THE OOCYTE AS A TRANSMITTER AND MEDIATOR OF GENETIC DAMAGE TO OFFSPRING

by

CORY SANDS GRESHAM

(Under the Direction of Travis Glenn)

ABSTRACT

Although numerous chemical germ cell mutagens have been identified in research animals, no chemical mutagens have been positively identified in human germ cells. The paucity of effective methods to investigate mutations in human germ cells intensifies the need for germ cell mutation research in animal models. Furthermore, the vast majority of existing germline mutagenesis research has focused exclusively on male germ cells while very little is known about mutagenesis in female germ cells. The λ transgenic medaka (a small fish carrying the *cII* mutation reporter gene) was used to investigate the various roles of the oocyte in the transmission and repair of mutations from mutagen-treated parents to their offspring. To investigate the direct transmission of genetic damage to offspring via the oocyte, female transgenic medaka were exposed to the chemical mutagen, 1-ethyl-1-nitrosourea, and bred to wild-type males. Eighteen percent of the offspring (14/80) displayed an increased frequency of mutations in the *cII* gene (*cII* MF). This frequency of mutant offspring indicated that female germ cells were more sensitive to chemical mutagens than premeiotic male germ cells (18% vs. 4%, X^2, $p<0.01$), which contradicted past research. Further experiments revealed 2% of offspring derived from

treated oocytes showed increased *cII* MF in the unexposed, paternally derived *cII* gene. These untargeted mutations occurred in regions of DNA never exposed to the mutagen, a hallmark of genomic instability. Untargeted experiments were repeated in transgenic mice to further investigate untargeted mutations, and revealed a similar response in both fish and mammalian animal models, thus indicating a conserved mechanism. The untargeted mutations occurred post-fertilization, under the direction of the early embryo. Finally, the role of the oocyte's DNA repair enzymes in response to DNA damage in the early embryo was investigated. The frequency of affected offspring derived from oocytes lacking functional p53 was significantly higher than offspring derived from wild-type oocytes (36% vs. 11%, X^2, $p<0.01$). These experiments demonstrated that female germ-cells may be equally sensitive to chemical mutagens as male germ cells. Additionally, mutations observed in the offspring were fixed after fertilization, where DNA damage responses likely depended on the cellular processes of the oocyte.

INDEX WORDS: MUTATION, OOCYTE, MEDAKA, TRANSGENIC

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DEDICATION

This work is dedicated to my patient and loving wife, Ashley, and my father for all of his help editing this manuscript. Thanks to our very supportive families who have always been there for us.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Nearly 5% of all live births in the United States are affected by birth defects, chromosomal abnormalities, or other *de novo* genetic diseases, which cost billions of dollars in healthcare related expenses and often create lifelong afflictions in the affected children (1-3). These genetic diseases include some of the most salient disorders such as: various neoplasias, autism spectrum disorders, Down's syndrome, cystic fibrosis, and Tay-Sachs disease (4-9). Despite the financial, social, and medical impact of these genetic abnormalities, no single chemical has been definitively proven to induce mutations in human germ cells (10-12). In contrast, numerous chemical germ cell mutagens from both environmental sources (13, 14) and pharmaceuticals (15-20) have been identified in research animals (21, 22). DeMarini, a leader in the fields of environmental mutagenesis and genetic toxicology, recently proposed that evidence from studies of animal germ cells and epidemiological surveys is sufficient to declare the existence of human germ cell mutagens (12).

Investigating Mutations in Human Germ Cell: Challenges and Progress

A consortium of leading researchers concluded that the discrepancy between animal models and human research is due to 'technological limitations' for detecting germ cell mutations in humans, rather than human germ cells being resistant to mutagens (10). Studies of germ-cell mutagenesis in humans face numerous challenges such as: insufficient numbers of human subjects, insensitive methods to detect mutations,

inadequate length, intensity, and type of exposures, inadequate dosimetry (measurement and calculation of radiation exposure or dose received by a tissue) (23), and inability to efficiently detect *de novo* mutations in the human genome (10). The few studies that have been published on human mutations largely focused on two groups, [1] cancer/ chemotherapy survivors and their offspring as well as [2] survivors of large-scale radiation exposures and their offspring (e.g. atomic bomb detonations in Hiroshima and Nagasaki, Japan, and the accident at Chernobyl Nuclear Power Plant in Pripyat, Ukraine). Studies of chemotherapy and radiation survivors generally employed two methods to detect mutations in human germ cells; either direct detection of gene mutation and chromosome breakage in sperm, or analysis of change of length mutations in minisatellite regions of DNA in sperm or offspring (10). More recently, next-generation DNA sequencing has begun to shed light on *de novo* mutations (6-9).

Multiple chemotherapy agents (cyclophosphamide, mitomycin-C, chlorambucil, bleomycin, and procarbazine) have been shown to induce a significant increase in mutation rates in the sperm and offspring of treated male mice, (17, 18, 24, 25). An increased frequency of mutations can also be found in sperm derived from human chemotherapy patients (26, 27). In contrast to this *in vivo* evidence, a significant increase in adverse birth outcomes in the offspring of chemotherapy survivors has not been observed (10).

Despite the remarkably large, yet acute dose of radiation, there has not been any detectable increase in the frequencies of inherited genetic diseases or adverse pregnancy outcomes observed in the offspring of atomic bomb survivors (28). Children born to survivors of either detonation did not show a statistically significant increase in

stillbirths, childhood mortality (≤ 17 years of age), sex chromosome aneuploidy, or mutations resulting in electrophoretic variants (29). Children of atomic bomb survivors also displayed no significant increase in the frequency of mutations within minisatellite regions of DNA compared to age-matched controls (30). More recent analyses failed to correlate an increase in inherited multifactorial diseases such as Diabetes Mellitus or hypertension to atomic bomb exposure (31).

However, more convincing data demonstrating the existence of a human germ cell mutagen has come from minisatellite analysis of survivors or clean-up workers of the Chernobyl accident (12). Dubrova *et al*. showed an increase in the frequency of change of length mutations at multiple repetitive DNA sequences in both the sperm and progeny of exposed males (32-35). Aghajanyan and Suskov observed a similar effect in both exposed clean-up workers (liquidators) and their offspring, and further demonstrated that *in vitro* radiation of peripheral blood lymphocytes derived from children or exposed workers, induced more mutations than blood samples of control children (36). The absence of effective methods to investigate human germ cell mutagenesis presents a need for germ cell mutation research in animal models (37).

Significant challenges inhibit detection of germ cell mutations in human or animal populations. The transmission of genetic damage to offspring *in vivo* remains difficult to study due to multiple practical research challenges. First, mutations are exceedingly rare, around one spontaneous mutation per 10^5 to 10^7 loci (4, 5). To reliably detect significant numbers of mutations, assays must be able to efficiently screen very large numbers of loci. In addition, mutation assays must differentiate between mutant and non-mutant genes. This in itself is difficult as many mutations are silent and do not alter the

phenotype. The ideal mutation reporter gene must be able to harbor a mutation without selection, which would artificially increase or decrease mutation frequency (38). The mutation reporter gene would, ideally, be present in multiple different cells, tissues, and species facilitating direct comparison of mutation rates at many biological scales. Also required, is a reliable spontaneous mutation rate for precision and comparison with other research (11). Finally, the mutation assay should have the ability to determine the DNA sequence of the mutations thus providing insights into the mechanisms of mutagenesis and DNA repair.

Germ Cell Mutagenesis Animal Models and Assays: History and Characteristics

Most of our current understanding of germ line mutagenesis is based on studies of mutations in progeny of mutagen-treated adult mice (39-41). The principle assays employed include the mouse Specific Locus Test (SLT) (42), Dominant Lethal Assay (DL) (43), and Heritable Translocation Test (HT) (44). Figure 1.1 shows which tissues are sampled for each assay. Among those, the SLT is the best characterized, and has been extensively used to evaluate chemical and radiation mutagens (39, 45). The assay, developed by William Russell at Jackson Laboratories in Bar Harbor, Maine and later Oak Ridge National Laboratory in Tennessee, exploits phenotypic variations in offspring to indicate mutations in germ cells of the parents (42, 46). To conduct the assay, a wild-type adult is exposed to the compound or radiation of interest, and then bred to the tester strain of the opposite sex (42). The tester strain is the key to the assay as it is homozygous recessive at seven loci. Six loci correspond to coat color: *a* (nonagouti, chromosome 2), *b* (brown, chromosome 4), c^{ch} (chinchilla at white, chromosome 7), *d*

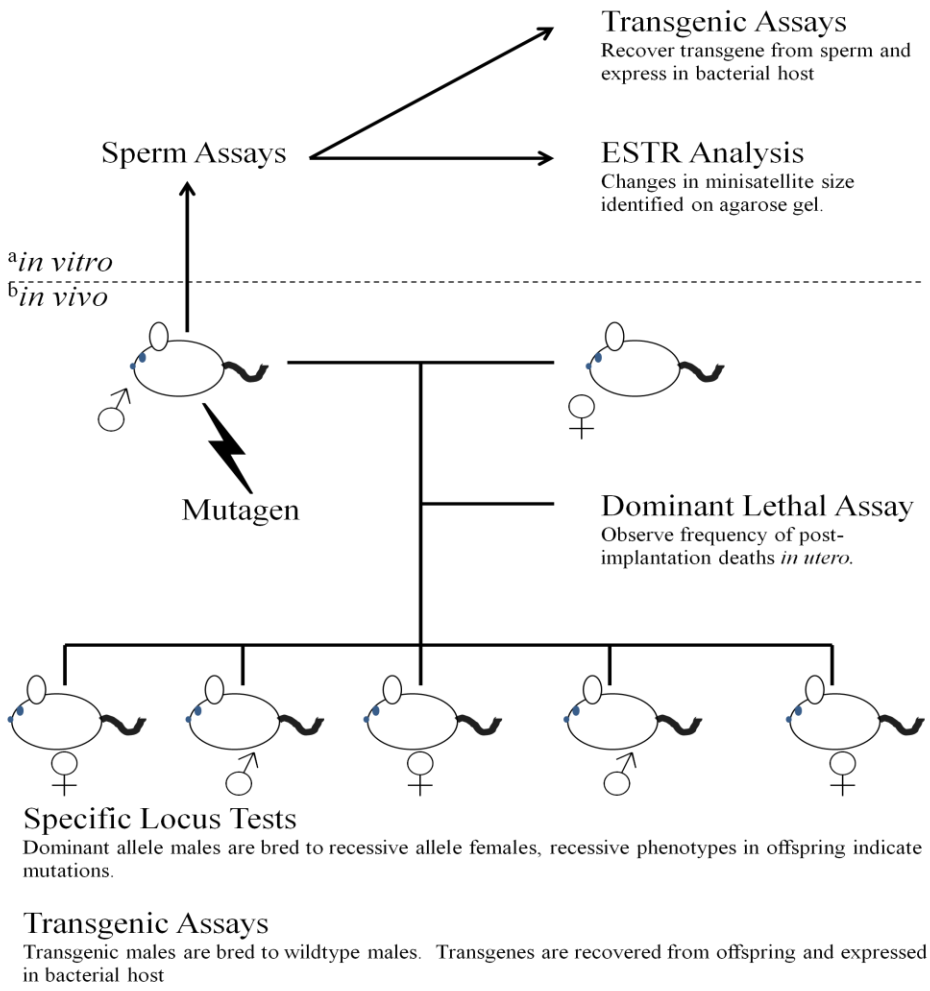

Figure 1.1 Rodent-based mutation assays for the detection of mutations in male germ cells. The adult male is dosed with the agent of interest. [a]Sperm is collected for *in vitro* analysis: for ESTR analysis, DNA extracted from sperm is digested with restriction enzymes and electrophoresed on agarose gels to identify change of length mutations. For transgenic analysis, DNA is extracted from sperm, transgenes are recovered and expressed in a bacterial host, resulting in a frequency of mutant transgenes vs. total recovered transgenes. [b]Dosed males are bred, producing an F_1 generation for *in* vivo analyses. The dominant lethal assay involves breeding dosed males, then sacrificing gravid females to count post-implantation, *in utero* deaths. Offspring could also be screened for recessive mutations in the SLT or in a paternally derived transgene.

(dilute, chromosome 9), *p* (pink-eyed dilution, chromosome 7), *s* (piebald-spotting, chromosome 14) and one loci corresponding to ear morphology, *se* (short-ear, chromosome 9) (42, 46). Offspring are screened visually for any recessive phenotypes. Any recessive phenotypes observed in the offspring indicate a mutation that occurred in the dominant allele of the wild-type parent which was treated with the agent of interest. The SLT has numerous benefits that helped it become the "workhorse" for studying germ cell mutagenesis. Firstly, the information obtained is particularly relevant to human genetic hazards, as the test identifies transmitted mutational damage in a mammal. The assay also permits the detection of mutations in one generation(42). Results are obtained without pathological or molecular processing, such as dissection or DNA extraction. F_1 mice only have to be carefully observed for changes in ear length and coat color or texture. A relatively large number of loci can be screened quickly, as a single technician can evaluate 2,000 loci per hour (47). The SLT can also be combined with other biological endpoints to correlate findings by other methods (45). For example, using this assay, mutation rates in offspring of a treated adult can be compared to tumor formation, thus evaluating a correlation between the mutagenic effects and carcinogenic effects of a specific agent. A particular benefit considering the current work, is that either the female or male parent can be wild-type, thus either male or female germ-cells can be exposed to the test mutagen.

Despite the numerous advantages of this assay, multiple limitations have severely limited the application of the SLT such that it is no longer commonly used (39, 48). Firstly, Russell created the recessive tester strain by breeding mice with unusual appearances which he obtained from private mouse collectors and fanciers (46).

Therefore, the recessive phenotypes he selected for were common enough to appear spontaneously in the collections of amateur fanciers (46). As a result, some of the seven endogenous recessive loci have very high mutation rates (up to 10 fold greater than spontaneous mutant frequencies in *Drosophila* (49)) and may not represent the mutation rates of the entire genome (45). Russell countered this argument stating the SLT is less concerned with presenting a perfect reflection of genomic mutation rates, but rather responsiveness to mutagens, and defining the parameters and important variables of mutagenesis (46). Another disadvantage of the SLT, is the difficulty recovering the type and location (spectra) of mutations. Because the seven loci are relatively large, ranging from 600 bp to >5,000 bp (50), performing detailed sequence analysis can be labor and resource intensive (45). This problem is compounded when attempting to thoroughly describe the spectra of spontaneous mutations. It is also possible that non-mutational events, or mutations outside the seven loci may be falsely scored as mutants. Finally, the SLT relies on rearing F_1 offspring to weaning and specific locus mutations with adverse heterozygous effects may not survive long enough to be scored.

The most prohibitive factor, however, when conducting the SLT is the enormous number of offspring that must be produced and scored. The SLT only detects forward, loss-of-function mutations at the seven specific loci which have visible, recessive phenotypes (45). Therefore only seven of the thousands of endogenous loci are screened per individual offspring. As a result, analysis of a single test agent necessitates tens of thousands of animals which quickly becomes prohibitively expensive and labor intensive (11, 40). For example, Russell's first published SLT study utilized 85,875 mice. Given

the restraints of the SLT, new assays to detect mutations in the progeny of exposed adults are warranted.

A similar assay was developed using the common fruit fly, *Drosophila melanogaster*. H.J. Muller first employed the *Drosophila* Sex-Linked Recessive Lethal assay (SLRL) to investigate the mutagenic effects of X-rays in 1927 (51). The assay became increasingly popular following the Second World War (52). In 1947, Auerbach *et al.* identified mustard gas as the first ever chemical mutagen using *Drosophila* (53).

Similar to the SLT, the *Drosphila* SLRL assay relies on phenotypic changes to offspring to identify mutations in germ cells. Specifically, this assay identifies lethal mutations on the X chromosome of male *Drosophila* flies exposed to the test agent (54). Briefly, wild-type (canton-S) male flies are treated with the agent in question and bred to females with homozygous, sex-linked, recessive phenotypic markers. The most often used female strain is the "Basc" strain which carries the apricot eye (w^a) and bar eye (B) markers, as well as several inversions to prevent crossing over with the male's X chromosome (55). The F_1 generation is mated to each other and missing male phenotypes are noted in the F_2 generation. If a mutation occurs in the treated X chromosome of the F_0 male, non-bar-eyed males will be missing from the F_2 generation, as their sole X chromosome contains a lethal mutation (54, 56).

The sex-linked recessive lethal assay has several advantages over the mouse specific locus test including a shorter generation time (~10 days), low cost of culture media, high fecundity, and the assay requires only simple laboratory facilities (55). *Drosophila* are also capable of performing many of the same bio-activation reactions as the mammalian liver (55). Additionally, the SLRL assay is estimated to screen 800 loci

on the X chromosome (~80% of the chromosome) which represents approximately 20% of the entire genome (56). Many mutant strains including DNA repair enzyme knockouts are available in *Drosophila* (57-59) allowing researchers to evaluate a certain enzyme's role in germ cell mutagenesis.

As with any mutation assay, the SLRL test faces several shortcomings. For example, sequencing individual mutations, and thus determining the mutational spectra of the agent in question is not possible with the SLRL assay and would require additional mutation assays. Also, *Drosophila* are an invertebrate animal with rapid gametogenesis and a short life cycle, precluding investigations of certain germ cell development stages. Russell *et al.* 1958 showed chronic low doses of radiation were less mutagenic than acute doses, even if total dose was the same (49). Chronic exposures are not possible in *Drosophila* due to their short lifespans, so all exposures are acute. The Russells had now demonstrated that "the genetic hazards, at least under some radiation conditions, may not be as great as those estimated from the mutation rates obtained with acute radiation" (i.e. *Drosophila*) (49).

Transgenic Mutation Assays

Transgenic mutation animal models offer practical alternatives that avoid the limitations of *Drosophila* and are much less resource intensive than the SLT. In the last quarter century, a new generation of animal mutation models were developed based on permanent integration of prokaryotic vectors into the animal's genome (60). In 1988 Gossen *et al.* successfully integrated a λ bacteriophage vector containing the bacterial *LacZ* gene into BALB/c x DBA/2 mice (shuttle vector λgt10LacZ) (61). The *LacZ* gene encodes β-galactosidase, a necessary gene for the metabolism of lactose in *E. coli*. *LacZ*

served as a mutation reporter gene that could be mutated in the mouse and be "shuttled" to a *LacZ*$^{-}$ *E. coli* host via the λ phages. The *E.coli* were cultured in media lacking glucose, but containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which releases a blue dye when digested by β-galactosidase. Thus, blue plaques indicate a functional, wild type *LacZ* locus was recovered from the mouse tissue, whereas colorless plaques indicate the presence of mutated *LacZ*. Gossen *et al.* demonstrated an increased frequency of *LacZ* mutations in mice treated with 1-ethyl-1-nitrosourea. Other transgenic rodent mutation models based on λ phage shuttle vectors were developed and marketed shortly after including the Muta^{TM}-Mouse (*LacZ* based assay described above, Hazelton Research, Denver, CO), the Big Blue® Mouse (*LacI* mutation target gene of the λLIZ vector in C57BL/6 or B6C3F1 mice, Stratagene, La Jolla, CA) (62).

Transgenic mutations assays possess several advantages for detecting mutations in somatic (and later germ-cell) tissues. As the mutation target genes (*LacZ*, and later *LacI* and *cII*) are stably integrated into the animal's endogenous DNA, they are present in the nucleus of the animal cell, the most relevant location to detect mutations. Also as a result of permanent genomic integration, every cell in the animals' body contains the target gene, allowing determination of mutant frequencies from nearly any tissue. This is crucially important for regulatory testing purposes because previous *in vivo* somatic mutation tests sampled a narrow range of tissues or cell types (liver, bone marrow and peripheral blood), and salient mutagens can be specific to other tissues (62).

Transgenic assays also allow efficient screening of large numbers of target loci per animal (10^5 to 10^6 in the *cII* assay (40, 63, 64)), increasing statistical power while vastly reducing the number of research animals consumed (65). For example, the SLT

screens seven loci per rodent, whereas the *cII* mutation assay usually screens ~200,000 loci per Big Blue mouse (based on 40 transgenes per haploid genome), thus screening approximately 30,000 fold more loci per animal. Due to the large number of loci screened per animal, Carr and Gorelick estimated as few as eight animals are needed per treatment group to generate a statistically significant increase in somatic mutations (65).

The numerous practical advantages of these systems has lead to a rapid increase in utilization of transgenic models for regulatory and mechanistic mutagenesis research (66). Transgenic mutation assays have been particularly insightful in determining complex mechanistic questions left unresolved by conventional mutation assays (62). For example, tamoxifen, a commonly used treatment for certain breast cancers, was known to be a potent liver carcinogen in rats (67, 68), yet appeared non-mutagenic on *in vitro* assays (69), and only weakly mutagenic in hepatocyte unscheduled DNA synthesis assays (70). Using the Big Blue® Mouse, Davies *et al.* not only identified tamoxifen as a potent hepatocyte mutagen, but also proved the induced mutations were primarily G:C→T:A transversions (71, 72). The mutational spectra information provided evidence that tamoxifen induces mutations by forming a bulky adduct and then an apurinic site. In another example, o-anisidine, a known carcinogen of the urinary bladder was determined to be weakly mutagenic using *in vitro* analysis, and non-genotoxic to the urinary bladder using multiple *in vivo* assays (unscheduled DNA synthesis, micronucleus induction, and DNA single strand breaks) (73). However, the same researchers were able to identify o-anisidine induced mutations in *lacI* genes recovered from urinary bladders, providing a possible mechanism of carcinogenesis (74).

However, these assays also face certain shortcomings. Identifying mutant versus non-mutant phages required distinguishing between blue and clear plaques. The observation that partially blue and clear plaques (sectored plaques) appear commonly (75) makes discerning mutant plaques more difficult. Sectored plaques present a source of error, which requires additional plating to resolve (76). Plating the λ phages for the *LacI* and *LacZ* assays requires large plates and relatively high volumes of media. These assays also require more molecular biology equipment and training than the SLT.

