-0.24	0.01	0.07
Gaps+Breaks	-0.02	0.03	0.062
Total Exchanges – (Gaps+Breaks)	0.02	0.02	0.035**
Total Aberrations	0.004	0.04	0.92

*[1]For an F-test of the linear contrast within the 2X4 ANOVA.
*[2]Observed mean difference is the observed mean for F1 offspring receiving the 21 + 500 cGy minus the observed mean for F1 offspring receiving the 2 + 500 cGy dose.
**Statistically significant difference at the α=0.05 level.

APPENDIX 1 – Preparation of Whole Chromosome-Specific Painting Probes for Japanese Medaka (*Oryzias latipes*)

Introduction

In the Glue Grant submitted to the Department of Energy we proposed to investigate GI by assessing the possible transmission of damage done to germ cells from irradiated males to their unirradiated F1 offspring thru the presence of symmetrical translocations. In order to measure the symmetrical translocations it was necessary to prepare FISH whole chromosome-specific painting probes prepared by microdissection and PCR.

Attempts at preparing the FISH whole chromosome-specific painting probes through microdissection and PCR showed that hybridization of the probe was possible, achieving ~80% coverage on chromosome #1, however full coverage of the chromosome was never achieved. The PCR product produced during the labeling or specific steps of the PCR protocol produced DNA products ~300 bp in length, and the desired product size was ~500-800 bp in length. Ultimately the signal produced during hybridization was often weak and didn't achieve full coverage on the chromosome. The protocol that produced the best hybridization signal is presented below. Hopefully modifications can be made to this protocol and full coverage can be achieved.

Metaphase Cell Collection, Fixation and Slide Preparation for Male Testes

Six-month to one year old adult male medaka were euthanized and testes removed using methods approved by the Animal Care and Use Committee at Colorado State University (Protocol Nos. 01-189A-01 and 03-181A-02). The testes were rinsed 2X in Phosphate Buffer Solution (Dulbecco's PBS) and placed in a 15 ml tube containing 1 ml of fresh PBS. The tissue was dissociated using liberal pipeting or by homogenizing in a Dunce Homogenizer. The cells were centrifuged at 1000 RPM for 10 minutes. The

supernatant was removed and 1 ml of 0.05% trypsin and 0.02% EDTA-Na in PBS was added for 5 minutes at 33°C. The trypsinization process was stopped by adding 4 ml of fresh L-15 media (Leibovitz's L-15 (Sigma, St. Louis, MO) cell media supplemented with 20% fetal bovine serum, streptomycin (50μg ml^{-1}) and penicillin (50 U ml^{-1}). Mitotic cells were collected by adding colcemid (0.1 μg ml^{-1}) (GIBCO, Grand Island, NY) to the cell suspension for 3 hours. The cells were centrifuged at 1000 RPM for 10 minutes. The supernatant was removed and the cells resuspended in 10 ml of hypotonic solution (75 mM KCl) for 20 minutes. To fix the cells, fresh methanol:acetic acid (3:1 vol/vol) was added drop-wise (~1 ml) to the hypotonic solution and allowed to sit at room temperature for 5 minutes. The cell suspension was centrifuged at 1000 RPM for 10 minutes. The cells were fixed an additional 2X with methanol:acetic acid (3:1 vol/vol) for 5 minutes and centrifuged at 1000 RPM for 4 minutes. The fixed cells were dropped onto cold wet slides and allowed to air dry. Once dry the slides were stained in freshly prepared 10% Giemsa in pH 6.8 buffer for 10 minutes.

Microdissection

Metaphase chromosomes, dropped onto micro cover slip slides, were dissected using an Eppendorf Micromanipulator, Model 5171 (Eppendorf North America, Inc., Madison, WI), mounted on a Zeiss Axiovert 100 inverted microscope (Carl Zeiss, Inc., Thornwood, NY) (1-3). Glass capillary tubes, ~1.2 mm diameter, were pulled into needles using a Kopf needle puller (Tujunga, CA), and sterilized with germicidal UV light in a Stratagene Stratalinker 1800 UV irradiator (Stratagene, La Jolla, CA) for 20 minutes.