In 1996 Jakubczak *et al.* developed a transgenic mutation assay based on the λLIZ vector of the Big Blue® system that exploited the *cII* gene of λ phage (38). This new approach was able to overcome some limitations of previous based transgenic assays which used the Lac operon (*LacZ* and *LacI*). The *cII* gene is a part of the *cro* operon of λ and, once transfected into *E. coli* helps determine whether the phage will enter the lytic cell cycle (resulting in a plaque on the *E. coli* lawn) or the lysogenic cell cycle (indistinguishable from the lawn). By plating each sample under both selective (*cII*$^-$ phages form plaques) and non-selective conditions (all phages form plaques), the frequency of mutant phages within the total phages recovered can be determined (38). This assay relies on the presence or absence of a plaque rather than color, and can be plated at much higher densities, which reduces time and resource costs. However, the assay does require accurate incubation temperatures and advanced molecular biology training to culture and transfect the *E. coli*.

The *cII* mutation assay is a well-characterized animal mutation model for somatic mutation analysis, and has recently been employed for germ cell mutation studies (40, 63, 77-79). The present studies used the λ transgenic medaka in which each cell contains

~75 copies of the mutation target transgene per haploid genome (80), and Big Blue® transgenic mice in which each cell contains ~40 copies of the transgene per haploid genome (81, 82). These models allow screening of millions of loci per animal greatly reducing the animal resources necessary. Recent publications employing the λ transgenic medaka to investigate mutations passed via germ cells demonstrated that only a few hundred offspring are required per treatment group (40, 83). In contrast, the specific locus test requires hundreds of thousands of animals per treatment group (84, 85).

Mutations are also detected in single genes which are the ultimate endpoints of DNA damage and repair. However, because the *cII* gene is not expressed in the host animal, mutations in the target gene persist and accumulate over time without selection. Additionally, spontaneous mutant frequencies, which are necessary for comparing treatment groups are quickly determined. Mutations can be characterized at the molecular level through rapid cloning and DNA sequencing, yielding mutational spectra that may help disclose mechanisms of mutagen action. Mutations in the *cII* gene can be correlated with other endpoints such as neoplasm formation, or developmental abnormalities (86, 87).

Mutations in Female Germ Cells

Very little is known about mutagenesis in female germ cells as the vast majority of research examining the transmission of genetic damage to offspring focused only on male germ cells (88). Few chemical mutagens have been tested in both male and female germ cells and the few that have been examined were analyzed with the Specific Locus Test (SLT) or the Dominant Lethal Assay (DL) (89). To date, only six chemicals have been evaluated in female germ cells for mutagenic effects using the SLT: bleomycin,

chlorambucil, ethyl-nitrosourea, mitocyin C, procarbazine, and triethylenemelamin (90). In contrast to many of the radiation-based studies, results of chemical mutagen analyses indicate that female germ cells can be more or less sensitive than male germ cells and therefore results from male studies cannot be extrapolated to female germ cells. For example, although procarbazine, triethylenemelamin, and 1-ethyl-1-nitosourea induced more mutations in offspring derived from treated male germ cells than female germ cells (91-95), both sexes appeared equally sensitive to chlorambucil (18, 96). Meanwhile, female germ cells appear more sensitive to bleomycin than male germ cells (19). Further studies employing the dominant lethal assay have identified at least three chemicals that will induce mutations in female germ cells, but not male germ cells (hyacinthine methanesulfonate, cisplatin, and adriamycin) (97, 98).

Results from the SLT provide an estimate of the spontaneous mutant frequency of female germ cells that is lower than male counterparts (85). The mutation response to radiation exposures was also lower than male germ cells (85, 99-102). The relative resistance of female germ cells to DNA damage from radiation exposures has been attributed to differences in oogenesis and spermatogenesis. Female germ cells are DNA repair competent from stem cells throughout oogenesis to mature oocytes, in contrast, postmeiotic male germ cells (spermatids and spermatozoa) are largely unable to repair damaged DNA (103-105).

Inconsistencies between male and female germ cell responses to mutagens provide further evidence that male germ cells cannot be used as surrogates for female germ cells when evaluating the genetic health risk of suspected chemical mutagens. Therefore, female germ cells must be independently evaluated. The goal of this work

was to investigate selected functions of the female germ cell in the transmission of mutations from parent to offspring using transgenic animal models.

Oogenesis and Gestation Present Unique Challenges to Investigating Mutations Transmitted by Female Germ Cells

Analysis of mutations transmitted by female germ cells is also necessary because differences in gametogenesis and gamete function lead to unique patterns of mutations in the offspring of mutagen-exposed females compared to males. Despite the need for mutation analyses in female germ cells, oogenesis presents many practical challenges to investigation. For example, the supply of male gametes is seemingly inexhaustible whereas females are born with a finite pool of potential gametes (106). This discrepancy is exacerbated in mammals wherein only a few oocytes mature during each estrus cycle. Thus sampling large numbers of oocytes requires large numbers of adult females (85). Additional offspring can be obtained from a single male by adding more females, yet more offspring cannot be produced from a single female by adding more males.

In addition, mitosis and meiosis of male germ cells are discrete processes (107), which allows researchers to sample progeny derived from cells of each relevant stage of spermatogenesis. Response to DNA damage is known to change throughout gametogenesis, thus researchers often desire offspring derived from germ cells treated with a suspected mutagen at a certain stage. Offspring collected 20 to 60 days (including a 20 day gestation) following dosing of the male mouse for example, are derived from male germ cells that had completed meiosis at the time of dosing thus postmeiotic spermatids and spermatozoa germ cells were exposed. Offspring collected >60 days following dosing are derived from premeiotic spermatogonial stem cells at the time of

dosing. In contrast, mitosis of female germ cells occurs only during fetal development. Meiosis begins while the female is still an embryo *in utero,* and meiosis in the oocytes is arrested at prophase I until sexual maturity. Upon sexual maturity, a few oocytes are recruited each cycle to resume Meiosis I, and begin Meiosis II. The second Meiosis is only completed after the oocyte is fertilized (108). For these reasons, treating certain stages of development in female germ cells is nearly impossible.

Japanese Medaka as Animal Models of Germ Cell Mutagenesis

The biology of the λ transgenic medaka overcomes many of the problems which hinder rodent female germ cell mutation models. Medaka are small (2-4 cm) and easy to culture in the lab with relatively low costs. A short generation time (sexually mature by 6-8 weeks of age) allows for rapid assessment of genetic damage through multiple generations. There are also many transgenic inbred and mutant strains of medaka, including strains in which DNA repair enzymes are knocked out. The biology and development of the Japanese medaka is well understood (109, 110). Additionally, the genomes of multiple medaka strains have been sequenced (111, 112) and several assays have demonstrated the value of medaka as a germ cell mutagenesis model (113-115). Shima and Shimada developed a specific locus assay based on two strains of medaka (113). Winn *et al.* developed the λ transgenic medaka, in which the λLIZ vector, identical to the transgene of the Big Blue® Mouse, is stably integrated into the medaka genome (80). Tsyusko *et al.* identified nine minisatellite regions of the Japanese medaka and designed a germline mutation assay similar to ESTR analysis of rodents (116).

In contrast to the small litter size and 20-day gestational period of rodent animal models, the female medaka extrudes batches of ~20 eggs every day with no gestation

time, allowing for large numbers of oocytes to be collected from a single female (117). Therefore λ transgenic female medaka provides an efficient, cost-effective alternative to rodent female germ cell mutation animal models.

Research Objectives and Rationale for Each Experiment

This work seeks to better understand the role of the female germ cells in transmission of genetic damage from parent to offspring by separately exposing male and female germ cells to a known mutagen and assaying the kind and frequency of mutations in the offspring. First, transgenic female medaka were exposed to 1-ethyl-1-nitrosourea (ENU) and mutations were analyzed in the offspring to investigate the direct transmission of genetic damage to offspring via the oocyte. These experiments (contained in chapter two of this work) tested two hypotheses. First, that the range of *cII* mutant frequencies (*cII* MF) will indicate the presence and frequency of non-mutant offspring, mosaic mutant offspring, and whole body mutant offspring. This experimental design is nearly identical to Winn *et al.* 2008, in which **male** medaka were exposed to ENU and offspring were screened from mutations (40). Thus, the frequency of mutant offspring derived from ENU-treated **female** germ cells (chapter two) can be compared to the frequency of mutant offspring derived from mutagen-treated premeiotic and postmeiotic **male** germ cells (40). We hypothesize there will be no significant difference in the frequency of mutant offspring derived from mutagen treated male or female germ cells.

The oocyte has significant functions and responsibilities in addition to simply contributing genetic material to the next generation (118) and these other functions may greatly affect mutagenesis of offspring. The oocyte must provide almost every necessary component to complete early cellular divisions in the developing embryo, including

maternal mRNA transcripts, and tRNA stored during oogenesis (118) because transcription and translation are uncoupled in the early embryo (119). DNA repair in the pre-implantation embryo is also believed to rely significantly, if not completely, on the oocyte (119, 120). Therefore the oocyte could not only directly transmit DNA damage to offspring via the maternal DNA, but also mediate mutations by inappropriate DNA repair or error-prone DNA synthesis to the unexposed paternally derived DNA of offspring.

To investigate the role of the oocyte in mediating mutations to the unexposed, paternally derived DNA of offspring, mutagen-treated wild type female medaka were bred to untreated transgenic male medaka to examine the induction of untargeted mutations by female germ cells to the male genome (presented in chapter three). The mutations observed in offspring were necessarily fixed after fertilization. The oocyte has functions in addition to contributing genetic information, including guiding cell division (DNA replication) and DNA damage response. Under these conditions, it is possible for a damaged oocyte to induce mutations in both haploid genomes- the DNA from both the maternal and paternal genome. This experiment tests the ability of mutagen-exposed oocytes to induce "untargeted" mutations in the unexposed DNA contributed by the sperm. All mutant offspring were hypothesized to be mosaic mutant offspring because untargeted mutations must occur in the embryo, rather than the gamete. Further, it is hypothesized that a statistically significant increase in the frequency of untargeted mutant offspring derived from mutagen treated female germ cells versus male germ cells will appear. Finally, it is hypothesized the mutational spectra derived from mutant offspring will be significantly different from an ENU induced spectra, but not significantly

different from mutant offspring generated by male untargeted mutagenesis studies, as determined by hypergeometric analysis.

The characteristics of untargeted mutagenesis were further explored in a transgenic rodent model, the Big Blue® mouse (chapter four). These experiments sought to determine if previous results investigating untargeted mutagenesis are unique to fish, and what features were conserved. Specifically this research tested the following hypotheses: first, there will be no statistically significant difference in the frequency of mutant offspring generated by mutagen treated fish versus mice. Second, it was hypothesized that there will be no statistically significant difference in the mutational spectra of mouse and fish mutant offspring, as determined by hypergeometric analysis.

Finally, the role of DNA repair mechanisms in the oocyte was investigated. The role of maternal p53 in DNA repair and the transmission of mutations to offspring was elucidated by analyzing mutations in offspring derived from $p53^{-}$ oocytes and mutagen-treated male germ cells. Cells confronted with damaged DNA have evolved four complex systems to cope with or repair the damage: 1) multiple DNA repair enzymes, 2) cell cycle control checkpoints which delay cell division allowing more time for DNA repair, 3) apoptosis, and 4) DNA damage tolerance to allow replication to continue (121). An embryo derived from a mutagen-treated gamete certainly qualifies as a cell with significant DNA damage, but the rapid progress of embryogenesis may inhibit these four systems. Although mRNA profiles of embryos demonstrate that a multitude of genes involved in DNA repair are expressed in early embryogenesis (122), research with mouse and human embryos indicate that transcription and translation are uncoupled until at least the 2-4 cell stage (123). Taken together, Jaroudi and SenGupta conclude “although many

of the genes required for DNA repair are expressed, the embryo's ability to repair DNA may be highly limited" (119). Cell cycle control may not be feasible in the early embryo either, due to rapid cell division. Cell cycle control checkpoints may not be active until after zygotic gene expression has begun, usually the 4-8 cell stage in human embryos (124, 125). As with cell cycle control, apoptosis is not likely in the early stages of embryo development. For example, it is not observed in human embryos until after blastocyst formation (70 to 100 cells in humans) (126). Given these constraints, it is unlikely that apoptosis, or cell cycle arrest is a likely mechanism to respond to DNA damage in the early stage embryo.

DNA repair enzymes may be the most important embryonic tool to respond to DNA damage. As transcription and translation are uncoupled for multiple rounds of cell division, the functional DNA repair enzymes in the early embryo were supplied by the oocyte during oogenesis (123). Any new DNA repair enzymes translated which are translated in the embryo, must use transcripts from the oocyte. p53 is a very important enzyme for DNA damage response and repair, as well as embryonic development, therefore we hypothesize that maternal p53 function (contributed by the oocyte) alters the embryo's response to DNA damage.

In recent years, the *p53* gene has been shown to be a key regulator in a wide range of cellular processes, including cell cycle control, DNA repair, genome stability, programmed cell death, differentiation (127), senescence (128) and angiogenesis (129, 130). In addition *p53* is a major component of DNA damage response pathway in mammalian cells.

Analyzing mutations in offspring derived from $p53^-$ oocytes and mutagen-treated male germ cells reveals the role of maternal p53 on the transmission of genetic damage to offspring (chapter five). In these experiments, the female parent, and thus the oocyte, carried a loss-of-function mutation in the p53 gene. The resulting protein, once transcribed, is severely truncated and non-functional, as it lacks the DNA-binding domain. These female knock-out medaka were bred to transgenic male medaka, and the offspring are collected before and after exposing the males to ENU. In this scenario, the sperm cell introduced damaged DNA to the embryo, which has no functional p53. Therefore the embryos must complete DNA replication and cell division in the absence of functional p53. Controls include offspring derived from p53 competent mothers. This procedure allowed testing whether offspring derived from $p53^-$ oocytes will have a higher spontaneous mutant frequency than offspring derived from $p53^+$ oocytes, and second, if offspring of $p53^-$ oocytes and mutagen treated male germ cells will have a higher frequency of mutant offspring than offspring derived from $p53^+$ oocytes and mutagen treated male germ cells.

The present work seeks to understand better the role of the female germ cells in transmission of genetic damage from parent to offspring by separately exposing male and female germ cells to a known mutagen and assaying the kind and frequency of mutations in the offspring. The research objectives established for this study were:

1) To understand better the direct transmission of genetic damage from mutagen-treated oocytes to the maternally derived DNA of the offspring,

2) To investigate the induction of untargeted mutations by mutagen-treated female germ cells to the unexposed, paternally derived genome of offspring,

3) To compare and contrast untargeted mutagenesis between a transgenic rodent model, the Big Blue® mouse, versus a transgenic fish animal model, the λ transgenic medaka, and

4) To examine the role of functional maternal p53 on the mutant frequency of offspring derived from mutagen-treated male germ cells.

The results of these experiments will contribute to understanding the diverse roles of oocytes in the transmission of DNA damage from parent to offspring. These insights could strengthen the evidence that female germ cells are sensitive and uniquely responsive to chemical mutagens. Therefore, analyses of female germ cells should be included in risk assessments, and studies of inherited diseases and reproductive health.

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CHAPTER 2

MUTANT OFFSPRING DERIVED FROM MUTAGEN-TREATED, λ TRANSGENIC FEMALE MEDAKA ARE PRIMARILY MOSAIC MUTANT OFFSPRING[1]

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ABSTRACT:

Investigating mutations in offspring transmitted by female germ cells is hampered by practical challenges including the finite pool of female germ cells, and complexity of oogenesis. Further, estimation of genetic health risks based on analyses of male germ cells is not consistently applicable to female germ cells. To address the need for improved approaches to study mutagenesis mediated by female germ cells, we used a fish model that carries the *cII* mutation target transgene. Mutations in the *cII* genes carried by offspring of 1-ethyl-1-nitrosourea (ENU)-treated λ transgenic females and untreated, wild-type male medaka were quantified and described. Offspring that exhibited *cII* mutant frequencies (MF) greater than 3-fold above controls were scored as mutant offspring, and comprised up to 17.5% of offspring, which is comparable to frequencies of mutant offspring derived from mutagen-treated male germ cells. Mutant offspring exhibited a wide range of *cII* MFs, from ~3 to 500-fold (*cII* MF= 9.0×10^{-5} to 1.6×10^{-2}) induction compared to controls. Characterization of mutational spectra revealed that both whole body and mosaic mutant offspring were produced, with mosaic mutants comprising the vast majority (93%) of mutant offspring. Whole body mutants were generated from mutations fixed either in gametes, or at the one-cell stage, whereas mosaic mutants were generated from mutations fixed at or after the two-cell stage of development. Further, these data show that fixation of persistent DNA damage in mature oocytes was delayed until after fertilization. Consequently, as shown in male germ cells, post-fertilization processes contributed by the maternal genome may act in the mutagenesis of early stage embryos.

INTRODUCTION:

Oogenesis, the production and maturation of female germ cells, is a complex, and lifelong process which hampers experimental research on female germ cells compared to males and provides a developmental basis for understanding the transmission of genetic damage to offspring via female germ cells. Female humans and rodents are born with a finite pool of potential gametes, of the ~6 million germ cells, only 300 to 400 will ever mature, in contrast to males, whom continuously produce spermatocytes throughout their reproductive lifespan (1). Oogenesis begins in the ovary of a female fetus, when germinal cells undergo multiple mitotic divisions and oogonia transform to oocytes. The new oocytes are arrested in prophase I of the first meiotic division (dictyotene phase arrest). By birth, all oocytes are arrested and remain so through pre-pubescent life, until meiosis I resumes at sexual maturity (2). During this arrest, oocytes increase in size and accumulate nutrients.

The oocytes are arrested prior to diakinesis, when chromosomes are condensed into heterochromatin. The structure of chromatin (the organization of DNA around histone proteins) is important when considering mutation frequencies, as mutation rates vary depending on the availability of a base pair to the mutagen (3, 4). DNA tightly associated with histones is protected from certain types of DNA damage, however, that same tight association is known to impede DNA repair (5, 6).

In mammals, only a few (1 to 14 per gestation, depending on species) of the arrested germinal cells mature during each estrus cycle, complete the first meiotic division just prior to ovulation, separate from the polar body, and immediately begin meiosis II (1). The second polar body is ejected and the second meiotic event is

completed only after the oocyte is successfully fertilized (1). In contrast, spermatocytes are continuously derived from spermatogonial stem cells in as little as 35 days in mice and 64 days in humans. Spermatogonial stem cells are slow-cycling, repair competent cells that generate other stem cells and spermatogonia (7).

The complex process of oogenesis makes collecting large numbers of offspring or oocytes for analysis from selected females very expensive time consuming (8). As a result of these challenges, the vast majority of germ-cell mutation research using animal models has focused exclusively on male germ cells (8-10).

However, multiple studies have shown male and female germ cells respond differently to chemical and radiation mutagens (11-13). Therefore, results from analyses of mutagen treated male germ cells are not sufficient to estimate genetic risk to female germ cells, necessitating an efficient animal model for detecting mutations passed to offspring by female germ cells.

Very few chemical mutagens have been tested in both male and female germ cells and the few that have been examined were analyzed with the Specific Locus Test (SLT) or the Dominant Lethal Assay (DL) (10). Results from the SLT provide an estimate of the spontaneous mutant frequency of female germ cells that are lower than male counterparts (12). The mutation response of female germ cells to radiation exposures was also lower than male germ cells (12, 14-17). The relative resistance of female germ cells to DNA damage from radiation exposures has been attributed to differences in oogenesis and spermatogenesis. Female germ cells are DNA repair competent from stem cells throughout oogenesis to mature oocytes, in contrast, postmeiotic male germ cells (spermatids and spermatozoa) are largely unable to repair damaged DNA (18-20).

To date, only six chemicals have been evaluated in female germ cells for mutagenic effects using the SLT: bleomycin, chlorambucil, ethyl-nitrosourea, mitocyin C, procarbazine, and triethylenemelamin (9). In contrast to many of the radiation-based studies, results of chemical mutagen analyses do not clearly indicate that male germ cells are more sensitive than female germ cells and therefore results from male studies cannot be extrapolated to female germ cells. For example, although procarbazine, triethylenemelamin, and 1-ethyl-1-nitosourea induced more mutations in offspring derived from treated male germ cells than female germ cells (21-25), both sexes appeared equally sensitive to chlorambucil (13, 26). Meanwhile, female germ cells appear more sensitive to bleomycin than male germ cells (27). Further studies employing the dominant lethal assay have identified at least three chemicals that will induce mutations in female germ cells, but not male germ cells (hyacinthine methanesulfonate, cisplatin, and adriamycin) (28, 29). Inconsistencies between male and female germ cell responses to mutagens provide further evidence that male germ cells cannot be used as surrogates for female germ cells when evaluating the genetic health risk of suspected chemical mutagens.

Previous analyses of mutations in female germ cells observed multiple mosaic mutant offspring (12, 30). Mosaicism refers to a percentage of an individual's cells containing a mutation while the other cells do not. The frequency of mosaic mutant offspring derived from mutagen-treated parents may be greatly underestimated. Although mosaics have been observed in many studies of germ line mutagenesis, these individuals are often excluded from analysis (14, 30-32). Mosaicism remains an important topic of research as many diseases including osteogenesis imperfecta,

achondroplasia, autism, abortion and multiple neoplasias have been linked to mosaicism (33-35).

The biology of the female medaka, a small fish native to Southeast Asia, overcomes many of the problems which have limited the usefulness of rodents as female germ cell mutation models. For instance, female medaka extrude ~20 eggs daily with no internal gestation time, allowing for reasonably large numbers of oocytes or embryos to be collected quickly from a single female. In contrast, mice require a 19-21day gestation period to produce a litter of 4 to 15 pups (36). Medaka fry can be easily reared in large numbers to appropriate size for DNA analysis.

The λ transgenic medaka, which carries the *cII* mutation target gene, offer practical solutions to the challenges of detecting mutations in the offspring of mutagen treated rodent female germ cells. First, mutation assays must efficiently screen very large numbers of loci because mutations are exceedingly rare (1 spontaneous mutation per 10^5 to 10^7 loci), as well as be able to differentiate mutant genes from the overwhelming majority of non-mutant genes. To overcome these challenges, transgenic animal models, such as the λ transgenic medaka, incorporate a prokaryotic vector, harboring a mutation target gene in their genome. Millions of these target genes can be analyzed in a single animal, greatly reducing the number of animals required (31, 37).

The unique biology of female germ cells, and their response to chemical mutagens, necessitates an efficient animal model to investigate mutations passed to offspring via oocytes. Reports demonstrating the efficient detection of mutations in the offspring of mutagen treated male medaka (37-39) indicate the female medaka could also be an efficient animal model to investigate mutations transmitted via the oocyte.

We hypothesized that exposing transgenic females to a known chemical mutagen, ENU, and breeding to wild-type males will induce mutations in the offspring that can be detected and described with the *cII* mutation assay. Two specific hypotheses were tested: first, that the range of *cII* mutant frequencies (*cII* MF) will indicate the presence of non-mutant offspring, mosaic mutant offspring, and whole body mutant offspring, second, there will be no statistically significant differences in the frequency of mutant offspring derived from mutagen-treated female germ cells versus mutagen-treated premeiotic and postmeiotic male germ cells.

MATERIALS AND METHODS:

Animals

Wild-type (CAB strain) and λ transgenic medaka (strain λ 310), which contain the λLIZ bacteriophage vector harboring the *cII* gene mutation target gene (homozygotes ~150 λ copies /diploid genome), were obtained from in-house stocks at the Aquatic Biotechnology and Environmental Laboratory, University of Georgia (31). Fish were maintained on a 12 hr light-dark cycle, except breeding pairs maintained on a 16:8 light-dark cycle in dechlorinated municipal water at 24° C and fed 1 to 2 times daily with brine shrimp nauplii (Artemia Great Salt Lake, UT) and supplemented 1 to 2 times daily with a commercial flake diet. All animal care protocols were approved by The University of Georgia Institutional Care and Use Committee.

Mutagen Treatment

Adult transgenic females (λLIZ homozygous) were independently exposed to 100 ppm of the germ cell mutagen, 1- ethyl-1- nitrosourea (ENU) in their ambient water for one hr on two consecutive days. Females were bred to untreated, wild-type males for

seven days prior to exposure thus yielding control offspring and 35 days following exposure yielding treatment offspring. Therefore, control and treatment offspring were siblings. All medaka were bred in a group setting (multiple males and females) so the precise lineage of each offspring is unknown. Approximately 50 control offspring were collected to determine the spontaneous *cII* MF (SMF) and to compare the SMF to the historical spontaneous *cII* MF. One-hundred offspring were collected following ENU exposure (treatment group) to provide sufficient data for comparing frequencies of mutant offspring to previously published work (37). All progeny were hemizygous for the *cII* target gene, which was contributed only by the female parent (Figure 2.1). Collected embryos were allowed to hatch and were cultured under standard conditions for 14-21 days, flash frozen, and stored at -80° C prior to mutation analysis.

DNA Extraction

Genomic DNA was isolated from individual siblings using previously described procedures (31, 40). Briefly, tissues from whole animals were digested with proteinase (1X SSC, 20% SDS, 20mg/ml proteinase K) and extracted with equal volumes phenol:chloroform. DNA was precipitated using ethanol then resuspended in Tris-EDTA buffer (pH7.5).

cII Mutation Assay

Mutations in the offspring of mutagen exposed females were analyzed using a positive-selection assay in which the *cII* gene was the mutation target gene (41). The assay is based on the role of the *cII* protein in the commitment of bacteriophage λ to the lysogenic or lytic cycle in *E. coli*. Selection of mutant *cII* genes was facilitated using a specialized *E. coli* strain (G1250 , *hfl*$^{-}$) that extends the longevity of the *cII* protein

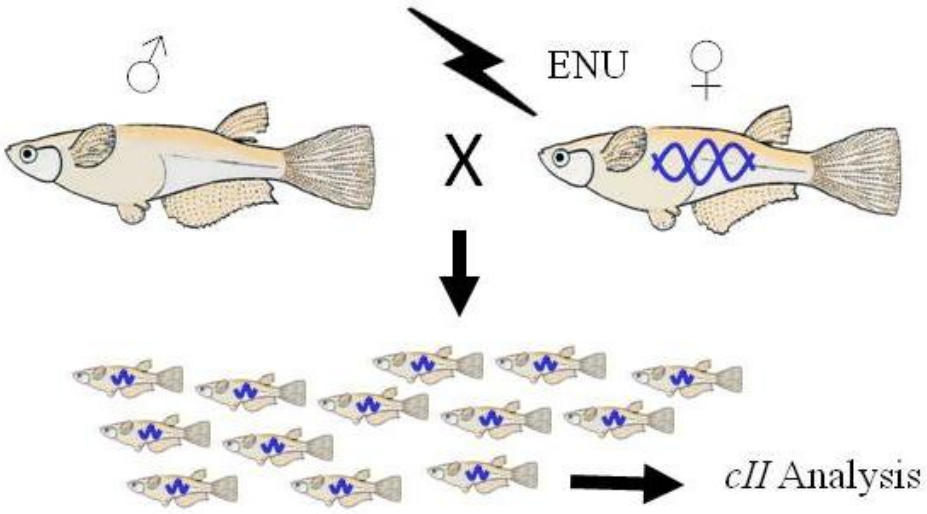

Figure 2.1 Schematic of the breeding design used to collect hemizygous offspring of mutagen treated λ transgenic females and wild-type males. Offspring were collected prior to and for 35 days following exposure of the females to 100 ppm ENU for one hour on two consecutive days. Mutations in the offspring were detected in the *cII* mutation target gene, which was derived from the female parent.

product. Genomic DNA was incubated with packaging extracts to simultaneously excise and package the λ*LIZ* vector into viable bacteriophage. The packaged phage particles were then allowed to infect and lyse the *E. coli* host. The frequencies of *cII* mutants (*cII* MFs) were calculated by dividing the total number *cII* mutant plaque forming units (PFUs) on the selective screening plates by the total λ *cII* phages on the permissive titer plates. To select λ*cII*$^-$ mutants (screening plates), the packaged phages were mixed with *E. coli* cells, plated, and incubated at 24° C for 40hr. The phages with wild-type *cII* became lysogenic, and were indistinguishable from the *E. coli* lawn, whereas phages that carried a mutation in the *cII* gene formed easily counted plaques in the bacterial lawn when incubated at 24° C. To quantify the total λ*cII* recovered (λ*cII*$^-$ mutants and λ*cII*$^+$ non-mutants) (permissive titer plates), packaged phages were diluted and mixed with *E. coli* cells, plated, and incubated at 37° C for 15hr. All packaged phages entered the lytic cycle forming a plaque. DNA samples with *cII* MFs greater than three standard deviations above the mean spontaneous *cII* MF (3×10^{-5}) were designated as mutant offspring (Mutant Offspring *cII* MF $\geq 9.0 \times 10^{-5}$). This conservative definition of mutant offspring is similar to previous studies using λ transgenic medaka (31). Frequencies of mutant offspring were compared using Chi-Square analysis.

Mutational Spectra

DNA mutations from offspring with elevated *cII* MFs were sequenced and the resulting spectra were compared to spontaneous and previously published spectra to identify unique characteristics of mutagenesis in female germ cells. To characterize the spectra of *cII* mutations from offspring with elevated *cII* MFs, we used standard methods to isolate individual mutant plaques. The λ *cII* mutants were characterized using DNA

sequencing methods previously described (31). Individual λ *cII*-mutant plaques were cored at random from mutant screening plates and purified on G1250 *E. coli* cells. Individual plaques were excised and the *cII* gene amplified by PCR. PCR products were electrophoresed on a 1% agarose gel, cleaned with QIAquick PCR purification kit (Qiagen, Valencia CA), then diluted to 0.1μg/μL using Tris-EDTA. Diluted PCR products were labeled (BigDye Terminator Cycling DNA Sequencing Kit; Applied Biosystems, Foster City, CA) and then purified again using DyeEx 2.0 spin columns (Qiagen). Samples were analyzed using an automated DNA Sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). Spectra were compared using previously published hypergeometric analysis methods (42).

RESULTS:

Mutant Offspring Were Derived From ENU-treated Female Germ Cells

To establish the frequency of spontaneously mutated *cII* genes in offspring (spontaneous *cII* MF), 47 offspring derived from 5 males and 14 females were collected prior to mutagen exposure and analyzed for mutations in the *cII* target gene. These offspring, which serve as the control population, displayed a mean *cII* MF of 3.3×10^{-5} with a range of 1.6×10^{-5} to 5.7×10^{-5} (SD= 0.95×10^{-5}) (Appendix A). No mutant offspring were identified in the control population, nor in the control populations of any previous studies conducted at ABEL ((31, 37), unpublished data, University of Georgia).

Eighty-two offspring derived from embryos collected after mutagen exposure were analyzed and displayed a mean *cII* MF of 34.1×10^{-5}, with a range of 4×10^{-6} to 1.6×10^{-2} (SD=179.7×10^{-5}). Figure 2.2 compares the *cII* MF of offspring collected prior to and following exposing female medaka to ENU and Appendix B lists the *cII* MFs

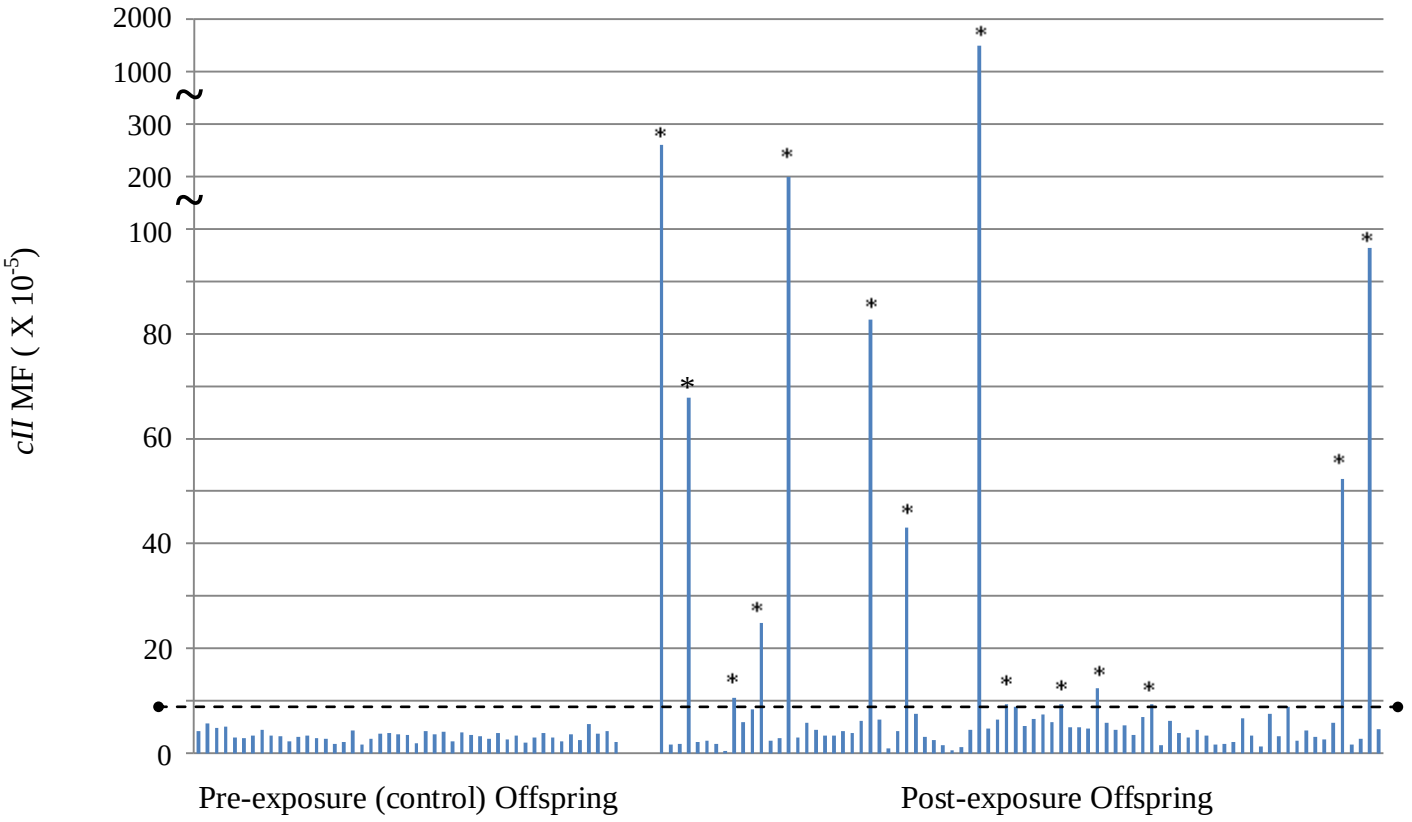

Figure 2.2 *cII* mutant frequencies of control offspring (n=47) and offspring derived from ENU-treated oocytes (n=80). Offspring with a *cII* MF greater than or equal to 9.0 X 10^{-5} (hashed line) were scored as mutant offspring and identified with an asterisk. The axis is broken twice to accommodate the wide range of *cII* MFs.

of all offspring collected following ENU exposure. Mutant offspring were distinguished from non-mutant offspring by elevated *cII* MFs of $\geq 9.0 \times 10^{-5}$ that is, greater than 3 standard deviations above the mean control *cII* MF. Using these criteria, 14 mutant offspring derived from ENU treated females were identified (figure 2.2). The *cII* MF of the 14 mutant offspring varied greatly, ranging from 3 to 500 fold elevation above the spontaneous *cII* MF. The majority (79%, 11/14) of mutant offspring displayed a *cII* MF $<9.9 \times 10^{-4}$, followed by offspring with a *cII* MF between 9.9×10^{-4} and 9.9×10^{-3} (14% 2/14), and finally one offspring with a *cII* MF $>9.9 \times 10^{-3}$ (7%, 1/14).

The frequency of mutant offspring (MOF) derived from ENU treated female germ cells was 17.5% (14 mutant offspring / 82 total offspring sampled) (Appendix B). This MOF is not significantly different than the previously published MOF (37) derived from ENU treated postmeiotic male germ cells (17.5 % vs. 11%, X^2, p=0.20). In contrast, the MOF derived from ENU treated oocytes was significantly higher than the previously published (37) MOF derived from ENU treated premeiotic male germ cells (17.5% vs. 3.5% X^2 p<0.001).

Magnitude of the cII MF Indicated that the Majority of Mutant Offspring Were Mosaic Mutant Offspring

Non-mutant offspring carry only spontaneous mutations, and thus display a low *cII* MF ($\approx 3.0 \times 10^{-5}$) (figure 2.3). By definition, whole body mutant offspring carry the same mutation in every cell. The medaka offspring carry ~75 copies of the *cII* target gene, thus, a whole body mutant offspring, with one mutation in every cell, would have a *cII* MF of ~1/75 or 1.3×10^{-2}. Whole body mutant offspring are generated when a mutation is fixed either in the gamete or the zygote, and this mutation is clonally

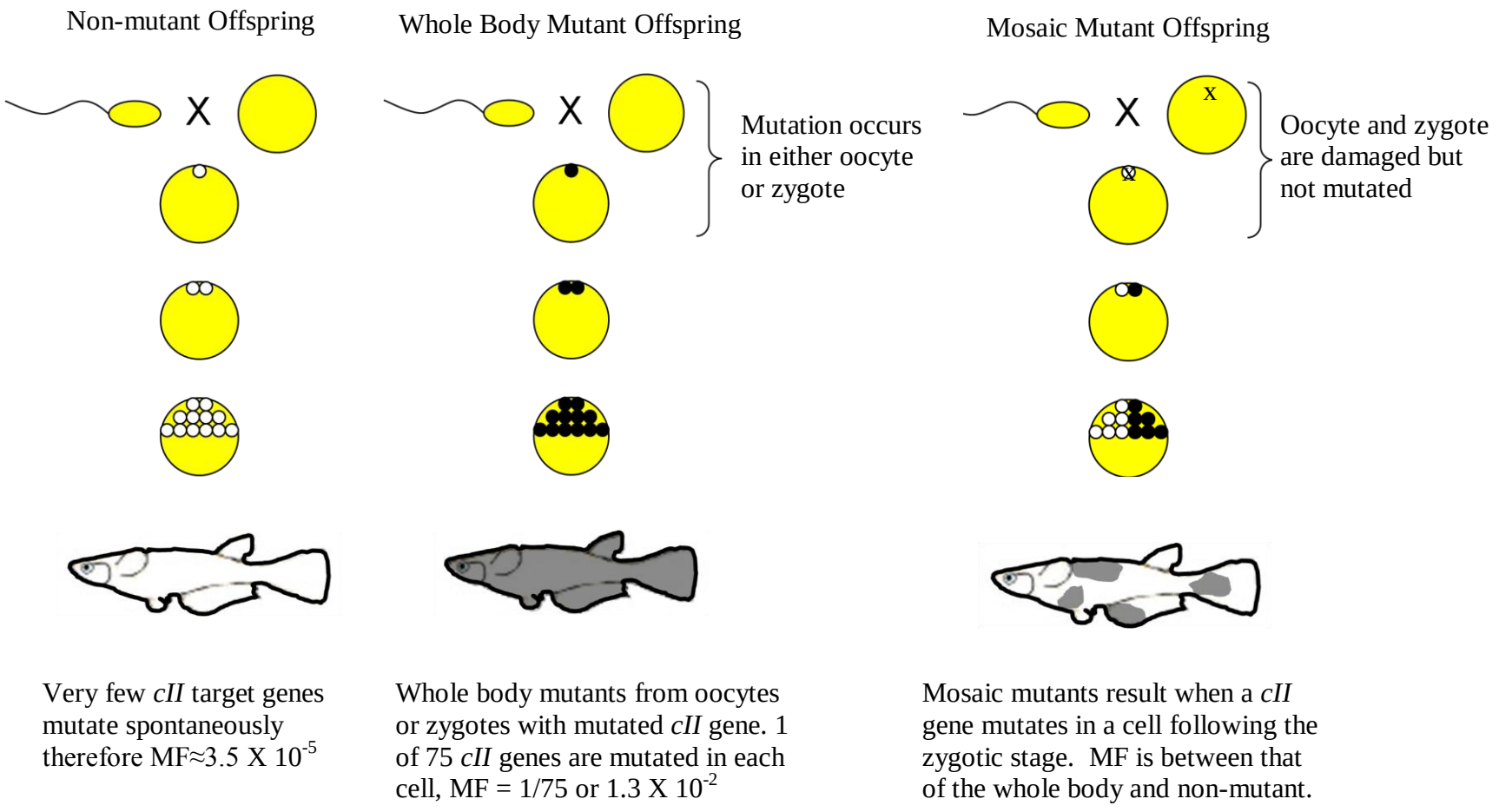

Figure 2.3 Illustration contrasting formation of non-mutant, whole body mutant and mosaic mutant offspring. Mutations fixed in the oocyte or zygote are expanded by mitotic division, resulting in a whole body mutant offspring. Mutations fixed after the first embryonic cell division are present in only a fraction of the daughter cells, creating mosaic mutant offspring.

expanded to all daughter cells as the embryonic cells divide (figure 2.3, (43, 44)). One presumed whole body mutant offspring was detected among the 82 treated offspring that were analyzed (figure 2.2, Appendix B). In contrast, mosaic mutant offspring contain mutations in a fraction of their cells, and display a *cII* MF between the spontaneous and whole body levels (3.0×10^{-5} < Mosaic *cII* MF < 1.3×10^{-2}). Mosaic mutant offspring could arise if the mutation is fixed sometime after the first cell division wherein only the daughter cells of the original mutant cell will carry the mutation (figure 2.3)(14, 45). The vast majority (93% 13/14) of mutant offspring derived from ENU treated females were mosaic mutant offspring (Appendix B).

Spectra of cII Mutations Supported Mosaic or Whole Body Mutant Offspring Status

One hundred and twenty two mutant λ *cII* genes isolated from seven individuals (~17 genes/individual, Table 2.1) were sequenced to characterize mutations carried by offspring derived from ENU treated oocytes. The spectra of mutations generally confirmed mutant offspring status as whole body or mosaic. DNA sequence analysis of mutant *cII* genes recovered from the suspected whole body mutant offspring (Table 2.1, individual *a*) disclosed a single mutation, which is consistent with clonal expansion of a single mutation. Mutational spectra of mosaic mutant offspring with *cII* MF >5×10^{-4} (table 2.1, individuals B, C, D) consist entirely of a single clonally expanded mutation. Mutational spectra of the remaining offspring displayed both independent and clonally expanded mutations (Table 2.1, Individuals E and F).

A Distinctive Mutation Was Revealed in the Offspring of an ENU-treated Female

Individual (g) displayed two distinct mutations at the same base pair (bp 73). These mutations occur at uneven frequencies; most were G→T transversions, [73%

Table 2.1 Mutational spectra of seven offspring derived from ENU-treated female medaka and wild-type males. *cII* MF, base pair of the *cII* gene, and mutation type are also listed.