Whole-Chromosome Painting Probe Preparation

Ten chromosomes, each of chromosomes Number 1, were collected by microdissection and placed into sterilized 200 µl flat top PCR microcentrifuge tubes. The chromosomes were then amplified by degenerative oligonucleotide primer (DOP) Polymerase Chain Reaction (PCR) using techniques previous described by (4). The amplification was done in a 20-µl volume containing 2 µl ThermoSequenase polymerase (4 U μl^{-1}) (USB, Cleveland, Ohio), 2 µl ThermoSequenase reaction buffer, 2.5 mM each dTTP, dCTP, dATP, and dGTP (Sigma-Aldrich, Inc., Saint Louis, MO), 2 µM 6-MW Telenius (DOP) primer (5´-CCGACTCGAGNNNNNNATGTGG-3´) (MacroMolecular Resources, Fort Collins, CO), and 10 µl mineral oil to prevent evaporation. The reaction was run in an MJR PTC-100 Programmable Thermal Controller (MJ Research, Boston, MA) using a heating, annealing and extension profile of 95°C for 3 minutes, 10 cycles at 94°C for 1 minute 30 seconds, 30°C for 1 minute 30 seconds, and a ramp of 0.2°C per second up to 72°C 2 minutes, 50 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 3 minutes, followed by 72°C for 5 minutes and held at 4°C until removed.

The products were verified by running 4 µl of sample with 0.8 µl of loading dye on a 1.5% Agarose Gel for 60 minutes at 90 Volts and comparing to a DNA ladder. Products were then labeled in a 25-µl volume containing 2.5 µl ThermoSequenase polymerase (4 U μl^{-1}) (USB), 2.5 µl ThermoSequenase reaction buffer, 2.5 mM each dTTP, dCTP, dATP, and dGTP (Sigma-Aldrich, Inc., Saint Louis, MO) 2 µM 6-MW Telenius DOP primer (MacroMolecular Resources, Fort Collins, CO), 1 µl of DNA sample and 10 µl of mineral oil to prevent evaporation. In addition, 125 nmol Dioxigenin-11-dUTP (Boehringer, Mannheim) was added. The thermal profile for the

labeling consisted of 1 cycle at 95°C for 5 minutes, 30 cycles at 94°C for 1 minute, 56°C for 1 minute, and 72°C for 3 minutes, followed by 72°C for 5 minutes and held at 4°C until removed.

Fluorescence in situ Hybridization (FISH)

FISH was carried out essentially as described by Pinkel and coworkers (5) and by Lichter and coworkers (6). Target slides onto which the hybridization probe was placed were prepared by denaturing the chromosomes on slides in a coplin jar containing 70% formamide in 2X saline sodium citrate (SSC) at pH 5.8 for 2-5 minutes at 70°C. The slide was quickly transferred to ice cold 70% ethanol for 2 minutes, followed by transfer to 85% and then 100% room temperature ethanol and finally by drying with a clean air jet. Approximately 40 µl of the hybridization mixture containing 50% formamide, 10% dextran sulfate, 2X SSC (pH 6.3), 1 µl of probe PCR labeled with Dioxigenin-11-dUTP and 1 µl of salmon sperm Cot DNA (see following subsection for details on Cot preparation) was denatured for 10 minutes at 84°C and then reannealed for 30-60 minutes at 37°C. The probe hybridization mixture was then placed on the slide, covered by a 22 x 50 mm cover slip and sealed with rubber cement. Hybridization was allowed to occur for 24-36 hours at 37°C. After probe hybridization to the denatured chromosomes, the cover slips were removed, the slides were washed in two changes of 50% formamide in 2X SSC, pH 5.8, at 42°C and then were washed twice more in 2X SSC, pH 6.3. and twice in 1X PN buffer for 3-5 minutes each, also at 42°C.

For probe detection, PN buffer containing 5% non-fat dry milk was applied for 5 minutes at room temperature, then the solution was drained from the slide and a solution of 200 µg ml^{-1} Anti-Digoxigenin-Rhodamine in distilled water was applied to the slide.

The slide was incubated for 1 hour at 37°C, and then rinsed in two changes of 1X PN buffer at 42°C for 3-5 minutes each. For microscopy, the slides were counterstained with a solution of slowfade containing 4,6-diamidino-2-phenylindole (DAPI) at a concentration of 2 g ml^{-1} under a cover slip. Slides were then viewed under a fluorescence microscope with appropriate excitation and emission filters.