Individual	*cII* Mutant Frequency	Base Pair	Mutation	no. of Sequences
A	1.6 X 10-2	124	A→G	19
B	2.5 X 10-3	64	G→T	16
C	6.8 X 10-4	91	G→T	20
D	5.2 X 10-4	5	T→A	18
E	2.5 X 10-4	139	T→A	14
		3	G→A	1
		179	Insert G	1
F	9.3 X 10-5	179	Insert G	3
		179	Delete G	2
		185	T→G	3
		1	A→G	1
		25	G→A	1
		101	G→C	1
		115	C→G	1
		221	T→C	1
		234	T→G	1
		242	Delete A	1
G	7.5 X 10-5	73	G→T	11
		73	G→A	4
		25	G→A	1
		112	T→C	1
		179	Insert G	1

(11/15)] and the remaining (27%) were G→A transitions (table 2.1). The probability that two independent mutations would occur at the same base is extremely small.

DISCUSSION:

Mutant offspring that were derived from mutagen-treated, λ transgenic female medaka were found to be primarily mosaic mutant offspring. This finding concurs with the results of more traditional assays (SLT) which examined progeny from mutagen treated females and found a surprisingly high frequency of mosaic mutant offspring (reviewed in (12)). The high proportion of mosaics found in this work and others (37) indicates mutations in offspring are fixed in the early embryo, rather than the gamete. The high frequency of mutant offspring derived from treated oocytes relative to treated premeiotic male germ cells demonstrates that despite continuous DNA repair activity, oocytes are sensitive to chemical mutagens and efficient transmitters of induced DNA damage to offspring (37). The high sensitivity of female germ cells to chemical mutagens conflicts with the most directly applicable studies of ENU treated female mice (21, 25). The ability to obtain hundreds of offspring from a few females in a matter of days, as well as screen 10^5 to 10^6 loci from each offspring reveals several advantages of using the λ transgenic medaka over more traditional rodent based assays to investigate mutagenesis in female germ cells.

Establishing a consistent spontaneous mutant frequency is essential for studies of suspected mutagens, because the frequency of induced mutations is determined by subtracting spontaneous mutations from the total observed mutations (12). The frequency of spontaneous *cII* mutations (*cII* SMF) occurring in offspring derived from λ transgenic females (3.2×10^{-5}) was similar to the historical somatic *cII* SMF obtained

from the whole bodies of λ transgenic medaka (3.2×10^{-5} vs. 3.0×10^{-5} (31)). The *cII* SMF derived from offspring of λ transgenic female medaka was not significantly different from the *cII* SMF derived from offspring of λ transgenic male medaka, (3.2×10^{-5} vs. 2.9×10^{-5} t-test, $p>0.05$ (37)). This finding would imply that both male and female germ cells repair spontaneous DNA damage with approximately the same fidelity.

In contrast, similar analyses performed with the mouse SLT indicated a significantly lower SMF in female germ cells than males [1.6×10^{-6} vs. 5.3×10^{-5} (12, 46)], implying the female germ cells are either more resistant to spontaneous DNA damage (the long dictyotene arrest decreases metabolism and perhaps free radical development), or they are better at repairing the damage (oocytes remain DNA repair competent throughout oogenesis). Several possible explanations could account for this discrepancy between the medaka and mouse mutation models including species differences and the type of information each assay gives. The two animal species could have significantly different SMFs, though this explanation is unlikely given the somatic *cII* SMF is remarkably similar to the historical SLT SMF [2.9×10^{-5} vs. 5.3×10^{-5} (31, 46)]. In fact, the SLT estimate of female germ cell SMF is lower than all of the other SMF estimates discussed above. Close examination of how the SLT female SMF was derived provides another explanation. This estimate was determined by screening 3,753,449 loci and observing only six mutations [$6/3{,}753{,}449 = 1.6 \times 10^{-6}$ (12)]. The entire estimate of female SMF is based on only six total mutations. The authors note the low frequency of observed mutations creates very wide confidence intervals, and careful consideration employed before using the SLT female SMF (12).

Comparison of spontaneous mutant frequencies also illustrates some of the practical differences between the two assays. Russell and others screened 536,207 offspring to determine a SMF, whereas this work only analyzed 47. The almost insurmountable difference in sample sizes nearly disappears when the actual number of mutations observed and total loci screened by each assay are compared. The mouse SLT found six mutations in 5.4×10^5 loci versus the 2,006 mutations found in 6.3×10^7 loci screened in the transgenic medaka. Despite the advantage that screening for SLT mutations is rapid, and requires little training compared to the *cII* mutation assay, the transgenic medaka model is able to produce the necessary offspring in less time, analyze far more loci per offspring, and consume far less offspring.

Although the mean *cII* MF of offspring derived from ENU treated female medaka is reported, the wide standard deviation (1.8×10^{-3}) indicates a wide variation among individual *cII* MFs. Therefore, the mean is not particularly useful for describing the effects of a given mutagen as the presence of the whole body mutant offspring greatly skews the mean. The offspring *cII* MFs appear to display a Poisson process, in which the probability of producing a mutant offspring is consistent though uncommon (17%). Differences in how a chemical mutagen affects the mean *cII* MF versus the frequency of mutant offspring produced are crucial to understanding the genetic health risk to offspring. For example, a risk assessment based on the mean *cII* MF would conclude all offspring will receive a certain amount of genetic damage (whether or not that level of damage is acceptable). In reality, the majority of offspring would not display any significant change, while a small percentage is drastically affected. For this reason,

frequencies of mutant offspring are thoroughly discussed (the percentage of affected offspring) as opposed to the mean *cII* MF.

Seventeen percent of offspring derived from ENU treated female λ transgenic medaka displayed significantly elevated *cII* MFs indicating female germ cells are as sensitive to ENU as postmeiotic male germ cells and more sensitive than premeiotic male germ cells. Determining the relative sensitivity of each germ cell is important for several reasons. Responses to chemical and radiation mutagens are known to vary widely depending on the stage of gametogenesis at the time of exposure (22, 37, 47). Also, due to the inherent challenges of working with female rodents, analyses of male germ cells are used as surrogates for the lack of information on female germ cells.

Previous studies comparing the relative sensitivities of male and female germ cells to chemical mutagens fail to demonstrate that one sex is consistently more sensitive to chemical mutagens (Table 2.2). The most directly applicable study to our work used the mouse SLT to compare the relative sensitivities of male and female germ cells to ENU, and found that, in contrast to our work, pre- and postmeiotic male germ cells are more sensitive to ENU than female germ cells (21, 25). However, data from *Drosophila* sex-linked recessive lethal assays (21, 48) indicate female germ cells are more sensitive to ENU concurring with our work.

Possible explanations for the discrepancy between results from the mouse SLT and λ transgenic medaka include different ENU doses and routes of administration (160 mg/kg ENU via intraperitoneal injection versus 100 ppm ENU dissolved in ambient water, respectively), physiologic/metabolic differences between species, as well as the mutation assays. The mutant frequency estimate derived from the female mouse SLT is

Table 2.2 Relative sensitivities of male and female germ cells to various mutagens. One sex does not appear consistently more sensitive than the other.

Mutagen	Model	Relative Sensitivity			Reference
		Oocyte	Post-meiotic Male Germ cell	Pre-meiotic Male Germ Cell	
ENU	Medaka λ *cII*	++	++	+	Current work (37)
ENU	Medaka SLT	NT	++	+	(38, 45)
ENU	Mouse SLT	+	++	++	(12, 21)
ENU	Drosophila SLRL	++	+	+	(48)
ENU	Drosophila SLRL	+	++	+	(49)
Procarbazine	Mouse SLT	+	++	++	(21, 22)
TEM	Mouse SLT	+	+	+	(21)
TEM	Mouse SLT	+	++	++	(23, 24)
Bleomycin	Mouse DL	++	-	-	(11)
Bleomycin	Mouse SLT	++	+	+	(13, 50)
Chloroambucil	Mouse SLT	++	++	++	(13, 26)

- Negative
+ Mildly Positive
++ Positive
NT Not Tested

based on eight total mutations, which produces very wide confidence intervals. The results reported here do not definitively prove that one sex is always more sensitive to chemical mutagens, but rather illustrate that male and female germ cells must both be evaluated to more accurately estimate genetic health risks of chemical mutagens.

The ability of the *cII* mutation assay (and other transgenic mutation assays) to disclose the frequency of mutations in each individual offspring (i.e. mutation load, what proportion of *cII* genes were mutated in each individual) was especially insightful. The vast majority (94% 17/18) of mutant offspring derived from ENU-treated female λ transgenic medaka were mosaic, carrying a mutation in only a portion of their cells. Although mosaic mutant offspring have been documented in the offspring of mutagen treated male and female adults (12, 30, 37, 45), such offspring are often underreported or excluded from analysis (12, 32). For example, Barnett *et al.* exposed male Big Blue® mice to ENU and observed that 0.8% (5/597) of the offspring displayed an increased frequency of mutations in the *lacI* gene (32). However, only offspring with *lacI* MF high enough to indicate a whole body mutant offspring were included in analysis (i.e. 0.8% of offspring were whole body mutant offspring). If mosaic mutant offspring are excluded from our results, and only whole body mutant offspring are compared (as in Barnett *et al.*), the frequency of mutant offspring derived from ENU-treated mouse spermatogonial stem cells and ENU-treated medaka female germ cells are not significantly different [0.8% (5/597) vs 1.3% (1/80) X^2, $p>0.2$ (32)]. Many mouse specific locus tests also ignore mosaic mutant offspring (12, 14).

Whether or not to include mosaic offspring in analyses depends on the question the researcher is attempting to answer. Whole body mutant offspring necessarily result

when a mutation is fixed in the gamete or zygote, and then expanded to all cells through mitotic division. Therefore analyses which only identify whole body mutant offspring give an accurate estimate of how many mutations were fixed in the germ cell/gamete of interest. On the other hand, inclusion of mosaic mutant offspring gives more complete information on the total genetic health risk to offspring.

The high frequency of mosaic mutant offspring observed in this study concurs with Winn *et al*., and Shima and Shimada, as many more mosaic offspring than whole body mutants were observed (37, 45). In contrast, historical work with *Drosophila* indicates many more whole body mutant offspring are generated than mosaic mutant offspring (51, 52). Russell also reports the majority of mutant offspring derived from SLT experiments were whole body mutant offspring (14). The discrepancy in the proportions of mosaic versus whole body mutant offspring remains unclear. It could be due to differences in animal model, as Winn and Shima and Shimada employed the Japanese medaka whereas historical work employed *Drosophila* or mice. Winn, Shima and Shimada also used mutation assays is which mosaicism is immediately obvious (λ*cII*, medaka SLT), whereas the Drosophila SLRL assay cannot easily detect mosaicism.

The relatively high proportion of mosaic mutant offspring supports the need to evaluate mosaicism in mutagenesis research and genetic health risk assessments. Distinguishing between whole body and mosaic mutant offspring may have severe consequences not only for the proband (first affected member of a group), but also the following generations. For instance, while whole body mutants have at least a 50% chance of passing the mutation to their offspring (F_2 generation, depending if the individual is homozygous or heterozygous for the mutation, and which allele is

contributed to the gamete) it is more likely for the mutation to be identified by clinical screening because the mutation is present in every cell. Mosaic mutant offspring may be less likely to pass the mutation on (germ cells may not be mutated), however, they are also less likely to display a phenotype and thus the mutation identified prior to conceiving offspring.

Somatic and germinal mosaicism has been linked to various diseases. For instance, colorectal cancer has been linked to somatic mosaic mutations of the *mutS* loci. Multiple diseases are known to follow aberrant inheritance, which describes the existence of an allele or a disease genotype in a child that is not detected in either parent. There are two possible mechanisms of aberrant inheritance: 1) a *de novo* mutation occurred during the offspring's embryonic development, or 2) one or both parents are germinal mosaics. Mohrenweiser described a case study of two siblings affected by *osteogenesis imperfecta* while neither parent was affected (33). Following genetic screening, the causative mutation was present in the father's hair roots and lymphocytes, but not dermal fibroblasts. Further genetic analysis revealed 10% of his sperm also carried the mutation (33). In another example, mosaicism was identified in the spermatogonial stem cells of an unaffected father with three achondroplastic children (34). In each case, the mutation must have occurred early in the father's life to have such varied distribution. Similar cases have been reported in which the parents displayed no clinical evidence of hemophilia, but their offspring do. In one case, the mother was mosaic for a loss of function mutation in clotting factor IX, in another family, the mother was mosaic for a factor VIII deficiency in her oogonia, but not somatic cells (33). Parental mosaicism can provide an origin of disease that was previously thought to be due to *de novo* mutations in

the proband. It is believed that parental mosaicism is responsible for 5-15% of cases where a proband is affected by a single gene, dominantly inherited disorder (33, 53). Additionally, sporadic low-level mutations (mosaicism) were observed in the medaka data (Table 2.1) and such mutations have been linked to autism spectrum disorders (54-56).

Mosaic mutant offspring are interesting not only in their relation to disease, but also because mosaicism provides some information on how mutations arise in offspring. Mosaic individuals carry identical mutations in a fraction of their cells (table 2.1). Two possible mechanisms for producing mosaic mutant offspring are widely discussed in the literature. In the first, mosaic mutant offspring are the result of a mutation on only one strand of DNA in the gamete or zygote. Thus, in the next round of cell division, one daughter cell would carry the mutation and the other wouldn't; as the embryo develops, 50% of the cells contain the mutation, and 50% are wild type. Russell *et al.* found support for this mechanism with the observation that mosaic mutant offspring produced by the specific locus test appeared to be 50% mutant cells and 50% wild type (14). In the second mechanism, which is discussed in the results section, DNA damage is present in the gamete, but is not fixed as a mutation until at least after the first cell division. In this case, one of the early cells would have the mutation and the others would not. All daughter cells derived from the original mutant cell would carry the mutation. If the mutation was fixed at the four cell stage, for example, then 25% of the total cells would carry the mutation, or at the 8 cell stage, 13% would carry the mutation, thus this mechanism accommodates a wider range of mutant frequencies. In contrast to Russell's findings, Shima and Shimada, Winn *et al.* and the present work found the mutant

frequencies of mosaic mutant offspring to be in proportions other than 50-50 (37, 45). Though never published on its own, Shima and Shimada report irradiating two cell embryos produced only mosaic mutant offspring, and never whole body mutant offspring (45). It would be very interesting to know if the mutant frequencies of the irradiated embryos approached the 50-50 ratio.

The high frequency of mosaic mutant offspring (figure 2.2) shows that post-fertilization processes have a significant impact on the mutational burden of offspring. Grenier *et al.* demonstrate that early embryos begin responding to DNA damage between the two and eight cell stage as evidenced by an increase in yH2AX foci (indicative of double strand breaks) and increased reactivity of p53 binding proteins (53BP1) (57). Therefore, these results indicate mutagenesis within the early embryo as well as the gametes must be studied to more fully understand the transmission of genetic damage from parent to offspring.

Mutational spectra results (table 2.1) display an increased frequency of clonally expanded and independent mutations, and supported the status of mutant offspring as whole body or mosaic mutant offspring. As *cII* MFs increased, the mutational spectra included a higher proportion of clonal mutations and fewer independent mutations (table 2.1). As expected, the mutational spectra derived from the whole body mutant offspring consisted of a single mutation, consistent with clonal expansion of a mutation fixed in either the gamete or zygote. Conversely, mosaic mutant offspring, with lower *cII* MFs, corresponded to more diverse mutational spectra with fewer clonal mutations and a higher proportion of independent mutations. Individuals B, C, and D have a *cII* MF that would indicate they are mosaic, yet those spectra consist of single clonally expanded

mutations, indicative of whole body offspring (table 2.1). Mosaic mutant offspring with very high *cII* MFs (>5.0 X 10^{-4}) displayed mutational spectra consisting of a single mutation (table 2.1, individuals B, C, and D)). The spectra of individuals B, C, and D likely included independent mutations at relatively low frequencies, because the small sampling of only ~20 sequenced *cII* genes per offspring would not likely include such independent mutations by random chance. Interestingly, even the most highly mosaic offspring still displayed clonally expanded mutations, indicating some fixation in early development.

One mutant offspring (individual G, table 2.1) displayed two independent mutations at unequal frequencies at the same base pair (base 73). The probability of such an event occurring spontaneously in the same individual is exceptionally low. These mutations are noteworthy as they are extremely rare in spontaneous or chemically induced mutational spectra. This double, or delayed mutation was reported in the mutational spectra of 23% (3/13) of the mutant offspring derived from ENU-treated, λ-transgenic male medaka germ cells.

Mutant offspring that were derived from mutagen-treated, λ transgenic female medaka were found to be primarily mosaic mutant offspring. This finding concurs with the results of more traditional assays (SLT) which examined progeny from mutagen treated females and found a surprisingly high frequency of mosaic mutant offspring (reviewed in (12)). The high proportion of mosaics found in this work and others (37) indicates mutations in offspring are fixed in the early embryo, rather than the gamete. The high frequency of mutant offspring derived from treated oocytes relative to treated premeiotic male germ cells demonstrates that, despite continuous DNA repair activity,

oocytes are sensitive to chemical mutagens and are efficient transmitters of induced DNA damage to offspring (37). This finding conflicts with the most directly applicable studies of ENU treated female mice (21, 25). Further research is needed to resolve the discrepancy in the sensitivity of female germ cells between studies.

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CHAPTER 3

MUTAGEN TREATED FEMALE MEDAKA INDUCE UNTARGETED MUTATIONS IN THE UNTREATED, PATERNALLY DERIVED, DNA OF OFFSPRING[1]

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ABSTRACT:

The oocyte performs multiple functions to ensure normal embryonic development. Chemical mutagen exposure of the oocyte is known to induce mutations in the directly-exposed, maternally derived DNA of offspring. However, such exposure could also induce untargeted mutations in the unexposed paternally derived DNA of offspring, due to the oocyte's functions guiding initial embryonic cell divisions. These so called untargeted mutations were identified and characterized in the paternally derived genomes of the offspring of 1-ethyl-1-nitrosourea (ENU) treated female medaka. Untreated, λ transgenic, male medaka were bred to ENU-treated, wild type females and mutations were detected in the paternally derived transgene of offspring, which was never exposed to the mutagen. Of the 295 offspring analyzed, 2.4% (7/295) showed an elevated *cII* mutant frequency. This frequency of untargeted mutant offspring was not significantly different from the frequency of untargeted mutant offspring derived from ENU-treated male germ cells. The results indicated that mutagen damaged oocytes were capable of transmitting mutations to the unexposed, paternally derived DNA of offspring.

INTRODUCTION:

Our understanding of the transmission of genetic damage to offspring, and the associated genetic health risks, is based primarily on studies in which one parent is exposed to a mutagen and mutations in offspring are identified in the DNA inherited only from the exposed parent (i.e., targeted mutations). However, *in vitro* and *in vivo* studies have demonstrated that both chemical and radiation mutagens can induce mutations in the DNA of a parent that was never exposed to the mutagen when the DNA is examined in offspring with one exposed and one unexposed parent (1-5). These untargeted mutations are not well understood and may represent a significant, and unaccounted for health risk to humans.

Previous studies (discussed in Chapter 2) examined mutations in the offspring of 1-ethyl-1-nitrosourea (ENU) treated female λ transgenic medaka. These treated oocytes contained the *cII* mutation target gene which allowed examination of the effect of chemical mutagens on the directly exposed, maternally derived DNA (i.e. targeted mutations). The majority of the observed mutant offspring (94%, 13/14) were mosaic mutant offspring which indicated mutations in these offspring were fixed after fertilization (6-8). The observation that mutations in offspring were fixed following fertilization, argues for shifting the focus of study from the gamete to the early embryo. Here, we hypothesize DNA damage in the oocyte will induce mutations in the unexposed DNA of offspring contributed by the male germ cell.

Untargeted and delayed mutagenesis are the hallmarks of Genomic Instability (3, 9). Untargeted mutagenesis has been frequently reported, (2, 10, 11), yet the mechanisms remain poorly understood (3). Our previous research has used λ transgenic medaka to

investigate untargeted mutagenesis in the maternally derived genome of offspring derived from mutagen treated male germ cells (Unpublished data, Aquatic Biotechnology and Environmental Laboratory). Specifically, male medaka were exposed to the germ cell mutagen ENU, bred to λ transgenic females, and mutations were analyzed in the untreated, maternally derived genome of offspring (figure 3.1). The resulting fry were then analyzed for mutations in the transgenes derived from the untreated ova. When postmeiotic male germ cells were so treated, 2.3% (16/705) of the offspring showed an increased frequency of untargeted mutations, whereas when premeiotic germ cells were exposed, 3.9% (26/673) of offspring showed an increase in untargeted mutations. These results and those of similar experiments clearly demonstrate mutagen treated male germ cells will induce untargeted mutations in the untreated, maternally derived DNA of offspring (1). Niwa concludes DNA damage in zygotes activates an indirect mutational mechanism which persists at least until day 12 of gestation in mice (12). However, it remains unknown if mutagen treated **female** germ cells can induce untargeted mutations in the **paternal** genome of offspring.

The goal of this study was to better understand Genomic Instability, specifically untargeted mutations, by exposing female λ transgenic medaka to ENU and detecting mutations in the unexposed, paternally derived DNA of offspring. We hypothesized this treatment would result in a higher frequency of mutant offspring compared to untargeted mutant offspring derived from ENU treated male germ cells because the oocyte not only contributes genetic information to offspring but also provides most of the cellular machinery of early embryonic cell division.