Cot DNA preparation

The blocking Cot DNA was prepared by mixing Salmon Sperm DNA, 1 mg ml^{-1}, (Sigma-Aldirch, Inc., Saint Louis, MO) with distilled water. The solution was sonicated to approximately 500 bp. The weight of the sample was verified by running 4 µl with loading dye on a 1.5% Agarose Gel for 45 minutes at 90 Volts and comparing the size of the sample to a DNA ladder. The sample was then placed in boiling water for 30 minutes, transferred to a 65°C water bath for 4 minutes, and NaCl solution was added to achieve a final concentration of 0.3 M. Reannealing was allowed to proceed for 5.9 minutes. An equal volume of ice cold 10X S1 Nuclease Buffer (0.5 M NaCl, 2mM $ZnSO_4$, 0.06 M NaOAc (pH 4.2), 10% Glycerol) was added along with 1 unit of S1 nuclease per µg of DNA and allowed to incubate for 30 minutes at 37°C. An equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added for a final extraction and centrifuged at 2750 RPM for 10 minutes. The supernatant was collected and ½ the volume of 7.5 M Ammonium Acetate added along with twice the volume of 100% Ethyl Alcohol. The solution was centrifuged for 2 minutes at 2750 RPM and the supernatant removed. The pellet was washed with 70% Ethanol, air dried briefly and resuspended in 0.5 ml of TE Buffer (1 mg ml^{-1}).

Recipes

20X SSC (Saline Sodium Citrate)
3 M NaCl
0.3 M Na Citrate

10X PN Buffer, pH 6.8
500 ml 1M Na2HPO4, dibasic
100 ml 1M NaH2PO4, monobasic
Add monobasic to entire dibasic until you reach (pH 8) (~45-50 ml). Then add 0.05% Nonidet NP-40 (same as IGEPAL CA-630, Sigma-Aldrich, Inc., Saint Louis, MO)

References

1. X.Y. Guan, J.M. Trent and P.S. Meltzer, Generation of band-specific painting probes from a single microdissected chromosome. *Human Molecular Genetics* **2**, 1117-1121 (1993).

2. M. Muhlmann-Diaz, A.T. Christian and J.S. Bedford, Chromosome microdissection, In *Radiation Research 1895-1995 Congress Proceedings* (Hagen, U., Harder, D., Jung, H., and Streffer, C., Eds.), pp. 468-469. International Congress of Radiation Research, Wurzburg,Germany, (1995).

3. K.J. Bussey, Chromosome microdissection: on the cutting edge. *Applied Cytogenetics.* **22**, 30-36 (1996).

4. H. Telenius, N.P. Carter, C.E. Bebb, M. Nordenskjold, B.A. Ponder, A. Tunnacliffe, Degenerate oligonucleotide-primed PCR: general amplification of target DNA by a single degenerate primer. *Genomics.* **13**,718-725 (1992).

5. D. Pinkel, T. Straume and J.W. Gray, Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. *Proc. Natl. Acad. Sci.* **83**, 2934-2938 (1986).

6. P. Lichter, T. Cremer, J. Borden, L. Manuelidis and D.C. Ward, Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridization using recombinant DNA libraries. *Hum. Genet.* **80**, 224-234 (1998).

APPENDIX 2 - Multiple Comparison Test of Data

For the genomic instability and adaptive response experiments multiple breeding pairs were established and the data generated was a measure of cytogenetic damage in F1 offspring from either unirradiated or chronically irradiated parents. In the original statistical design, multiple F1 embryos were to be collected from the same breeding pair and the data from the individual embryos was going to be pooled together and analyzed. The mean aberration frequency for each breeding pair would then be compared against each other to establish breeding pair effects. However, a large degree of mitotic variability was measured among the individual embryos from the same breeding pair which complicated the statistical analysis.

In order to account for the high degree of variability in the mitotic indices a decision was made to change the experimental unit from the breeding pair to the individual embryo and to include only those embryos having 5 or more score able cells in the statistical analysis. Changing the experimental unit to the embryo resulted in unequal sample sizes among the groups. The statistical test used to account for the unequal sample size is known as a non-orthogonal linear contrast. Simply stated this test is a comparison of means by using an analysis of variance test.

The analysis of data was conducted at the same time for both experiments and included an F-test for the linear comparison of means from 2 fixed and 4 independent variables. The 2 fixed variables are F0 Unirradiated, written as F0=N, and F0 Irradiated, written as F0=Y. The four independent variables are F1 Unirradiated, written as F1=N, F1 embryos receiving 2 cGy + 500 cGy, written as F1=A2C, F1 embryos receiving 21 cGy + 500 cGy, written as F1=A21C, and F1 embryos receiving the 500 cGy dose alone, written as F1=C. The mean aberration frequencies for each case are tested in a 2X4

Analysis of Variance test which can be easier to comprehend if you view these variables in a table such as the one below.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated				
F0 Irradiated				

In this type of design 12 different hypotheses could be tested to determine significant differences based on observed mean aberration frequencies. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the standard error or, more appropriate by an F-test computed for the linear contrast (Littell et al. 1996).

Explanations of specific comparisons

Chapter 2 – Genomic Instability

In Chapter 2 three different hypotheses were tested to determine if a genomic instability could be measured using the G2 Chromosome assay. The information reported below will describe the hypothesis tested in each table.

Table 2-4 : A test of the null hypothesis of equal frequencies of aberrations in unirradiated offspring (ie. F1=N) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) using a linear combination of mean aberrations. The null hypothesis has the form H0: (F0=Y)-(F0=N) = 0 when F1=N. The linear contrast of the means has the expected value of 0 (ie. Equal means for F0=Y and F0=N) when the null

hypothesis is true. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the standard error or, more appropriate by an F-test computed for the linear contrast (Littell et al. 1996). For this test, observed means significantly >0 indicate greater frequencies of aberrations among unirradiated offspring when their parents had been irradiated.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated	**X**			
F0 Irradiated	**X**			

Table 2-6: A test of the null hypothesis of equal frequencies of aberrations in F1 offspring receiving the 500 cGy challenge dose of gamma rays (ie. F1=C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) using a linear combination of mean aberrations. The null hypothesis has the form H0: {(F1=C)-(F1=N)@F0=Y}-{(F1=C)-(F1=N)@F0=N}=0. The linear contrast of the means has the expected value of 0 (ie. Equal means for F0=Y and F0=N) when the null hypothesis is true. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the standard error or, more appropriate by an F-test computed for the linear contrast (Littell et al. 1996). For this test, observed means significantly >0 indicate greater frequencies of aberrations among offspring when their parents had been irradiated.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated				**X**
F0 Irradiated				**X**

Table 2-7: A test of the null hypothesis of equal frequencies of aberrations in F1 offspring receiving the 500 cGy challenge dose of gamma rays (ie. F1=C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) versus frequencies of aberrations in unirradiated offspring (ie. F1=N) originating from unirradiated (ie. F0=N) and irradiated parents (F0=Y) using a linear combination of mean aberrations. The null hypothesis has the form H0: (F1=C) – (F1=N) = 0 when F0=Y and F0=N. The linear contrast of the means has the expected value of 0 (ie. Equal means for F0=Y and F0=N) when the null hypothesis is true. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the standard error or, more appropriate by an F-test computed for the linear contrast (Littell et al. 1996). For this test, observed means significantly >0 indicate greater frequencies of aberrations among offspring receiving the 500 cGy challenge dose versus unirradiated offspring.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated	**X**			**X**
F0 Irradiated	**X**			**X**

Chapter 3

In Chapter 3 four different hypotheses were tested to determine if an adaptive response could be measured using the G2 Chromosome assay. The information reported below will describe the hypothesis tested in each table.

Table 3-2: A test of the null hypothesis of equal frequencies of aberrations in unirradiated offspring (ie. F1=N) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) using a linear combination of mean aberrations. The null hypothesis has the form H0: (F0=Y) – (F0=N)=0 when F1=N. The linear contrasts of means has the expected value of 0 (ie. Equal means for F0=Y and F0=N) when the null hypothesis is true. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the statistical error or, more appropriate by an F-test computed for the linear contrast (Littell et al. 1996). For this test, observed means significantly >0 indicate greater frequencies of aberrations among unirradiated offspring when their parents had been irradiated.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated	**X**			
F0 Irradiated	**X**			