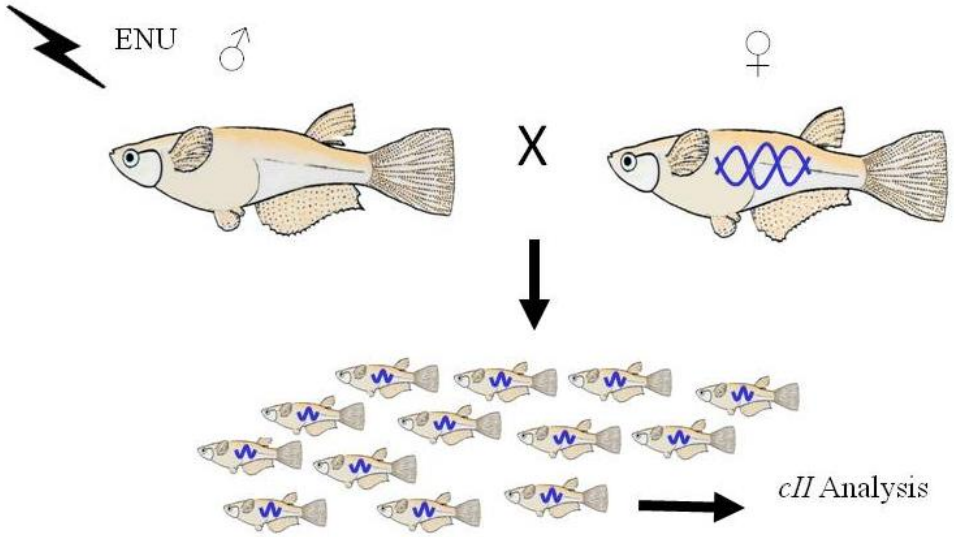

Figure 3.1: Illustration of the breeding scheme used to investigate untargeted mutations in offspring of medaka derived from ENU-treated, non-transgenic male germ cells and λ transgenic untreated females. Offspring were analyzed for untargeted mutations in the untreated, maternally derived transgene with the *cII* mutation assay.

Oocytes supply the embryo with many maternal gene products until full zygotic gene activation is achieved (13). Early embryogenesis is dependent on the accumulation of RNAs and proteins in the ooplasm during oogenesis (14, 15). These ooplasmic elements are also responsible for resetting global epigenetic imprinting necessary for normal embryonic differentiation (16).

Maternal mRNA already present in the oocyte at fertilization is the only template for translation until the two-cell stage of zygotic development (17-19). Therefore this mRNA would be exposed to the ENU in the adult female. Singer *et al*. demonstrated ENU will alkylate multiple oxygen molecules in mRNA (20), while Kanduc *et al*. (21) showed ENU can alkylate guanosine to 1-7 diethyl guanosine in tRNAs, and possibly further disrupt translation. Therefore, in addition to directly damaging DNA present in the oocyte, mutagen exposure to oocytes could alter transcription and translation in the early embryo, impeding DNA repair and replication. We hypothesized that ENU treated oocytes will generate more untargeted mutant offspring than similarly treated male germ cells because ENU treated oocytes will carry damaged DNA and RNA. This hypothesis was tested by exposing wild type female medaka to ENU and breeding them to untreated, λ transgenic males. Mutations were detected in the paternally derived, thus not directly exposed, transgene of offspring, using a positive-selection assay based on the *cII* gene as the mutation target gene.

MATERIALS AND METHODS:

Animals

Wild type (CAB strain) and λ transgenic medaka (strain λ 310), which contain the λLIZ bacteriophage vector harboring the *cII* gene mutation target gene (homozygotes

~150 λ copies /diploid genome), were obtained from in-house stocks at the Aquatic Biotechnology and Environmental Laboratory, University of Georgia (22). Fish were maintained on a 12 hr light-dark cycle, except breeding pairs maintained on a 16:8 light-dark cycle in dechlorinated municipal water at 24° C and fed 1 to 2 times daily with brine shrimp nauplii (Artemia Great Salt Lake, UT) and supplemented 1 to 2 times daily with a commercial flake diet. All animal care protocols were approved by The University of Georgia Institutional Care and Use Committee.

Mutagen Treatment

Adult wild type females were independently exposed to 100 ppm of the germ cell mutagen, ENU in their ambient water for one hr on two consecutive days. Females were triple rinsed with distilled, deionized water, and allowed to decontaminate in individual tanks for four hours following ENU treatment to allow the ENU time to degrade (half-life of ENU in living tissue is 34 mins and 1.5 hours in water, pH 7.0) (23). Females were bred to untreated, λ transgenic males for seven days prior to exposure thus yielding control offspring, and continuously from 1 to 35 days following exposure yielding treatment offspring. Therefore, control and treatment offspring were siblings. Offspring derived from three breeding pairs were analyzed by *cII* mutation analysis. Approximately 50 control offspring were collected to determine the spontaneous *cII* MF (SMF) and compared to the historical spontaneous *cII* MF. Approximately one-hundred offspring were collected from each breeding pair following ENU exposure (treatment group) to provide sufficient data for comparing frequencies of mutant offspring to previous work. All progeny were hemizygous for the *cII* target gene, which was contributed only by the male parent (Figure 3.2). Collected embryos were allowed to

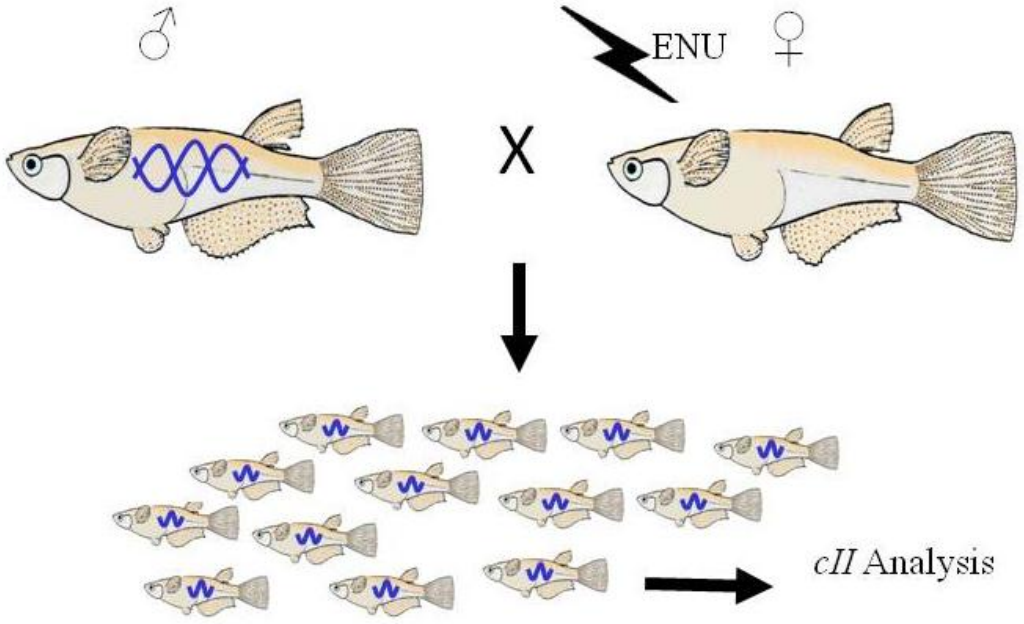

Figure 3.2 Illustration of breeding scheme to investigate untargeted mutations in medaka offspring derived from ENU-treated, wild type **female** germ cells. By design, mutations in offspring will only be detected in the paternally derived transgene, which was never directly exposed to the mutagen. Mutations in offspring collected prior to treatment reflect spontaneous mutant frequencies in the paternally derived *cII* gene, whereas *cII* mutant frequencies of offspring after exposure indicate the sum of the spontaneous *cII* MF and any untargeted mutations.

hatch and were cultured under standard conditions for 14 to 21 days, flash frozen, and stored at -80° C prior to mutation analysis.

DNA Extraction

Genomic DNA was isolated from individual siblings using previously described procedures (22, 24). Briefly, tissues from whole animals were digested with proteinase (1X SSC, 20% SDS, 20mg/ml proteinase K) and extracted with equal volumes phenol:chloroform. DNA was precipitated using ethanol then resuspended in Tris-EDTA buffer (pH7.5).

cII Mutation Assay

Mutations in the offspring of mutagen-exposed females were analyzed using a positive-selection assay in which the *cII* gene was the mutation target gene (25). This assay is based on the role of the *cII* protein in the commitment of bacteriophage λ to the lysogenic or lytic cycle in *E. coli*. Selection of mutant *cII* genes was facilitated using a specialized *E. coli* strain (G1250, *hfl*$^-$) that extends the longevity of the *cII* protein product. Genomic DNA was incubated with packaging extracts to simultaneously excise and package the λ*LIZ* vector into viable bacteriophage. The packaged phage particles were then allowed to infect and lyse the *E. coli* host. The frequencies of *cII* mutants (*cII* MFs) were calculated by dividing the total number *cII* mutant plaque forming units (PFUs) on the selective screening plates by the total λ *cII* phages on the permissive titer plates. To select λ *cII*$^-$ mutants (screening plates), the packaged phages were mixed with *E. coli* cells, plated, and incubated at 24° C for 40hr. The phages with wild type *cII* became lysogenic, and were indistinguishable from the *E. coli* lawn, whereas phages that carried a mutation in the *cII* gene formed easily counted plaques in the bacterial lawn when

incubated at 24° C. To quantify the total λ *cII* loci recovered (λ *cII*$^-$ mutants and λ *cII*$^+$ non-mutants) (permissive titer plates), packaged phages were diluted and mixed with *E. coli* cells, plated, and incubated at 37°C for 15hr. All packaged phages, regardless of whether a mutation has occurred or not, entered the lytic cycle forming a plaque.

Mutational Spectra

DNA mutations from offspring with elevated *cII* MFs were sequenced and the resulting spectra were compared to spontaneous and previously published spectra to identify unique characteristics of mutagenesis in female germ cells. To characterize the spectra of *cII* mutations from offspring with elevated *cII* MFs, we used standard methods to isolate individual mutant plaques. The λ *cII* mutants were characterized using DNA sequencing methods previously described (22). Individual λ *cII*-mutant plaques were cored at random from mutant screening plates and purified on G1250 *E. coli* cells. Individual plaques were excised and the *cII* gene amplified by PCR. PCR products were electrophoresed on a 1% agarose gel, cleaned with QIAquick PCR purification kit (Qiagen, Valencia CA), then diluted to 0.1μg/μL using Tris-EDTA. Diluted PCR products were labeled (BigDye Terminator Cycling DNA Sequencing Kit; Applied Biosystems, Foster City, CA) and then purified again using DyeEx 2.0 spin columns (Qiagen) Samples were analyzed using an automated DNA Sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems). Spectra were compared using previously published hypergeometric analysis methods (26).

Testing for Treatment Effects

The *cII* MFs of the control sample were tested for normal distribution by the Shapiro-Wilk test, $\alpha = 0.01$. For a normally distributed control population, the empirical

rule states that less than 0.15% of individuals will display a *cII* MF greater than 3 standard deviations above the mean (>5.2 X 10^{-5}). If there were no treatment effect, then less than 0.15% of the treatment sample would also display a *cII* MF > 5.2 X 10^{-5}. Therefore, if more than 0.15% of the treatment sample displayed a *cII* MF > 5.2 X 10^{-5} there was a treatment effect. The frequency of untargeted mutant offspring derived from mutagen treated **female** medaka was compared to the frequencies of untargeted mutant offspring derived from mutagen treated **male** medaka by Chi-Square analyses.

RESULTS:

Mean cII MF of Offspring Derived From Untreated Female Medaka.

Two of the three breeding pairs produced 63 offspring prior to ENU exposure, which were analyzed to determine the spontaneous *cII* MF (SMF) (Appendix C). *cII* mutation analysis revealed a mean *cII* MF of 2.2 X 10^{-5} (n=63) and a standard deviation of 1.0 X 10^{-5}. One pair had a *cII MF* range of 1.4 to 4.8 X 10^{-5} and the *cII MF* range of the other pair was from 0.1 to 4.5 X 10^{-5}.

cII MF of Offspring Derived From Mutagen-Treated Female Medaka.

Following exposure of the breeding females, 295 offspring were collected and analyzed from three breeding pairs. The mean *cII* MF of this sample was 2.5 X 10^{-5} with a standard deviation of 1.3 X 10^{-5}. Appendix D includes the offspring ID, number of *cII* mutants, PFUs and *cII* MF of offspring collected following mutagen treatment of the female medaka.

Of the 295 offspring sampled, 2.4% (7/295) displayed an increased *cII* MF (>5.2 X 10^{-5}) and were identified as mutant offspring. This frequency of mutant offspring was not significantly different from the frequency of mutant offspring derived from

postmeiotic untargeted male [2.4% vs. 2.3%, $X^2 = 0.01$, $\alpha > 0.1$ (Unpublished data, Aquatic Biotechnology and Environmental Laboratory, University of Georgia, Athens, Georgia)]. Nor was this frequency of mutant offspring significantly different from the frequency of mutant offspring derived from premeiotic untargeted male [2.4% vs. 3.9%, $X^2 = 1.38$, $\alpha > 0.1$ (Unpublished data, Aquatic Biotechnology and Environmental Laboratory, University of Georgia, Athens, Georgia)].

Magnitude of cII *MF and DNA Sequencing Indicates All Mutant Offspring are Mosaic Mutant Offspring.*

The range of *cII* mutant frequencies of the mutant offspring was 5.4 to 12.4 X 10^{-5} (appendix D), which represents a 2 to 5.6 fold elevation over the mean *cII* MF of the control offspring. Whole-body mutants would be expected to have a MF of ~ 1.3 X 10^{-2}. Thus, the observed mutant offspring are likely all mosaic mutant offspring because all *cII* MFs of the mutant offspring are < 1.3 X 10^{-2}.

DNA sequencing of a mutant offspring provided further evidence of mosaic mutations. Twenty-two mutant *cII* mutation target genes were sequenced from individual 1yyyy (appendix D, *cII* MF= 12.4 x10^{-5}). Nineteen of the 22 sequences (86%) contained the C→G mutation, indicating clonal expansion of this single mutation. Table 3.1 shows the mutational spectra of offspring 1yyyy.

DISCUSSION:

Mutagen treated female medaka induce untargeted mutations in the untreated, paternally derived, DNA of offspring. Previous experiments demonstrated ENU-treated female medaka transmit mutations to the DNA in offspring which they inherit from her

Table 3.1: Spectrum of untargeted mutations from offspring 1yyyy derived from an ENU-treated wild type female and a λ-transgenic male. These mutations were sequenced from the *cII* transgene, derived from the unexposed male parent. The C→ G transversion at base 172 comprises 91% (20/22) of the spectrum, indicating this mutation was fixed early in development and clonally expanded. Mutations at base pairs 40 and 274 represent independent mutations.

Individual	***cII* MF**	**Base Pair**	**Mutation**	**# of Seq.**
1yyyy	1.2×10^{-4}	172	C→G	20
		40	G→A	1
		274	C→G	1
			Total	22

(thus directly exposed to the mutagen). The current study provides evidence that these offspring also contain induced mutations in the unexposed DNA derived from the male parent. Data from both experiments confirm that mutagen treated oocytes mediate mutations in the directly exposed and unexposed regions of DNA of the offspring.

All untargeted mutant offspring were mosaic mutant offspring, indicating that the mutations were fixed after fertilization. The frequency of mutant offspring among all of the offspring analyzed is similar to previous untargeted experiments using the λ transgenic medaka, and was lower than similar experiments using minisatellite analysis in mice. To most accurately estimate the total genetic health risk to offspring of mutagen exposed parents, risk assessments should include targeted and untargeted effects.

The mean *cII* MF of the control offspring (spontaneous mutant frequency of this experiment) was significantly lower than the historical spontaneous *cII* MF [SMF; mean control SMF = 2.2×10^{-5}, std dev = 1.0×10^{-5}, n=63 vs. historical SMF = 2.9×10^{-5}, std dev = 1.2×10^{-5}, n=73 student's t-test $p<0.001$ (27)]. Table 3.2 compares the mean and standard deviation of the SMF of this experiment to published estimates of the *S*MF of the λ transgenic medaka. The lower SMF was due to an unavoidable change in the reagents used to make the media on which the *E. coli* are grown. Prior to beginning *cII* analysis, the casein digest, which provides nutritional support to the G1250 *E. coli*, became unavailable by the manufacturer. The substitute nutrition source depressed the formation of mutant plaques under selective conditions, resulting in a consistent, although lower, SMF (appendix C). Assuming all samples (treatment and control) were affected the same by the change in reagents, the *cII* MF of treatment offspring would also

Table 3.2: Comparison of mean and standard deviations of *cII* MFs for untreated, whole λ transgenic medaka. All *cII* analyses were performed at the Aquatic Biotechnology and Environmental Laboratory, University of Georgia, Athens, GA using identical protocols.

Mean *cII* MF (X 10^{-5})	**Standard Deviation (X 10^{-5})**	**Reference**
2.2	1.0	Present Work
2.9	1.2	(27)
2.9	-	(22)
3.0	1.3	(28)

be falsely depressed. The frequency of mutant offspring estimates are not affected because mutant offspring are defined relative to the SMF within this experiment.

The frequency of untargeted mutant offspring derived from mutagen treated female germ cells was not significantly different from the MOF of either premeiotic or postmeiotic mutagen treated male germ cells (i.e., just as many untargeted mutant offspring are observed regardless of which gamete was exposed). The data failed to support the hypothesis that mutagen treated oocytes would induce more untargeted mutations than similarly treated male germ cells. There are several mechanisms that could explain these results. The chemical mutagen ENU may have disrupted both mRNA, and tRNA in the oocyte, but the affected molecules could have been replaced prior to fertilization. It is also possible that critically damaged oocytes may have undergone selection, and been removed from the germline prior to fertilization. Russell *et al.* provided some evidence of oocyte selection, as chlorambucil was shown to kill immature oocytes (6). Alternatively, fertilized oocytes with damaged DNA, mRNA, and tRNA may not have been able to successfully complete embryogenesis, and therefore the offspring died prior to *cII* mutation analysis. Generoso *et al.* provided evidence for the embryonic death explanation in that *D. melanogaster* offspring derived from ENU-treated oocytes show a statistically significant increase frequency of fetal death (29). Finally, the ENU may not have seriously damaged cellular components of the oocyte (mRNA, tRNA etc) or if they had, the damage does not affect how the developing embryo responds to DNA damage.

The frequencies of untargeted mutant offspring observed in studies of λ transgenic medaka (~2%) appear lower than previous estimates using ESTR analysis.

Niwa and Kominami report around 20% of offspring derived from irradiated fathers showed mutations in **maternally** derived minisatellites. However, multiple key differences between the studies preclude direct comparison, including: different mutation detection assays, the relatively high spontaneous mutant frequency (SMF) of ESTR (~10%), radiation exposure vs. chemical, and, of course fish vs. rodent animal models. To determine if our untargeted results are similar in mammals, and also more thoroughly describe untargeted mutagenesis in mammals with additional mutation burden and spectra data, untargeted experiments should be repeated in transgenic rodents.

Although untargeted mutagenesis has been observed *in vivo* before, our study provides additional mutational burden data and the spectra of the untargeted mutations in the λ transgenic medaka. The magnitudes of *cII* MFs indicate that, as expected, every mutant offspring was a mosaic, We expected mosaics in this experiment because untargeted mutations cannot be fixed prior to fertilization, (i.e., the transgene had no chance to interact with exposed cellular components until fertization), thus there is no route to whole-body mutants. The untargeted mutations are direct evidence of global errors in DNA repair and replication occurring in the early embryo as a response to introduced DNA damage.

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CHAPTER 4

MUTAGEN TREATED MALE GERM-CELLS MEDIATE UNTARGETED MUTATIONS TO THE MATERNAL GENOME OF OFFSPRING: COMPARISON OF TRANSGENIC RODENT AND FISH MODELS [1]

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ABSTRACT:

Recent experiments have shown that mutagen exposure in a parent can induce mutations in regions of the offspring's DNA that were never directly exposed to the mutagen. We sought to compare the frequency and spectra of untargeted mutagenesis in two transgenic animal mutation models, the Big Blue mouse and the λ transgenic medaka (a small fish). Female Big Blue transgenic mice were bred to ENU-dosed C57BL/6 males and the resulting offspring were analyzed for untargeted mutations in the untreated, maternally derived *cII* gene. Control offspring were collected prior to dosing whereas treatment offspring were derived from either pre- or postmeiotic male germ cells at the time of dosing. These results were compared to a similar experiment using the medaka animal model. The mean control *cII* mutant frequency (MF) was 3.8×10^{-5} (standard deviation of 1.7×10^{-5}) for the mouse model. Mutant offspring were defined by *cII* MF $> 8.8 \times 10^{-5}$ and the frequency of mutant offspring (MOF) derived from ENU treated postmeiotic germ cells was 3.5% (6/171) with a range of *cII* MF from 8.9×10^{-5} to 14.1×10^{-5}. This frequency of mutant offspring was not significantly different from the MOF of medaka (3.5% vs. 2.3% X^2=0.86, p> 0.25). The mutant medaka spectra was significantly different from the spontaneous spectra of Big Blue mice (Cochran-Armitage p= 0.02), but not from the spectra of mutations derived from medaka untargeted mutant offspring (Cochran-Armitage, p=0.72). Of the 49 offspring derived from ENU-treated premeiotic germ cells one mutant offspring was identified, indicating an approximate MOF of 2%. Our results indicated untargeted mutagenesis occurs at similar frequencies and induces similar mutations in transgenic mice and medaka, which implies a common mechanism operating in both species.

INTRODUCTION:

Mutations present in offspring of chemically-treated parents are thought to occur by DNA damage in the parent being fixed as a mutation in germ cells and directly passed to offspring via the gametes (spermatozoa and oocytes) (1). This mechanism assumes mutations are the results of errors in the repair of damaged DNA (1). Therefore, mutations would occur exclusively in DNA directly exposed to the mutagen. According to this model, only genes inherited from an exposed parent are susceptible to an increased frequency of mutations (2).