Table 3-3: A test of the null hypothesis of equal frequencies of aberrations in offspring receiving the 2 cGy adapting dose and 500 cGy challenge dose (ie. F1=A2C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) and offspring receiving the 500 cGy challenge dose alone (ie. F1=C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) using a linear combination of mean aberrations. The null hypothesis has the form H0: (F1=A2C)-(F1=C) = 0 when F0= Y and F0=N. The linear contrasts of means has the expected value of 0 (ie. Equal means for F1=A2C and F1=C) when the null hypothesis is true. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the statistical error or, more appropriate by an F-test computed for the linear contrast (Littell

et al. 1996). For this test, observed means significantly >0 indicate greater frequencies of aberrations among offspring receiving the 2 cGy adapting dose and 500 cGy challenge dose verses those receiving the 500 cGy challenge dose alone regardless if their parents had been chronically irradiated or not.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated		**X**		**X**
F0 Irradiated		**X**		**X**

Table 3-4: A test of the null hypothesis of equal frequencies of aberrations in offspring receiving the 21 cGy adapting dose and 500 cGy challenge dose (ie. F1=A21C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) and offspring receiving the 500 cGy challenge dose alone (ie. F1=C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) using a linear combination of mean aberrations. The null hypothesis has the form H0: (F1=A21C)-(F1=C) = 0 when F0= Y and F0=N. The linear contrasts of means has the expected value of 0 (ie. Equal means for F1=A21C and F1=C) when the null hypothesis is true. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the statistical error or, more appropriate by an F-test computed for the linear contrast (Littell et al. 1996). For this test, observed means significantly >0 indicate greater frequencies of aberrations among offspring receiving the 21 cGy adapting dose and 500 cGy challenge dose verses those receiving the 500 cGy challenge dose alone regardless if their parents had been chronically irradiated or not.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated			**X**	**X**
F0 Irradiated			**X**	**X**

Table 3-5: A test of the null hypothesis of equal frequencies of aberrations in offspring receiving the 21 cGy adapting dose and 500 cGy challenge dose (ie. F1=A21C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) and offspring receiving the 2 cGy challenge dose and the 500 cGy challenge dose (ie. F1=A2C) originating from unirradiated parents (ie. F0=N) and irradiated parents (ie. F0=Y) using a linear combination of mean aberrations. The null hypothesis has the form H0: (F1=A2C)-(F1A21C)=0 when F0=Y and F0=N. The linear contrasts of means has the expected value of 0 (ie. Equal means for F1=A2C and F1=A2C) when the null hypothesis is true. Observed means that differ from 0 may be tested for significance using a t-test of the observed mean divided by the statistical error or, more appropriate by an F-test computed for the linear contrast (Littell et al. 1996). For this test, observed means significantly >0 indicate greater frequencies of aberrations among offspring receiving the 2 cGy adapting dose and 500 cGy challenge dose verses those receiving the 21 cGy challenge dose and 500 cGy challenge dose regardless if their parents had been chronically irradiated or not.

	F1 No Irradiation	F1 2 cGy +500 cGy	F1 21 cGy +500 cGy	F1 500 cGy
F0 Unirradiated		**X**	**X**	
F0 Irradiated		**X**	**X**	

APPENDIX 3 - Glossary of Terms

- Adaptive Response (AR) – a phenomenon whereby exposure to low doses of ionizing radiation prior to a large challenge dose reduces the amount of damage inflicted by the large challenge dose alone.

- Break- a separation of chromatid material at a distance greater than the diameter of the of the chromatid arm or completely separate from the chromatid axis.

- Chromatid -one of the paired chromosome strands, joined at the centromere, which make up a metaphase chromosome, resulting from chromosome reduplication during the S phase (DNA synthetic phase) of interphase.

- DOE – The U.S. Department of Energy (DOE) is a federal agency with the mission to advance the national, economic, and energy security of the United States; to promote scientific and technological innovation in support of that mission; and to ensure the environmental cleanup of the national nuclear weapons complex.

- Exchange- a rejoining of segments of differing chromatids appearing either as a symmetrical or assymetrical exchange of material.

- Gap – a visual non-staining region of the chromatid arm that is less than or equal to the diameter of the chromatid arm.

- Genomic Instability (GI) - A phenomenon whereby radiation-induced effects become evident in the progeny of *in vitro* or *in vivo* models, and become evident in the first or subsequent generations.

- Gray (Gy) - a unit used to measure a quantity called absorbed dose. This relates to the amount of energy actually absorbed in some material, and is used for any type of radiation and any material. One gray is equal to one joule of energy deposited in one kg of a material. Absorbed dose is often expressed in terms of hundredths of a gray, or centi-grays. One gray is equivalent to 100 rads.

- *In Vitro* – An experimental technique where the experiment is conducted outside the organism or cell; occurring in an artificial environment.

- *In Vivo* – An experiment technique where the experiment is conducted inside the organism or cell; occurring within the living body of the organism.

APPENDIX 4 – G2 Chromosome assay aberration data

Japanese Medaka G2 Chromosome Assay Data for Genomic Instability and Adaptive Response

F0	F1 Dose (cGy)	Breeding Pair No.	Embryo No.	No. of Cells Scored	Gaps	Breaks	Assymetrical Exchange	Symmetrical Exchange	Total Exchange	Total Aberrations
Unirradiated	0	1	1	37	0	0	0	0	0	0
Unirradiated	0	1	2	14	0	0	0	0	0	0
Unirradiated	0	1	4	6	0	0	0	0	0	0
Unirradiated	0	4	3	5	0	0	0	0	0	0
Unirradiated	0	4	6	12	0	0	0	0	0	0
Unirradiated	0	4	7	6	0	0	0	0	0	0
Unirradiated	0	5	1	25	1	1	0	0	0	2
Unirradiated	0	5	4	8	0	0	0	0	0	0
Unirradiated	0	16	1	5	0	0	0	0	0	0
Unirradiated	0	16	2	13	0	1	0	0	0	1
Unirradiated	0	16	4	14	0	0	0	0	0	0
Unirradiated	2 +500	3	4	17	1	1	1	0	1	3
Unirradiated	2 +500	3	9	5	0	0	0	0	0	0
Unirradiated	2 +500	9	3	6	0	0	0	0	0	0
Unirradiated	2 +500	9	9	5	0	0	0	0	0	0
Unirradiated	2 +500	9	10	6	0	2	0	0	0	2
Unirradiated	2 +500	10	1	17	0	3	1	0	1	4
Unirradiated	2 +500	10	5	9	0	4	0	0	0	4
Unirradiated	2 +500	12	1	12	0	0	0	0	0	0
Unirradiated	2 +500	12	2	5	1	0	0	0	0	1
Unirradiated	2 +500	12	5	5	0	0	0	0	0	0
Unirradiated	2 +500	12	6	6	7	1	0	0	0	8
Unirradiated	21 + 500	3	1	5	0	1	0	0	0	1
Unirradiated	21 + 500	3	3	10	0	3	0	1	1	4
Unirradiated	21 + 500	3	7	9	0	1	0	0	0	1
Unirradiated	21 + 500	4	4	8	0	1	0	1	1	2

Unirradiated	21 + 500	4	5	9	0	2	0	0	0	2
Unirradiated	21 + 500	4	7	15	0	1	0	0	0	1
Unirradiated	21 + 500	5	2	9	1	0	0	0	0	1
Unirradiated	21 + 500	7	3	7	0	1	1	0	1	2
Unirradiated	21 + 500	7	5	6	0	1	1	0	1	2
Unirradiated	21 + 500	9	6	10	0	0	0	0	0	0
Unirradiated	21 + 500	9	7	13	2	2	0	0	0	4
Unirradiated	21 + 500	9	9	6	0	1	0	0	0	1
Unirradiated	21 + 500	9	10	12	1	1	0	0	0	2
Unirradiated	21 + 500	11	2	15	0	1	0	0	0	1
Unirradiated	21 + 500	11	4	7	0	0	0	0	0	0
Unirradiated	21 + 500	11	6	8	1	0	0	0	0	1
Unirradiated	21 + 500	11	7	11	1	0	0	0	0	1
Unirradiated	21 + 500	11	8	7	0	0	1	0	1	1
Unirradiated	21 + 500	11	1	16	1	3	0	0	0	4
Unirradiated	21 + 500	11	2	9	1	0	0	0	0	1
Unirradiated	21 + 500	11	3	12	0	0	0	0	0	0
Unirradiated	21 + 500	11	4	23	0	2	0	0	0	2
Unirradiated	21 + 500	11	5	12	2	1	0	0	0	3
Unirradiated	21 + 500	11	6	7	0	0	2	0	2	2
Unirradiated	21 + 500	11	7	10	0	0	0	0	0	0
Unirradiated	21 + 500	11	8	29	1	4	0	1	1	6
Unirradiated	21 + 500	11	10	18	0	2	0	0	0	2
Unirradiated	21 + 500	12	2	5	0	1	0	0	0	1
Unirradiated	21 + 500	12	8	9	3	0	0	0	0	3
Unirradiated	21 + 500	15	1	6	0	3	0	0	0	3
Unirradiated	21 + 500	15	6	6	0	0	0	0	0	0
Unirradiated	21 + 500	16	1	14	0	0	1	0	1	1
Unirradiated	21 + 500	16	2	17	0	0	0	0	0	0
Unirradiated	21 + 500	16	3	13	2	1	0	0	0	3
Unirradiated	21 + 500	16	9	5	0	0	0	0	0	0
Unirradiated	21 + 500	17	1	37	1	1	0	0	0	2
Unirradiated	21 + 500	17	2	10	0	0	0	1	1	1