In vitro and *in vivo* data collected over the past three decades from chemical and radiation exposures contradict this dogma by demonstrating increased frequencies of mutations within regions of DNA never exposed to the mutagen. These observations lead to the theory of Genomic Instability (3). Genomic instability includes two principle concepts: 1) delayed effects, in which genetic abnormalities are detected in cells several generations after exposure, and 2) untargeted or bystander effects, in which genetic anomalies are observed in DNA that was never directly exposed to the mutagen (4, 5). The goal of this research was to compare one aspect of genomic instability, untargeted mutagenesis, in two distinct transgenic animal models, rodents and fish.

Early evidence of untargeted mutagenesis came in the form of *in vitro* cell cultures in which some, but not all, cells were exposed to α- particle radiation. In these experiments, the neighboring, unirradiated cells (bystander cells) showed increased frequencies of mutations (3). Nagasawa irradiated Chinese Hamster Ovary cells with α-particles, and although only an estimated 1% of the cells were actually transversed by an α-particle, 30% of the total cells contained Sister Chromatid Exchange mutations (SCE)

(6). Deshpande performed similar experiments in human lung fibroblasts, and observed increased frequencies of SCEs in cells where the α-particle did not hit the nucleus (7). Mutations observed in bystander cells are predominantly point mutations, whereas directly irradiated cells were partial or total gene deletions (8, 9), indicating a possible separate mechanism of mutagenesis in the bystander cells. Further experiments indicated low fluence α-particles also upregulated the tumor suppressor gene TP53 in bystander cells (10). Subsequent research revealed that oxidative stress played a significant role in signal transduction from the irradiated cell to bystander cells (11).

Untargeted mutagenesis was further evidenced by medium transfer experiments, in which the growth media of irradiated cells was transferred to non-irradiated cells, which induced mutations in the naïve cells (12, 13). Similar studies also indicated α-radiated cells released reactive oxygen species which may induce the SCEs in non-irradiated cells. Non-irradiated cells also upregulated AP endonuclease, an important enzyme in the base excision repair pathway (14). Although these experiments provided insight into the cellular mechanisms involved in untargeted mutagenesis, they were *in vitro* and later research has sought to understand untargeted mutagenesis in the entire animals (15).

Evidence of untargeted mutagenesis *in vivo* was first observed using Expanded Simple Tandem Repeat (ESTR) analysis, in which short repeated sequences were analyzed for change of length mutations via additions or deletions (16, 17). Niwa and Kominami irradiated male mice and observed that 20% of the offspring displayed an increased frequency of mutations in the *maternally* derived hypervariable minisatellites (18). Similarly, Barber *et al.* irradiated several strains of male mice and observed both

characteristics of genomic instability: increased mutation rates of ESTRs several generations following exposure as well as increased frequencies of mutations at unexposed maternally derived regions of DNA (19). Tsyusko *et al*. provided further evidence of untargeted mutagenesis in a non-mammalian animal model, the Japanese medaka (*Oryzias latipes*). They observed much higher frequencies of mutations in microsatellite regions of offspring derived from irradiated males than would be expected from direct interaction of radiation with DNA. This finding indicated an untargeted mechanism of mutagenesis (15). Although these experiments provided strong evidence of untargeted mutagenesis in the germ line of both mice and medaka, the repeated sequence analysis they used is limited to detecting only change of length mutations whereas multiple experiments of bystander effects have demonstrated untargeted point mutations (3). Also, repeated sequences such as minisatellite and tandem repeats display a high spontaneous mutation frequency (SMF) in both rodents and humans compared to other regions (20, 21). In fact, the Ms6hm locus, a commonly used mutation target gene for germ cell mutagenesis studies, has one of the highest SMFs of any loci in the mouse genome (22).

The present study investigated untargeted mutagenesis in the offspring of mutagen treated males in two diverse animal models using the *cII* transgenic mutation assay. The *cII* mutation assay can detect a variety of mutations including point mutations, and small insertions/deletions (23, 24) and has a SMF only slightly higher than endogenous genes (25-27). In contrast to previous studies which relied on radiation exposures, we employ a chemical mutagen to confirm that untargeted mutagenesis is not unique to radiation exposures. The *cII* assay detects mutations in the λ transgene of

offspring, and the DNA sequence of those mutations is easily obtained, providing mutational spectra data. The *cII* mutation assay also provides the frequency of mutated target genes to non-mutant genes, which provides information about the mutational burden of offspring. The present work compares the untargeted response in divergent animal models (i.e. rodent and fish animal models) carrying an identical mutation target gene to test the following hypotheses: 1) there is no significant difference in the frequencies of mutant offspring between transgenic mouse and fish, and 2) there are no significant differences in the mutational spectra derived from affected mice or fish. If these hypotheses are true, then untargeted mutagenesis is likely a highly conserved phenomenon across multiple vertebrate lineages and is applicable to human health risk assessments.

MATERIALS AND METHODS:

Animals

Male inbred mice (C57BL/6, eight weeks of age) and female homozygous transgenic female Big Blue mice (eight weeks of age) were obtained from Charles River Laboratories (Wilmington, MA) and Taconic Farms (Germantown, NY) respectively. The female Big Blue mice carry the λLIZ bacteriophage vector harboring 40 copies of the *cII* mutation target gene per haploid genome. Mice were maintained at 22°C, 40% humidity, on a 12 hour light/dark cycle and given free access to water and rodent chow (Purina 5001). Breeding or pregnant animals were maintained under identical conditions and fed free choice Purina 5015 chow. The University of Georgia Institutional Animal Care and Use Committee approved the protocol used in this research.

Male wild type and female homozygous λ transgenic female medaka were obtained from in house stocks. The female λ transgenic medaka carry the λLIZ bacteriophage vector harboring 75 copies of the *cII* mutation target gene per haploid genome. Medaka were maintained at 24°C, on a 12 hour light/dark cycle and fed brine shrimp nauplii 1 to 2 x per day. The University of Georgia Institutional Animal Care and Use Committee approved the protocol used in this research.

Mutagen Treatment

Adult male inbred mice or medaka were dosed once with the potent germ cell mutagen ethyl-*N*-nitrosourea (ENU, 150mg/kg body weight) via intraperitoneal injection as previously described (28-30) or ambient water (100 ppm ENU). Each male was bred to three, untreated females, and offspring were collected prior to and following mutagen dosing. The mating scheme is displayed in figure 4.1. Offspring collected prior to exposure served as sibling controls. Offspring collected 21-43 days following exposure were derived from spermatids or spermatozoa (postmeiotic germ cells) at the time of exposure and offspring collected >110 days after exposure were derived from spermatogonial stem cells (premeiotic germ cells) during the ENU exposure. Progeny were hemizygous for the λLIZ vector containing the *cII* mutant target gene (40 copies/ diploid genome), contributed by the female parent only. Visibly pregnant females were isolated for whelping and day-old pups were euthanized with CO_2 prior to flash freezing in liquid N_2. Pups were stored at -80°C before processing for mutation analyses.

DNA Extraction

Genomic DNA was isolated from entire individual pups by a modification of previously described techniques (31). Briefly, whole pups were mechanically

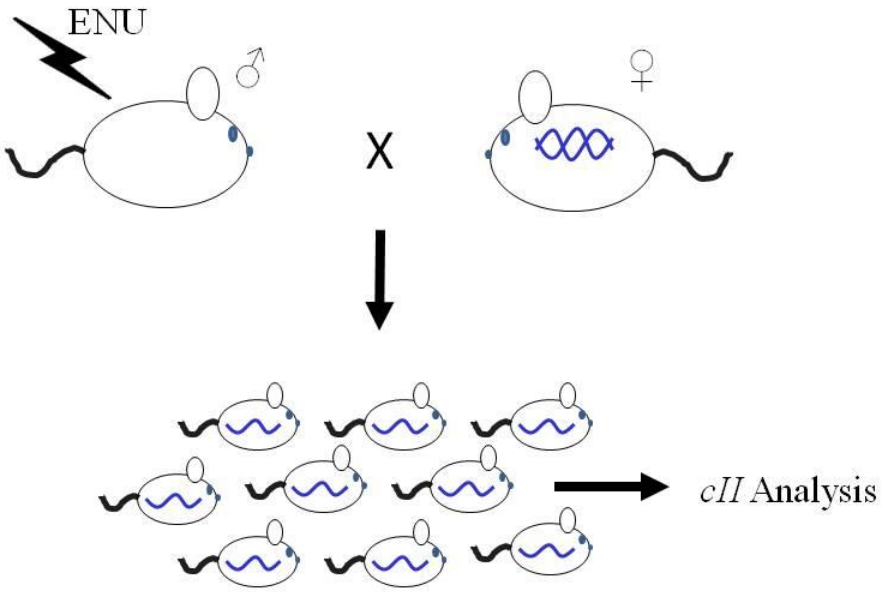

Figure 4.1 Mating scheme of C57BL/6 males and Big Blue® transgenic females. Offspring are hemizygous for the *cII* mutation target gene which is inherited solely from the unexposed female parent. Offspring were collected prior to and following exposure, to serve as control and treatment groups, respectively.

homogenized in 10 ml of homogenization buffer (1X SSC, 1.2% (w/v) SDS, 0.6 mg/ml proteinase K), and the tissue was digested at 50°C for 2 to 3 hrs. Following digestion, an additional 10 ml of buffer were added and DNA was extracted twice with equal volumes of phenol:chloroform. DNA was precipitated with ethanol and resuspended in Tris-EDTA buffer (pH 7.5).

cII Mutation Assay

Mutations in the offspring of mutagen-exposed females were analyzed using a positive-selection assay in which the *cII* gene was the mutation target gene (19). This assay is based on the role of the *cII* protein in the commitment of bacteriophage λ to the lysogenic or lytic cycle in *E. coli*. Selection of mutant *cII* genes was facilitated using a specialized *E. coli* strain (G1250, *hfl*$^-$) that extends the longevity of the *cII* protein product. Genomic DNA was incubated with packaging extracts to simultaneously excise and package the λ*LIZ* vector into viable bacteriophage. The packaged phage particles were then allowed to infect and lyse the *E. coli* host. The frequencies of *cII* mutants (*cII* MFs) were calculated by dividing the total number *cII* mutant plaque forming units (PFUs) on the selective screening plates by the total λ *cII* phages on the permissive titer plates. To select λ*cII*$^-$ mutants (screening plates), the packaged phages were mixed with *E. coli* cells, plated, and incubated at 24°C for 40 hr. The phages with wild type *cII* became lysogenic, and were indistinguishable from the *E. coli* lawn, whereas phages that carried a mutation in the *cII* gene formed easily counted plaques in the bacterial lawn when incubated at 24°C. To quantify the total λ *cII* loci recovered (λ *cII*$^-$ mutants and λ *cII*$^+$ non-mutants) (permissive titer plates), packaged phages were diluted and mixed with

E. coli cells, plated, and incubated at 37°C for 15 hr. All packaged phages, regardless of whether a mutation has occurred or not, entered the lytic cycle forming a plaque.

Mutant offspring were defined by an increased *cII* MF compared to spontaneous controls. DNA samples that exhibited *cII* MFs elevated three standard deviations above the mean of sibling controls were designated as mutant offspring by the following logic. The Shapiro-Wilk test was performed to determine if the control *cII* MFs were normally distributed. A normally distributed control data set allows the use of the Empirical Rule (68-95-99.7 rule). Therefore, 68% of the data is contained within one standard deviation of the mean, 95% of the data is contained within two standard deviations of the mean and 99.73% within three standard deviations of the mean. By definition, only 0.27% of the data lies above and below three standard deviations of the mean. This would leave only 0.14% of the normally distributed control sample *cII* MFs greater than three standard deviations above the mean. If there was no difference between control sample and treatment sample *cII* MF, only 0.14% of the treatment sample *cII* MFs would be greater than three standard deviations above the mean control *cII* MF. Therefore, any offspring with a *cII* MFs elevated three standard deviations above the mean of sibling controls were designated as mutant offspring.

Mutational Spectra

To characterize the spectra of *cII* mutations carried by selected individual offspring with elevated *cII* MFs, standard methods were employed to isolate individual mutant plaques, followed by PCR sequencing. The λ *cII* mutants were characterized using DNA sequencing methods previously described (33). Individual λ *cII*-mutant plaques were cored at random from mutant screening plates and purified on G1250 *E.*

coli cells. Individual plaques were excised and amplified by PCR. PCR products were electrophoresed on a 1% agarose gel, cleaned with QIAquick PCR purification kit (Qiagen, Valencia CA), diluted to 0.1μg/μL using Tris-EDTA. Diluted OCR products were labeled (BigDye Terminator Cycling DNA Sequencing Kit; Applied Biosystems, Foster City, CA) and then purified again using spin columns (DyeEx 2.0 Qiagen) Samples were analyzed using an automated DNA Sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems).

Statistical Analyses

Previously published statistical methods were used to test for normality, compare frequencies of mutant offspring and compare mutational spectra. The Shapiro-Wilk test was used to determine if the *cII* MFs of the control offspring were normally distributed. Significant differences of frequencies of mutant offspring were tested using *Chi-Square* analysis and mutational spectra were compared using the Cochran-Armitage test (34). Significance was determined at $\alpha=0.05$.

RESULTS:

Spontaneous cII Mutant Frequency (SMF)

Ten C57BL/6 mice successfully bred to 1, 2, or 3 Big Blue® transgenic females each, prior to ENU dosing. These pre-dose offspring served as sibling controls. The SMF of the Big Blue mouse as determined by averaging the *cII* MFs from 101 of these control offspring was 3.8×10^{-5} (standard deviation = 1.7×10^{-5}). Mutant offspring were distinguished from non-mutant offspring by a *cII* MF elevated above the SMF and mutant offspring are defined by a *cII* MF greater than three standard deviations above the SMF (8.8×10^{-5}). No mutant offspring were detected from the control group (appendix E).

Mutant Offspring Derived from ENU Treated Postmeiotic Male Germ Cells

Offspring derived from ENU treated postmeiotic male germ cells showed higher *cII* MFs in the unexposed, maternally derived target genes as compared to their pre-dose siblings. The same breeding groups used to produce control offspring were bred again 1 to 20 days following ENU dosing to produce offspring derived from ENU treated spermatids and spermatozoa. Table 4.1 summarizes the *cII* MFs of postmeiotic offspring and the *cII* MFs of all postmeiotic offspring are listed in Appendix F. Of the 171 postmeiotic offspring sampled, 3.5 % (6/171) displayed a *cII* MF >8.8 X 10^{-5}. The *cII* MF of the mutant offspring ranged from 8.9 X 10^{-5} to 14.1 X 10^{-5}, a 2.4 to 3.7 fold increase above mean control *cII* MF (3.8 X 10^{-5}).

Mutant Offspring Derived from ENU Treated Premeiotic Male Germ Cells

To produce offspring derived from ENU treated premeiotic male germ cells (spermatogonial stem cells) offspring were collected 110 or more days following ENU dosing. Table 4.2 summarizes the *cII* MFs of premeiotic offspring and Appendix G presents the *cII* MFs of all premeiotic offspring. Forty-nine offspring derived from premeiotic germ cells were collected and analyzed. Of these, one individual offspring displayed a *cII* MF >8.8 X 10^{-5}. The *cII* MF of the mutant offspring was 10.0 X 10^{-5}, a 2.6 fold increase above mean control *cII* MF (3.8 X 10^{-5}).

Spectra of Mutations Sequenced from Postmeiotic Mutant Offspring

A total of 138 mutant plaques were sequenced from the six mutant offspring derived from ENU treated, postmeiotic germ cells. Mutation spectra for each individual postmeiotic offspring are tabulated in Appendix H. The combined mutational spectra derived from postmeiotic mutant offspring was significantly different from previously

Table 4.1 Mutant and non-mutant rodent offspring derived from ENU treated, postmeiotic male germ cells. The ten ENU-treated male C57BL/6 mice produced 171 total offspring. The frequency of mutant offspring is shown in parentheses.

		No. of Offspring	
Male	**N**	**Non-mutant Offspring** *cII* MF<8.8 X 10^{-5}	**Mutant Offspring** *cII* MF≥8.9 X 10^{-5} (%)
1	10	10	0
2	6	6	0
3	29	28	1 (3)
4	15	15	0
5	19	17	2 (11)
6	22	22	0
7	14	14	0
8	8	8	0
9	24	22	2 (8)
10	24	23	1 (4)
Total (Overall %)	**171**	**165 (96.5%)**	**6 (3.5%)**

Table 4.2 Mutant and non-mutant rodent offspring derived from ENU treated, premeiotic male germ cells. Four ENU-treated male C57BL/6 mice produced only 49 offspring derived from ENU-treated premeiotic germ cells. The frequency of mutant offspring is shown in parentheses.

		No. of Offspring	
		Non-mutant Offspring	**Mutant Offspring**
Male	**N**	*cII* MF<8.8 X 10^{-5}	*cII* MF ≥8.9 X 10^{-5} (%)
3	14	14	0
6	20	20	0
9	5	5	0
10	10	9	1 (10)
Total (Overall %)	**49**	**48 (98%)**	**1 (2%)**

published spontaneous *cII* mutation spectra of Big Blue mice (Table 4.3,4, p= 0.02, (35)). Table 4.3 categorically displays the proportions of independent mutations and table 4.4 displays the spectra of mutations from mouse and fish mutant offspring, as well as spontaneous spectra for each species.

Several similarities and differences emerge when comparing the mutational spectra of mouse mutant offspring and spontaneous mutational spectra. First, mutant offspring spectra displayed a relative increase in the frequency of clonally expanded mutations when compared to the spontaneous spectra (Table 4.3). Additionally, mutant offspring spectra showed a relative increase in the frequency of frameshift mutations when compared to the spontaneous spectra (19% vs 11%). This caused the corresponding decrease in base substitutions (81% vs 86%) and complex mutations (mutations involving two or more adjacent base pairs) (0% vs 3%). The relative decrease in base substitutions was due to a decrease in transversions (32% vs 41%). The increase in frameshift mutations (11% vs. 18%) correlates to an increase in mutations at the 179-184 bp hotspot. This string of six guanine residues often results in small additions and deletions due to errors in DNA replication (36, 37).

Spectra of Mutations Sequenced from Premeiotic Mutant Offspring

Fourteen mutant plaques were sequenced from the only premeiotic mutant offspring and an additional 25 mutant plaques were sequenced from two other non-mutant premeiotic offspring that displayed an unusually elevated *cII* MF (*cII* MF=8.8 X 10^{-5}) (table 4.4). Although the latter offspring strictly failed to meet the criteria for mutant offspring based on *cII* MF, (*cII* MF $\geq$ 8.9 X 10^{-5}), their unusually high *cII* MF indicated the presence of untargeted mutations, and surpassed any control offspring.

Table 4.3 Frequencies of independent and clonally expanded mutations derived from ENU-treated pre and postmeiotic germ cells of transgenic mice and fish.

	Big Blue Mouse			λ Transgenic Medaka	
	Postmeiotic[a]	**Premeiotic[a]**	**Spontaneous[b]**	**Postmeiotic**	**Premeiotic**
Total Sequences	138	64	256	228	208
	% (n)	**% (n)**	**% (n)**	**% (n)**	**% (n)**
Independent	53% (73)	61% (39)	71% (182)	41% (93)	58% (120)
Clonal	47% (65)	39% (25)	29% (74)	59% (135)	42% (88)

[a]Present Study.
[b]Harbach *et al.* 1999 (35).

Table 4.4 Spectra of untargeted mutations derived from ENU-treated pre and postmeiotic germ cells of transgenic mice and fish. Spectra of spontaneous mutations for both species are also provided for comparison.

	Big Blue Mouse			**λ Transgenic Medaka**		
	Postmeiotic[a]	**Premeiotic[a]**	**Spontaneous[b]**	**Postmeiotic**	**Premeiotic**	**Spontaneous[c]**
Ind. Mutations	n= 73	n= 39	n=182	n=93	n=120	n=239
Base Substitutions	**81% (59)**	**79% (31)**	**86% (156)**	**67% (62)**	**80% (96)**	**82% (192)**
Transition	*49% (36)*	*59% (23)*	*44.5% (81)*	*31% (29)*	*47% (57)*	*41% (97)*
G→A	34% (25)	33% (13)	35.2% (64)	19% (18)	39% (47)	28% (67)
T→C	15% (11)	26% (10)	9.3% (17)	12% (11)	8% (10)	13% (30)
Transversion	*32% (23)*	*21% (8)*	*41.1% (75)*	*35% (33)*	*33% (39)*	*41% (97)*
G→T	12% (9)	15% (6)	19.2% (35)	13% (12)0	17% (20)	13% (31)
G→C	3% (2)	0% (0)	9.3% (17)	6% (6)	5% (6)	10% (23)
A→T	8% (6)	5% (2)	4.9% (9)	4% (4)	7% (8)	8% (18)
A→C	8% (6)	0% (0)	7.7% (14)	12% (11)	4% (5)	10% (23)
Frameshift	**19% (14)**	**18% (7)**	**11% (20)**	**23% (21)**	**19% (23)**	**18% (41)**
Insertion	*10% (7)*	*8% (3)*	*8% (15)*	*12% (11)*	*10% (12)*	*10% (23)*
Deletion	*10% (7)*	*10% (4)*	*3% (5)*	*11% (10)*	*9% (11)*	*8% (18)*
Complex	**0% (0)**	**0% (0)**	**3.2% (6)**	**11% (10)**	**1% (1)**	**3% (6)**

[a]Present Study.
[b]Harbach *et al.* 1999 (35).
[c]Winn *et al.* 2000 (36).

The limited spectra derived from premeiotic mutant offspring was compared to the published spontaneous *cII* mutation spectra (table 4.4). An increase in the frequency of clonally expanded mutations was observed when compared to the spontaneous spectra (39% vs 24%). Among the independent mutations, premeiotic mutant offspring showed a relative increase in frameshift mutations compared to the spontaneous spectra (18% vs 10.9%) and corresponding decrease in base substitutions (79% vs 89.6%). The proportion of transversions decreased compared to the spontaneous spectra (21% vs 41%).

Comparison and Contrast of Mutational Spectra Derived from Postmeiotic and Premeiotic Mutant Offspring

The mutational spectra derived from postmeiotic and premeiotic mutant offspring were more similar to each other than to the spontaneous mutation spectra. Both showed an increase in clonally expanded and frameshift mutations, and a decrease in base substitutions, particularly transversions (table 4.4). The mutational spectra of premeiotic mutant offspring showed an increase in transition mutations (59% vs 49%), especially T→C transitions (26% vs 15%) compared to the mutational spectra derived from premeiotic germ cells. The premeiotic mutant offspring also displayed fewer transversions compared to postmeiotic offspring (21% vs 32%), in particular the frequency of G→T transversions stayed relatively equal (12% vs 15%) while all other transversions (G→C, A→T, and A→C) decreased. There were no remarkable differences in the proportions of frameshift or complex mutations between premeiotic and postmeiotic offspring.

The *cII* mutation spectra of two postmeiotic offspring (Appendix H, Individuals 9w and 10v) and two premeiotic offspring (Appendix H, Individuals 10j and 6t) contain multiple identical mutations at the same base. These jackpot mutations are indicative of a single mutation occurring early in development and being clonally expanded through mitotic divisions. Clonal mutations between base pairs 179 and 184 were not considered true jackpot mutations, as this region is a mutation hotspot containing a string of six guanine nucleotides (36, 37).

Comparison of Untargeted Mutagenesis in the Mouse and Medaka Animal Mutation Models:

Similar Frequencies of Mutant Offspring Were Identified in Both the Mouse and Medaka Models

There was no statistically significant difference in the frequency of mutant offspring derived from ENU-treated fish or mouse postmeiotic germ cells (3.5% vs 2.3% X^2=0.86, p> 0.25). There was also no statistically significant difference in the frequency of mutant offspring derived from fish or mouse premeiotic germ cells (2% vs 3.9% X^2=0.42, p> 0.25). Table 4.5 compares frequencies of mutant offspring between Big Blue transgenic mice and the λ transgenic medaka.

The Magnitude of c*II Mutant Frequencies Were Similar in Fish and Mouse Mutant Offspring*

The *cII* MFs of mouse mutant offspring ranged 2.4 to 3.7 times the mean control *cII* MF. Similarly the *cII* MF of mutant offspring in fish ranged from 2.0 to 5.6 fold elevation above mean control, with the exceptions of two outliers (*cII* MF= 41.6 X 10^{-5} and 373 X 10^{-5}). It is unclear if the outliers were unique to the medaka or if they were

Table 4.5: Frequencies of untargeted mutant offspring derived from ENU-treated germ cells of transgenic mice and transgenic fish.

	Percentage of Mutant offspring	
	Big Blue Mice	**λ Transgenic Medaka**
Control	0% (0/101)[a]	0% (0/170)
Postmeiotic	3.5% (6/171)[b]	2.3% (16/705)
Premeiotic	2% (1/49)[c]	3.9% (26/673)

[a]Appendix E
[b]Appendix F
[c]Appendix G

common to both animal models. These outliers occurred at very low frequencies (2 mutant offspring with *cII* MF > 10 X 10^{-5} out of a total of 1,378 fish analyzed).

The Spectra of Mutations Were Similar Between Mouse and Fish Mutant Offspring

No statistically significant difference in the postmeiotic untargeted mutational spectra derived from fish and mouse mutant offspring was found (Cochran-Armitage, n_{fish}= 97, n_{mouse} =74, p=0.72). However, postmeiotic medaka mutant offspring showed an increase in complex mutations compared to the respective spontaneous spectra (11% vs 3%) unlike the postmeiotic mouse mutant offspring compared their respective spontaneous spectra (0% vs 3.2%) (table 4.4).

DISCUSSION:

Mutagen treated mice and medaka male germ cells induced untargeted mutations in the unexposed, maternally derived DNA of offspring at approximately the same rate. This finding failed to reject both hypotheses in that no significant differences were detected in the frequency of mutant offspring or the mutational spectra between transgenic mice and medaka. Such findings provide evidence of a common conserved mechanism that is responsible for inducing untargeted mutations in these diverse animal models.

The mean cII MF of control mice (SMF = 3.8 X 10^{-5}) found in this study is comparable to previously published SMFs. Table 4.6 compares the SMF determined in this work with the SMF of various studies using *cII* analysis of rodent tissues. The SMF reported here is lower than other estimates, a difference likely explained by several reasons. First, this study analyzed entire pups as opposed to certain tissues (e.g. liver) which often display higher cII MFs [table 4.6, (26, 36, 38)]. Another explanation for a

Table 4.6. Published spontaneous *cII* mutant frequencies from both Big Blue® and Muta™ Mouse transgenic mouse models.

Animal Model	Mean Spontaneous *cII* MF	Standard Deviation	no. of animals	Tissue	Age	Reference
Big Blue®	3.8×10^{-5}	1.68×10^{-5}	101	Whole Pup	24 hrs	This report
Big Blue®	5.7×10^{-5}	1.4×10^{-5}	6	Spleen	8 wks	Monroe *et al.* 1998(39)
	8.8×10^{-5}	4.3×10^{-5}	5	Spleen	12 wks	
Big Blue®	13.8×10^{-5}	7.9×10^{-5}	5	Liver	12 wks	Zimmer *et al.* 1999(26)
	5.8×10^{-5}	3.7×10^{-5}	5	Lung	12 wks	
Big Blue®	5.81×10^{-5}	2.5×10^{-5}	6	Liver	12-16 wks	Shane *et al.* 2000(38)
Muta^{tm}Mouse	5.5×10^{-5}	not reported	6	Intestine	12-16 wks	Swiger *et al.* 1999(24)

relatively low SMF of this study may be due to sampling unusually young pups (<24 hrs), because SMFs of untreated Big Blue mice was previously shown to increase with age [table 4.6, (39)].

The 3.5% (6/171) of postmeiotic offspring that displayed elevated *cII* MFs indicated a clear treatment effect. The estimated frequency of mutant offspring derived from pre- meiotic offspring (2%, 1/49) was based on a relatively small sample size available for premeiotic offspring (n=49). However, this MOF far exceeded the 0.15% anticipated in the absence of a treatment effect. The small sample size was due primarily to decreased fecundity of the aging breeder mice as they entered the third breeding cycle.

Comparing untargeted mutagenesis in the Big Blue mouse to the λ transgenic medaka reveals strikingly similar results as there were no significant differences in the frequency of mutant offspring (3.5% vs. 2.3%) or untargeted mutational spectra. The magnitudes of the *cII* MFs in the affected offspring were also similar between fish and mice. All mouse mutant offspring displayed *cII* MFs less than 1.0×10^{-4} as did the majority of medaka mutant offspring (13/16 postmeiotic mutant offspring and 24/26 premeiotic mutant offspring). However a few offspring with remarkably high *cII* MFs (> 1.0×10^{-3}) were observed in fish, but not in transgenic mice. This difference is likely due to the relatively small sample size of the mouse experiments and large sample size of the fish studies. Mice may also produce mutant offspring with remarkably high *cII* MFs but a much larger sample size would be required to detect them. Assuming the probability of detecting such an offspring is 1 in 700 (based on the frequency such offspring were observed in the fish study, and a binomial distribution) 1,600 individual

mouse offspring must be sampled for a 90% certainty of detecting at least one mouse mutant offspring with a *cII* MF >10 X 10^{-3}.

The MOF estimates from transgenic fish and mice, though similar, are lower than some previous estimates. For example, Niwa and Kominami (18) irradiated male mice (6Gy total γ-radiation), analyzed the maternally derived C3H/HeN allele of offspring and observed an increase in mutant frequency from 9.8% (spontaneous) to 20% (untargeted). Although this study and the transgenic mouse/medaka studies share a similar design in that the male parent is treated with a known mutagen and untargeted mutations are observed in the maternally derived loci of offspring, there are several key differences in the approach that could explain their higher frequency of mutant offspring. First, two entirely different mutation assays are used (*cII* vs. ESTR change of length analysis), so direct comparisons of mutant frequencies cannot be made. Second, no consistent mutagen dosing regimen was used between all three studies. ESTR analysis studies irradiated mice testicles with γ-radiation (18), whereas the transgenic studies employed the chemical mutagen ENU. Further, ENU was administered via intraperitoneal injection in transgenic mice, but added to the ambient water of transgenic fish and absorbed primarily via gills; this resulted in different absorption and distribution mechanisms of each study. It is worth noting that for each study, (ESTR in mice, transgenic mice, and transgenic fish) the selected doses have been previously shown to induce mutations in the offspring of treated male parents (33, 40, 41). For these reasons, direct comparisons of mutant frequencies cannot be made; the treatment effect must be compared to its corresponding control.

Niwa and Kominami (18) observed a two-fold increase in the untargeted mutant frequency above spontaneous (20% vs. 9.8%) when postmeiotic germ cells were treated. Similarly, at least a two-fold increase in the frequency of mutant offspring compared to spontaneous controls was observed in transgenic mice (3.5% vs. no spontaneous mutant offspring detected) and medaka (2.3% vs. no spontaneous mutant offspring detected). Niwa and Kominami failed to observe any untargeted effect in offspring when spermatogonial stem cells (premeiotic) were treated. In contrast, the frequency of mutant offspring derived from ENU treated premeiotic male germ cells of this study was greater than that of postmeiotic germ cells (2.3% vs. 3.9%) in the transgenic medaka (a MOF estimate could not be made for premeiotic mouse offspring due to the small sample size).

The mutation spectra of mouse mutant offspring derived from ENU treated postmeiotic germ cells are significantly different than the published spontaneous spectra. Mutant offspring showed an increase in frameshift mutations and relative decrease in complex mutations (involving two or more bases) and base substitutions. When comparing the untargeted mutational spectra of medaka mutant offspring derived from ENU-treated premeiotic germ cells a similar increase in frameshift and decrease in base substitutions is observed. DNA sequencing also confirmed all mutant offspring were mosaic mutant offspring. It is expected that the current experimental design would produce only mosaic mutant offspring. Mutations were detected in the maternally derived genome, which was first exposed to the damaged paternal genome during DNA replication of the first mitotic division of the embryo. Therefore mutations in the maternal genome (induced by the damaged female genome) are fixed during the first cell division, resulting in mosaic mutant offspring (figure 2.3). These results not only

indicate untargeted mutagenesis is common to diverse animal lineages such as mammals and fish, but provide further evidence that processes occurring post-fertilization have a significant impact on mutation fixation in offspring. These immediate post-fertilization processes may be under the guidance of the oocyte.

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CHAPTER 5

MATERNAL p53 FUNCTION ALTERS OFFSPRING RESPONSE TO DNA DAMAGE INTRODUCED BY POSTMEIOTIC, BUT NOT PREMEIOTIC MALE GERM CELLS[1]

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ABSTRACT:

Previous research indicated that the majority of mutations present in offspring to mutagen exposed parents were fixed in the early embryo, rather than the gamete. Given the constraints of rapid mitosis during early embryonic development, DNA repair is likely influenced by repair systems deposited during oogenesis and already present in the oocyte at fertilization. This study was conducted to determine the influence of maternally derived DNA damage response mechanisms on the frequency of mutant offspring derived from mutagen-treated male germ cells. Specifically, female medaka with nonfunctional p53 ($p53^{-/-}$ treatment) or wild type p53 ($p53^{+/+}$ control), were bred to ENU-treated, λ transgenic males. The frequencies of mutations in the *cII* target gene of offspring were compared. There was no difference in the frequency of mutant offspring derived from $p53^{-}$ vs. $p53^{+}$ oocytes and untreated male germ cells. Loss of oocyte p53 function increased the frequency of mutant offspring derived from ENU treated spermatids and spermatozoa (11% vs. 36%). Loss of oocyte p53 function did not increase the frequency of mutant offspring derived from ENU treated spermatogonial stem cells (3.5% vs. 4%). These results indicated the absence of functional maternal p53 decreased the offspring's ability to respond to DNA damage introduced by ENU-treated postmeiotic, but not premeiotic male germ cells. Therefore, spermatogonial stem cells are able to repair their ENU-damaged DNA such that maternal p53 does not affect the frequency of mutant offspring. The embryo's ability to repair DNA damage was strongly influenced by functional maternal p53.

INTRODUCTION:

Genetic damage can be passed from parent to offspring, resulting in serious diseases for the offspring. Several of our previous experiments demonstrate that the majority of affected offspring derived from mutagen treated parents are mosaic mutant offspring [(1) Chapter 2]. The presence of mosaic mutant offspring indicate that mutations observed in offspring were not fixed in either gamete, but rather fixed at some point after fertilization (1, 2). Therefore, the early stage embryo plays a major role in responding to DNA damage and ultimately determining the mutational burden of resulting offspring. The DNA repair mechanisms responsible for this reaction are thought to be largely under the influence of the ova. To determine the influence of maternal contributions in the early embryonic response to DNA damage, oocytes with no functional p53 protein were fertilized with mutagen-treated male germ cells and progeny were analyzed for mutations in the *cII* mutation target gene.

The early embryo (zygote to mid-blastula stage) is a unique time in vertebrate development and is particularly vulnerable to DNA damage. Cells confronted with damaged DNA have evolved several complex systems to cope with the damage: 1) DNA repair, 2) cell cycle control checkpoints, which delay cell division allowing more time for DNA repair, 3) translesion synthesis, in which error-prone polymerases attempt DNA replication through the damaged areas, and if all else fails, 4) apoptosis (3).

An embryo derived from a mutagen-treated gamete contains significant DNA damage, but the unique constraints of embryogenesis may inhibit the coping and repair systems. For example, embryonic mRNA profiles indicate several genes involved in DNA repair are expressed in the early embryogenesis (4). However, research with mouse

and human embryos indicates transcription and translation are uncoupled until the 2 to 4 cell stage, so these mRNA transcripts are not translated into functional enzymes. Therefore, the developing embryo must rely on maternal mRNAs and proteins present in the ooplasm (cytoplasm of the oocyte) which were added during oogenesis (5, 6). Non-homologous end joining and homologous recombination are both known to function by the two-cell zygote stage(7). Jaroudi and SenGupta concluded "although many of the genes required for DNA repair are expressed, the embryo's ability to repair DNA may be highly limited" (8). Another of example of rapid embryonic development constraining DNA damage response involves cell cycle control checkpoints. Cell cycle control may not be feasible in the early embryo due to the rapid cell division. For example, research with human embryos indicated cell cycle control checkpoints were not active until after zygotic gene expression had begun, the 4 to 8 cell stage in human embryos (9, 10). Like cell cycle control, apoptosis is not a likely DNA damage coping mechanism in the early stages of embryonic development either, because it is not observed in human embryos until after blastocyst formation (70 to 100 cells in humans) (11). Because of the constraints associated with embryogenesis, it is possible that the embryo's response to DNA damage largely relies on products present in the cytoplasm of the oocyte, which were deposited during oogenesis.

p53 is a tumor suppressor largely involved in DNA damage response that functions primarily through transcription activation. It is known as the Guardian of the Genome for its anti-neoplastic functions, for example 50% of all human tumors contain mutated p53 (12-14) and p53 is now known to be the most commonly altered gene in human tumors (13, 15-17). Many other functions have been documented for p53

including apoptosis, cell cycle control, DNA repair, and genomic instability (18-20). p53 is activated by multiple stresses including oxidative stress, hypoxia and DNA damage (18, 21).

We hypothesized that the rapid cell division and uncoupled protein synthesis of early embryonic development limit traditional DNA damage responses and force the embryo to rely on maternal contributions to cope with DNA damage. Further, maternal p53 is an important contribution to the ooplasm which facilitates the early embryo's response to DNA damage. Specifically, the goal of these experiments was to test the concept that loss of p53 function in the oocyte increases the frequency of mutant offspring derived from mutagen treated male germ cells. To evaluate this hypothesis, male λ transgenic medaka were bred to homozygous $p53^{-/-}$ females and the offspring were analyzed for mutations in the *cII* gene. In this breeding design, offspring develop from mutagen treated male germ cells and ova with no functional p53. The early embryo develops in an environment with damaged DNA and no functional p53 for several cell divisions. These experiments will test three hypotheses. First, offspring derived from $p53^{-}$ oocytes will have a higher spontaneous frequency of mutant offspring than offspring derived from $p53^{+}$ oocytes. Second, offspring of $p53^{-}$ oocytes and mutagen treated **postmeiotic** male germ cells will have a higher frequency of mutant offspring than those derived from $p53^{-}$ oocytes and mutagen treated male germ cells. Finally, offspring of $p53^{-}$ oocytes and mutagen treated **premeiotic** male germ cells will have a higher frequency of mutant offspring than those derived from $p53^{-}$ oocytes and mutagen treated male germ cells.

MATERIALS AND METHODS:

Animals

Wild type (CAB strain), λ transgenic, and $p53^{-/-}$ medaka were obtained from in-house stocks at the Aquatic Biotechnology and Environmental Laboratory, of the University of Georgia (22). The λ transgenic medaka (strain λ 310), contain the λLIZ bacteriophage vector harboring the *cII* gene mutation target gene (homozygotes ~150 λ copies /diploid genome). Homozygous p53 deficient ($p53^{-/-}$) medaka carry a mutation in glutamate 241 resulting in an early stop codon (23). The resulting truncated protein has no nuclear localization domain, and cannot bind DNA or polymerize into tetramers. Fish were maintained on a 12 hour light-dark cycle, except breeding pairs maintained on a 16:8 light-dark cycle in dechlorinated municipal water at 24°C, fed 1 to 2 times daily with brine shrimp nauplii (Artemia Great Salt Lake, UT) and supplemented 1 to 2 times daily with a commercial flake diet. All animal care protocols were approved by The Institutional Care and Use Committee of the University of Georgia.

Mutagen Treatment

Adult transgenic males (λLIZ homozygous) were exposed to 100 ppm of the potent germ cell mutagen, 1-ethyl-1-nitrosourea (ENU) in their ambient water for one hr on two consecutive days. Males were rinsed with untreated water, and quarantined for 4 hrs to allow for ENU degradation before returning to breeding tanks.

Breeding and Sampling Designs

Three breeding and sampling combinations were used. In the first design (figure 5.1), three unexposed $\lambda^{+/+}$, $p53^{+/+}$ male medaka were bred to multiple $\lambda^{-/-}$, $p53^{-/-}$ female medaka to determine if loss of p53 function within the oocyte would increase the

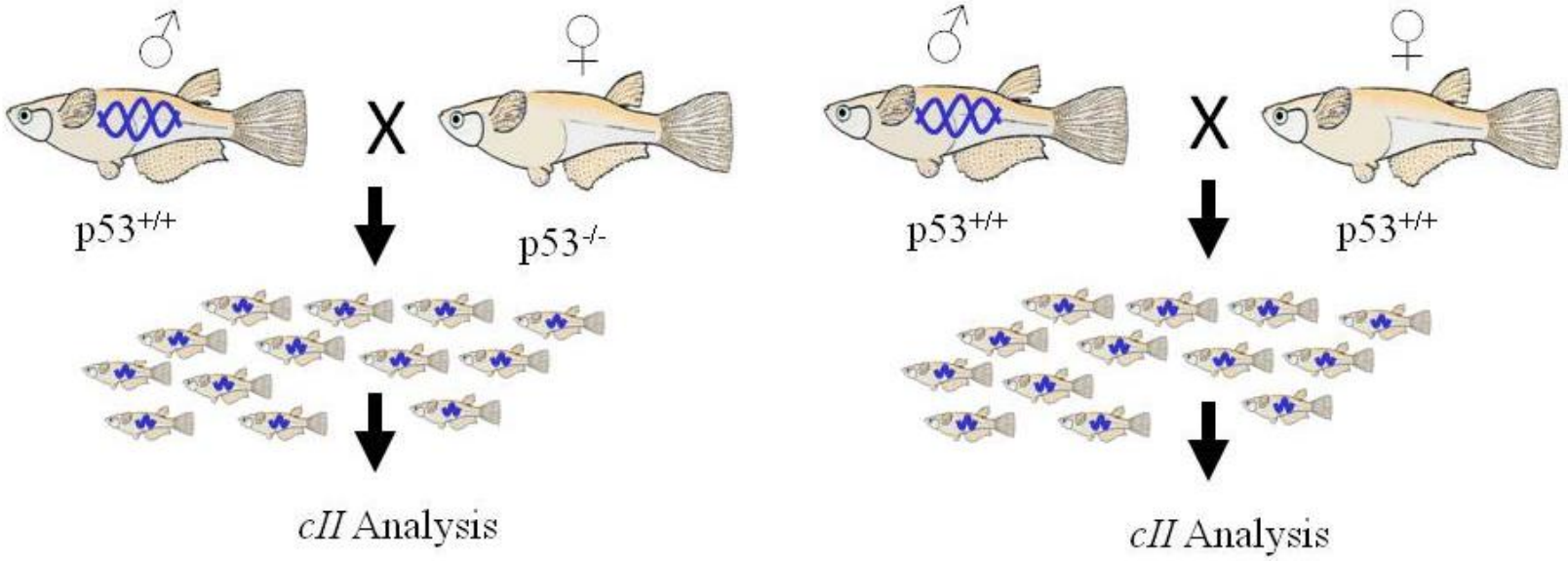

Figure 5.1 Illustration of the breeding scheme used to investigate the role of maternal p53 in coping with spontaneous DNA damage in offspring. a) λ transgenic, $p53^{+/+}$ male medaka were bred to $p53^{-/-}$, $\lambda^{-/-}$ female medaka. Offspring were analyzed for mutations in the paternally derived transgene using the *cII* mutation assay. b) comparison (control) offspring were generated by breeding λ transgenic, $p53^{+/+}$ male medaka to $p53^{+/+}$, $\lambda^{-/-}$ female medaka. The *cII* MFs were compared between the two groups of offspring.

spontaneous *cII* MF of offspring. The offspring of these pairings thus developed from an ovum without functional p53. Results were compared to the spontaneous *cII* MF of offspring derived from unexposed $\lambda^{+/+}$, p53 $^{+/+}$ male medaka and $\lambda^{-/-}$, p53 $^{+/+}$ female medaka. In the second design (figure 5.2), three ENU exposed $\lambda^{+/+}$, p53 $^{+/+}$ male medaka were bred to multiple $\lambda^{-/-}$, $p53^{-/-}$ females and the resulting offspring were collected 1 to 5 days following exposure. These offspring were derived from mutagen treated postmeiotic male germ cells (spermatids and spermatozoa) and ova lacking functional p53. Offspring were also collected prior to mutagen treatment to serve as sibling controls.