Unirradiated	21 + 500	17	3	16	1	1	0	0	0	2
Unirradiated	21 + 500	17	4	6	0	0	0	0	0	0
Unirradiated	21 + 500	17	5	6	0	1	0	0	0	1
Unirradiated	21 + 500	17	6	11	1	0	0	0	0	1
Unirradiated	21 + 500	17	7	6	0	1	0	0	0	1
Unirradiated	21 + 500	17	9	10	1	0	0	0	0	1
Unirradiated	21 + 500	17	10	66	0	0	0	2	2	2
Unirradiated	500	1	1	5	2	0	0	0	0	2
Unirradiated	500	1	2	16	1	0	0	0	0	1
Unirradiated	500	1	4	10	0	0	1	0	1	1
Unirradiated	500	1	5	7	1	0	0	0	0	1
Unirradiated	500	2	1	12	2	2	0	0	0	4
Unirradiated	500	2	2	27	0	1	3	1	4	5
Unirradiated	500	2	4	18	1	1	0	0	0	2
Unirradiated	500	2	6	10	0	2	1	0	1	3
Unirradiated	500	4	2	5	0	1	0	0	0	1
Unirradiated	500	4	3	11	1	0	1	0	1	2
Unirradiated	500	5	5	10	0	0	0	1	1	1
Unirradiated	500	5	6	11	1	1	0	0	0	2
Unirradiated	500	6	2	8	2	1	0	0	0	3
Unirradiated	500	6	4	10	0	0	0	0	0	0
Unirradiated	500	7	10	8	0	2	0	0	0	2
Unirradiated	500	9	6	8	0	0	0	0	0	0
Unirradiated	500	9	7	5	1	0	0	0	0	1
Unirradiated	500	9	10	12	1	0	0	0	0	1
Unirradiated	500	15	2	7	0	1	0	0	0	1
Unirradiated	500	15	5	8	1	1	1	0	1	3
Irradiated	0	4	3	12	0	0	0	0	0	0
Irradiated	0	4	5	17	0	0	0	0	0	0
Irradiated	0	4	8	13	0	0	0	0	0	0
Irradiated	0	4	9	9	0	0	0	0	0	0
Irradiated	0	5	4	8	0	0	0	0	0	0
Irradiated	0	5	5	4	0	0	0	0	0	0

Irradiated	0	5	7	9	0	0	0	0	0	0
Irradiated	0	6	1	14	0	1	0	1	1	2
Irradiated	0	6	2	17	0	0	2	0	2	2
Irradiated	0	8	1	11	0	0	0	0	0	0
Irradiated	0	8	2	6	0	0	0	0	0	0
Irradiated	0	8	9	8	0	0	0	0	0	0
Irradiated	0	18	1	10	0	0	0	0	0	0
Irradiated	0	18	4	13	0	1	0	0	0	1
Irradiated	2 +500	6	3	8	0	0	0	0	0	0
Irradiated	2 +500	6	4	12	0	3	0	0	0	3
Irradiated	2 +500	14	2	5	0	1	0	0	0	1
Irradiated	2 +500	14	10	8	1	2	0	0	0	3
Irradiated	2 +500	19	6	5	0	0	0	0	0	0
Irradiated	2 +500	19	10	5	0	1	0	0	0	1
Irradiated	2 +500	23	3	6	0	2	0	0	0	2
Irradiated	2 +500	23	4	7	1	0	0	0	0	1
Irradiated	2 +500	23	8	6	0	0	0	0	0	0
Irradiated	2 +500	23	9	7	1	0	0	0	0	1
Irradiated	2 +500	23	10	9	0	0	0	0	0	0
Irradiated	21 +500	1	7	13	5	0	0	0	0	5
Irradiated	21 +500	2	1	11	1	0	2	2	4	5
Irradiated	21 +500	2	3	5	1	0	0	0	0	1
Irradiated	21 +500	2	4	6	0	2	0	0	0	2
Irradiated	21 +500	2	7	27	0	0	0	1	1	1
Irradiated	21 +500	3	1	8	1	2	0	0	0	0
Irradiated	21 +500	4	3	5	0	0	0	0	0	0
Irradiated	21 +500	4	4	20	0	0	0	0	0	0
Irradiated	21 +500	5	1	8	3	4	0	0	0	7
Irradiated	21 +500	5	2	5	1	0	0	0	0	1
Irradiated	21 +500	5	3	11	2	0	0	0	0	2
Irradiated	21 +500	5	5	11	1	2	0	0	0	3
Irradiated	21 +500	6	5	5	0	2	0	0	0	2
Irradiated	21 +500	6	10	6	0	0	0	0	0	0

Irradiated	21 +500	7	1	13	1	7	0	0	0	8
Irradiated	21 +500	7	3	5	0	0	0	0	0	0
Irradiated	21 +500	7	6	12	0	1	0	0	0	1
Irradiated	21 +500	7	7	6	0	0	0	0	0	0
Irradiated	21 +500	7	8	5	0	1	1	0	1	2
Irradiated	21 +500	14	3	6	1	0	1	0	1	2
Irradiated	21 +500	14	4	5	1	0	0	0	0	1
Irradiated	21 +500	14	9	10		1	1	0	1	2
Irradiated	21 +500	15	1	5	0	0	0	0	0	0
Irradiated	21 +500	15	2	17	0	1	0	1	1	2
Irradiated	21 +500	15	4	10	1	0	0	0	0	1
Irradiated	21 +500	15	6	11	0	0	0	0	0	0
Irradiated	21 +500	15	7	12	0	1	0	0	0	1
Irradiated	21 +500	15	8	7	0	1	0	0	0	1
Irradiated	21 +500	15	10	9	0	1	0	0	0	1
Irradiated	21 +500	18	1	6	0	0	0	1	1	1
Irradiated	21 +500	18	2	10	1	0	0	0	0	1
Irradiated	21 +500	18	3	14	2	0	0	0	0	2
Irradiated	21 +500	18	4	6	1	0	0	0	0	1
Irradiated	21 +500	18	5	12	1	0	0	1	1	2
Irradiated	21 +500	18	6	14	0	0	0	0	0	0
Irradiated	21 +500	18	7	5	0	0	0	0	0	0
Irradiated	21 +500	18	8	5	1	1	0	0	0	2
Irradiated	21 +500	20	1	7	0	2	0	0	0	2
Irradiated	21 +500	20	5	11	2	2	0	0	0	4
Irradiated	21 +500	20	6	7	3	0	0	0	0	3
Irradiated	21 +500	20	8	7	1	4	1	0	1	6
Irradiated	500	4	3	7	1	1	0	0	0	2
Irradiated	500	4	4	16	0	0	0	0	0	0
Irradiated	500	4	6	6	2	0	0	1	1	3
Irradiated	500	4	7	6	0	1	0	0	0	1
Irradiated	500	5	3	7	1	3	1	0	1	5
Irradiated	500	5	5	11	0	2	0	0	0	2

Irradiated	500	9	2	8	2	1	0	0	0	3
Irradiated	500	9	5	5	1	0	0	0	0	1
Irradiated	500	9	9	11	0	0	1	0	1	1
Irradiated	500	15	4	10	2	1	0	0	0	3
Irradiated	500	15	5	8	0	0	0	0	0	0
Irradiated	500	15	6	8	0	0	0	0	0	0
Irradiated	500	15	7	6	0	0	0	0	0	0
Irradiated	500	15	8	23	0	0	0	0	0	0