The third design sought to determine if loss of p53 function within the oocyte would increase the frequency of mutant offspring derived from ENU treated spermatogonial germ cells. Three ENU treated $\lambda^{+/+}$, p53 $^{+/+}$ male medaka were bred to multiple $\lambda^{-/-}$, $p53^{-/-}$ females and offspring were collected > 60 days following exposure. These offspring were derived from ova lacking functional p53 and mutagen treated premeiotic male germ cells (spermatogonial stem cells). Offspring were also collected prior to mutagen treatment to serve as sibling controls.

In each breeding design, all progeny were hemizygous the *cII* target gene which was contributed only by the male parent and heterozygous for p53, as the female parent contributed only truncated p53. Collected embryos were allowed to hatch and cultured under standard conditions for 14 to 21 days (1). Any developmental abnormalities were recorded prior to the offspring being flash frozen, and stored at -80°C prior to mutation analysis.

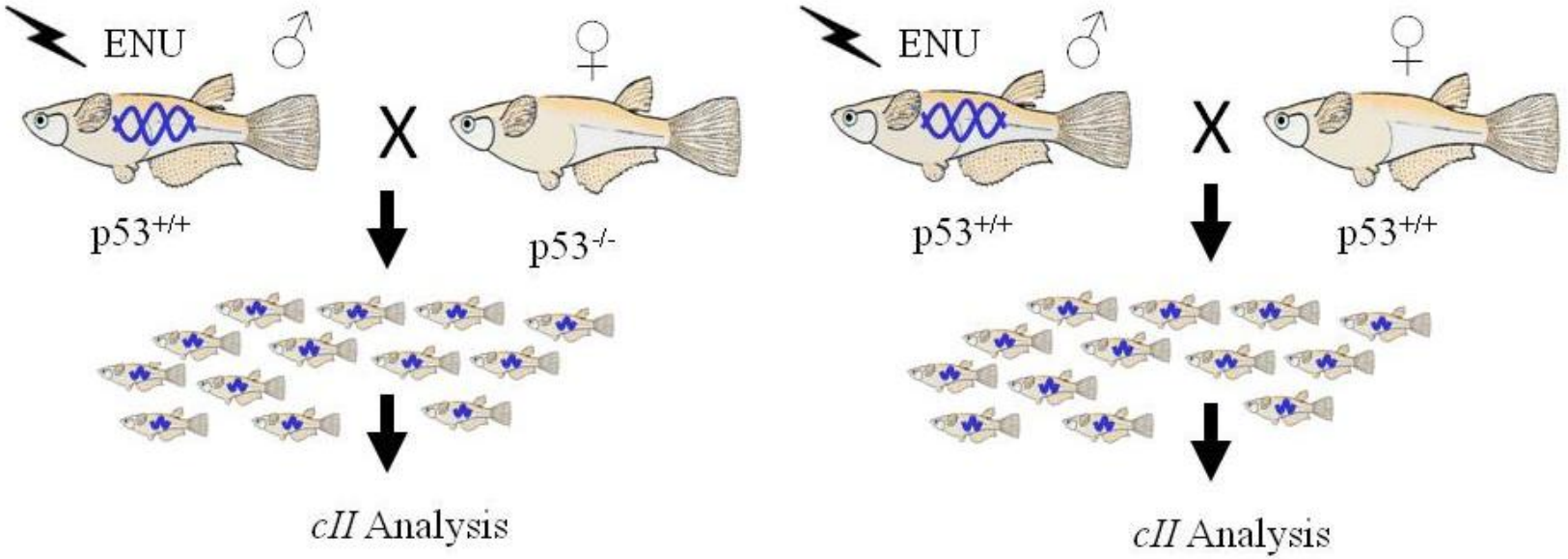

Figure 5.2 Illustration of the breeding scheme used to investigate the role of maternal p53 in coping with DNA damage introduced by the male gamete to offspring. a) ENU-treated, λ transgenic, $p53^{+/+}$ male medaka were bred to $p53^{-/-}$, $\lambda^{-/-}$ female medaka. Offspring were analyzed for mutations in the ENU-treated, paternally derived transgene using the *cII* mutation assay. b) comparison offspring were generated by breeding ENU-treated, λ transgenic, $p53^{+/+}$ male medaka to $p53^{+/+}$, $\lambda^{-/-}$ female medaka. The *cII* MFs were compared between the corresponding groups of offspring. Offspring collected 1 to 5 days following ENU exposure were derived from postmeiotic male germ cells at the time of exposure. Offspring collected >60 days following ENU exposure were derived from premeiotic male germ cells at the time of exposure.

DNA Extraction

Genomic DNA was isolated from individual siblings not showing developmental abnormalities using previously described procedures (22, 24). Briefly, tissues from whole animals were digested with proteinase (1X SSC, 20% SDS, 20mg/ml proteinase K) and extracted with equal volumes phenol:chloroform. DNA was precipitated using ethanol and resuspended in Tris-EDTA buffer (pH7.5)

cII Mutation Assay

A positive-selection assay based on the *cII* gene as the mutation target gene (25) was used to analyze mutations transmitted from mutagen exposed males to progeny. The assay is based on the role of the *cII* protein in the commitment of bacteriophage λ to the lysogenic cycle to *E. coli*. Selection of mutant λ*cII* is facilitated by using a specialized *E. coli* strain (G1250, *hfl*$^-$) that extends the longevity of the *cII* protein product. Genomic DNA was incubated with packaging extracts to simultaneously excise and package the λ*LIZ* vector into viable bacteriophage. The resulting packaged phage particles were then allowed to infect and lyse the *E. coli* host. To select λ *cII*$^-$ mutants, the packaged phages were mixed with *E. coli* cells, plated, and incubated at 24° C for 40hr. The phages with wild type *cII* became lysogenic and were indistinguishable from the *E. coli* lawn, whereas phages that carry a mutation in the *cII* gene formed plaques in the bacterial lawn when incubated at 24° C. The frequencies of *cII* mutants (MFs) were calculated by dividing the total number of *cII* mutant plaque forming units (PFUs) on the selective screening plates by the estimated total λ *cII* phages on the titer plates. DNA samples that exhibited MFs elevated three standard deviations above the mean of the control offspring, were designated as mutant offspring.

Mutational Spectra

To characterize the spectra of *cII* mutations carried by selected individual offspring with elevated *cII* MFs, standard methods were used to isolate individual mutant plaques, followed by PCR sequencing analysis. The λ*cII* mutants were characterized using DNA sequencing methods previously described (22). Individual λ *cII*-mutant plaques were cored at random from mutant screening plates, and purified on G1250 *E. coli* cells. Individual plaques were excised and amplified by PCR. The resulting PCR products were electrophoresed on a 1% agarose gel, cleaned with QIAquick PCR purification kit (Qiagen, Valencia CA), and diluted to 0.1μg/μL using Tris-EDTA. Diluted PCR products were labeled (BigDye Terminator Cycling DNA Sequencing Kit; Applied Biosystems, Foster City, CA) and purified again using spin columns (DyeEx 2.0 Qiagen.) Samples were analyzed using an automated DNA Sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems).

Statistical Analysis:

Frequencies of mutant offspring were compared using Chi-Square analysis.

RESULTS:

Maternal p53 Status in Response to Spontaneous DNA Damage

Forty-nine offspring were collected from $\lambda^{+/+}$, $p53^{+/+}$ male and $\lambda^{-/-}$, $p53^{-/-}$. These offspring displayed a mean *cII* MF of 1.7×10^{-5}, with a standard deviation of 1.3×10^{-5}. Individual *cII* MFs of each offspring is tabulated appendix J. No mutant offspring were detected among individuals derived from $p53^{-/-}$ ova, thus the mutant offspring frequency (MOF) is 0% (0/49). This MOF is identical to that of offspring derived from $p53^{+/+}$.

Table 5.1 summarizes the *cII* MF of offspring derived from $p53^{-/-}$ oocytes collected prior to and following ENU exposure.

Loss of Oocyte p53 Function Increases the Frequency of Mutant Offspring Derived from ENU Treated Spermatids and Spermatozoa

Fifty offspring derived from ENU treated $\lambda^{+/+}$, $p53^{+/+}$ male and $\lambda^{-/-}$, $p53^{-/-}$ females and collected 1 to 5 days following treatment exposure were analyzed for mutations in the *cII* gene (design two). Individual *cII* MFs can be found in appendix K. Of the 50 offspring, 10 individuals displayed a *cII* MF > 9.0 X 10^{-5}, thus meeting the criteria for mutant offspring. The resulting mutant offspring frequency of 20% (10/50) was significantly higher than the frequency of mutant offspring derived from ENU treated postmeiotic male germ cells and ova with functional p53 (20% vs 11%, X^2= 2.7 p<0.1, [(1) and Table 5.1 and 5.2]). The magnitude of *cII* MF among only the mutant offspring ranged from 1.0 x10^{-4} to 4.7 X 10^{-3}, representing a 3 to 157 fold elevation above the mean spontaneous *cII* MF of 3 X 10^{-5}.

Loss of Oocyte p53 Function Does Not Increase the Frequency of Mutant Offspring Derived from ENU Treated Spermatogonial Stem Cells

To determine if loss of p53 function within the oocyte would increase the frequency of mutant offspring derived from ENU treated spermatogonial germ cells, three $\lambda^{+/+}$, $p53^{+/+}$ male medaka were bred to multiple $\lambda^{-/-}$, $p53^{-/-}$ females and offspring were collected > 60 days following exposure. These offspring developed from mutagen treated premeiotic male germ cells and ova lacking functional p53. Appendix L lists the *cII* MF of offspring. Of the 101 offspring sampled, 4 individuals displayed a *cII* MF > 9.0 X 10^{-5}. The resulting MOF of 4% (4/101) was not significantly different than the

Table 5.1 Summary of *cII* MFs of offspring derived from p53 deficient oocytes and ENU treated male germ cells.

Male Germ Cell Treatment	Ova p53 status	Offspring analyzed	Range of *cII* MF				% Mutant Offspring
			0.1-8.9 X 10^{-5}[a]	0.9-9 X 10^{-4}[b]	0.1-9 X10^{-3}[b]	0.1-9 X 10^{-2}[b]	
			[number of offspring (% of total offspring)]				
0 ppm ENU	$p53^{-}$	49	49	0	0	0	0%
	$p53^{+}$	67	67	0	0	0	0%
100 ppm ENU Postmeiotic	$p53^{-}$	50	40	5 (10%)	5 (10%)	0	20%
	$p53^{+}$	139	124	8 (5.7%)	4 (2.9%)	3 (2.3%)	11%
100 ppm ENU Premeiotic	$p53^{-}$	101	97	0	4 (4.0%)	0	4%
	$p53^{+}$	319	308	8 (2.5%)	0	3 (0.9%)	3.5%

[a] non-mutant offspring
[b] mutant offspring

Table 5.2 Comparing frequencies of mutant offspring derived from $p53^{-/-}$, or $p53^{+/+}$ oocytes and ENU treated male germ cells.

	% Mutant Offspring	
Male Germ Cell Dose	**$p53^{-/-}$ ova**	**$p53^{+/+}$ ova**[a]
0 ppm ENU	0 (0/49)[b]	0 (0/67)[b]
100 ppm ENU, Postmeiotic	20.0 (10/50)	10.8 (15/139)
100 ppm ENU, Premeiotic	4.0(4/101)	3.5 (11/319)

[a] (1)

[b] (number of mutant offspring/ total number of offspring)

frequency of mutant offspring derived from ENU treated premeiotic male germ cells and ova with functional p53 (4% vs 3.5%, X^2= 0.06 p>0.25, (1), Table 5.2). The magnitude of the *cII* MF among the mutant offspring ranged from 1.5 x10^{-3} to 8.8 X 10^{-3}, a 52 to 292 fold elevation above the mean spontaneous *cII* MF. Although the absence of maternal p53 did not alter the frequency of mutant offspring, the magnitude of the *cII* MF of the mutant offspring was greatly increased. Mutant offspring derived from p53 deficient ova and ENU treated premeiotic male germ cells display greatly elevated *cII* MF.

DISCUSSION:

Offspring of unexposed, p53 deficient females and mutagen-treated males showed that the maternal p53 status influenced the offspring's ability to respond to DNA damage introduced by male germ cells. Absence of maternal p53 (the oocyte was $p53^-$) did not increase the frequency of mutations in offspring derived from untreated male germ cells (spontaneous DNA damage only) compared to offspring of $p53^+$ oocytes. However, the absence of maternal p53 (oocyte is $p53^-$) increased the frequency of mutant offspring derived from mutagen-treated **postmeiotic** male germ cells, indicating a decrease in the embryo's ability to respond to DNA damage appropriately. In contrast, the absence of functional maternal p53 (oocyte is $p53^-$) did not appear to alter the frequency of mutant offspring, derived from mutagen-treated **premeiotic** male germ cells.

By demonstrating that loss of oocyte p53 function did not increase the frequency of spontaneous mutant offspring, the results failed to support the first hypothesis. Specifically, offspring derived from untreated male germ cells had similar mutant frequencies regardless of oocyte p53 status. Therefore, sources of spontaneous DNA damage (e.g. oxidative stress) were not sufficient to produce mutant offspring in the

absence of maternal p53. This finding concurs with past research with DNA repair deficient animal models. Marchetti *et al* showed spontaneous DNA damage is not sufficient to increase the frequency of chromosomal abnormalities in offspring derived from female mice with deficient non-homolgous end-joining repair (26). Similarly, Buettner *et al.* showed that $p53^{-/-}$ Big Blue mice did not have a higher spontaneous *lacI* mutant frequency compared to $p53^{+/+}$ Big Blue mice in liver, spleen or brain (27). Although this work only analyzed three animals per treatment group, an excess of 8.0 X 10^6 loci were analyzed (27).

Other oocyte DNA repair mechanisms, not dependant on p53, could be sufficient to repair spontaneous DNA damage. For example, mismatch repair, non-homologous end-joining and other DNA repair mechanisms have been observed in *Xenopus* oocytes and their extracts (28-30). However, DNA repair processes such as nucleotide excision repair and base excision repair are regulated by p53 in certain situations (31).

Several forms of DNA damage are known to activate p53. UV radiation, which often produces pyrimidine dimers and occasional double strand breaks, is known to stabilize and activate p53 (32). Various other sources of double- strand breaks including ionizing radiations have also been found to activate p53(33-35). Once DNA damage is identified, p53 protein is rapidly collected and activated (36, 37). It is possible that the spontaneous DNA damage present in offspring derived from $p53^{-}$ oocytes and untreated male germ cells did not contain sufficient damage (e.g. double-strand breaks) to induce or activate p53.

Loss of oocyte p53 function increased the frequency of mutant offspring derived from ENU treated spermatids and spermatozoa (postmeiotic male germ cells). The

frequency of mutant offspring rose significantly (from 11% to 36%) when oocyte p53 was disabled, indicating that the oocyte p53 helped mitigate DNA damage in offspring derived from ENU treated postmeiotic male germ cells. These results supported the second hypothesis.

The maternally derived p53 likely achieves its DNA repair activity (thus decreasing frequency of mutant offspring) in a very short time period (minutes to hours). Expression of paternally derived genes begins at the two to four cell stage, thus paternal p53 could repair damage from then on, and compensate for the lack of competent, maternally derived p53. In cleavage stage rat embryos derived from irradiated males, increased activity of p53 binding proteins (53BP1) was observed between the two and eight cell stage(38). Although p53 appears active by the eight cell stage, the authors were unable to determine whether the maternal, paternal, or both copies of p53 was active.

By contrast, loss of oocyte p53 function did not increase the frequency of mutant offspring derived from ENU treated spermatogonial stem cells, thus not supporting the third hypothesis. These results implied that oocyte p53 does not mitigate DNA damage in offspring derived from ENU treated premeiotic male germ cells. Multiple mechanisms involved with spermatogenesis could explain why mutagen-treated premeiotic germ cells induce similar frequencies of mutant offspring regardless of maternal p53 status. For example, spermatogonial stem cells could undergo selection, in which severely damaged cells are removed (39, 40). Therefore the mature spermatozoa derived from the surviving stem cells would carry less DNA damage. Spermatogonial stem cells are also DNA repair competent thus allowing the damage to be repaired prior to meiosis (41, 42).

This study presents the first evidence that the early embryo's ability to repair DNA damage and suppress mutations is dependent on maternal p53. The frequency of mutant offspring derived from mutagen treated postmeiotic male germ cells increased from 11% to 36% in the absence of competent maternally-derived p53. Previous studies using physiologic endpoints rather than mutation assays, have documented other deleterious effects in offspring resulting from non-functional maternal, but not paternal p53. Levine *et al.* crossed $p53^{-}$ female mice to $p53^{+}$ males and although ovulation and fertilization of the $p53^{-}$ oocytes proceeded normally, embryo implantation and litter size decreased significantly (18). Interestingly, no deleterious effects were observed in the offspring derived from the reciprocal cross (male $p53^{-}$ and female $p53^{+}$), indicating maternal, but not paternal, p53 mitigates DNA damage in embryos.

The increased frequency of mutant offspring derived from p53 deficient female medaka and mutagen-treated males indicates that the λ transgenic and $p53^{-/-}$ medaka could be used for future research of environmental and germ line mutagens. The λ transgenic medaka is a sensitive and well established model for aquatic and germ line toxicology research (24, 43-45). The absence of complete p53 in oocytes did not increase the frequency of mutant offspring in untreated male germ cells, but did elevate the frequency of mutant offspring derived from ENU-treated, postmeiotic male germ cells. Therefore $p53^{-}$ oocytes and λ transgenic males could be used to evaluate suspected germ cell mutagens. Untreated control groups will not display elevated frequencies of mutant offspring due to spontaneous damage, thus *cII* MF of control offspring will be lower and consistent. A lower, repeatable, control *cII* MF will enhance the assays ability to detect smaller increases in *cII* MF of treatment groups.

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CHAPTER 6

CONCLUSIONS:

Analyses of mutations in the offspring of 1-ethyl-1-nitrosourea (ENU) treated λ transgenic medaka and mice revealed several major findings which contribute to understanding the oocyte's response to DNA damage. Oocytes with DNA damage will transmit this damage to offspring as well as induce new, untargeted mutations in the untreated, paternally derived DNA of offspring. Similarly, mutagen treated male germ cells will induce untargeted mutations in the unexposed, maternally derived DNA of offspring. These mutations are fixed not in the gamete, but in the early embryo. Further, maternal p53, present in the oocyte, proved essential for the embryo to properly respond to certain DNA damage introduced by the sperm.

The experiments reported here indicate that female germ cells are as sensitive to ENU as postmeiotic male germ cells, and more sensitive than premeiotic male germ cells. Although our work does not reinforce a particular consensus on the relative sensitivity, it further illustrates the need to include both female and male germ cells in the study of transmissible DNA damage and risk assessments.

Mutations in offspring were efficiently detected by the *cII* mutation assay. This assay allows for rapid screening of millions of copies of the transgene from a single animal, thus greatly reducing the number of animals required compared to previous, phenotype-based assays. Yet, this work revealed a difficulty of the assay, namely, replacing reagents as manufacturers discontinue or alter items. Finding a replacement

protein source after the manufacturer altered the casein source took months of research and testing.

Although the *cII* assay is relatively time consuming and reveals less information than sequencing the entire genome, the transgenic assay has certain advantages suited to the study of germline mutagenesis. High throughput sequencing is continuously becoming faster and less expensive, opening up a possible new technique to mutation detection and studying germ cell mutagenesis with distinct advantages. First, it would screen endogenous loci, to give an accurate mutation rate, and best characterize how and where an agent of interest (chemical, radiation) affects the genome. The lack of a shuttle vector, or prokaryotic vehicle, would eliminate *ex-vivo* mutations although sequencer errors would still exist. Complete genomic sequencing would also identify any hotspots. This technique would also allow for correlation of a given mutation and the subsequent pathology or endpoint of interest.

However, as with any assay, certain limitations exist. Genomic sequencing would produce a vast amount of data, and interesting patterns or anomalies could be much harder to identify. Rapid sequencing for mutations in offspring would still require breeding and dosing adult animals and extracting DNA from offspring. Perhaps the most limiting of all would be the difficulty of recognizing mosaic mutant offspring. "Deeper" sequencing (larger number of reads per nucleotide) is preferred to reduce sequencing error, however, sequencing software would not be able to distinguish error from low frequency mosaic mutations. Certain types of sequencing (e.g. capillary/polymer florescence) would be completely unable to detect rare mutations due to low signal to noise ratios. Detecting mosaicism would require comparing hundreds of reads to

determine the frequency of a given mutation. The vast majority of mutant offspring reported here were mosaic mutant offspring and would not be recognized by automated sequencers.

The vast majority of mutant offspring detected in all studies reported here were mosaic mutant offspring. This is a significant finding as mosaicism has been linked to multiple diseases including osteogenesis imperfecta, Ehlors-Danlos syndrome, autism, and multiple cancers. Additionally, mosaic mutations will not be passed to the next generation unless the mutation is present in a germinal cell. Subsequently, there is no way to know if the deleterious mutations will be passed on or not without sequencing the DNA of germinal tissue.

The high frequency of mosaic mutant offspring has an even greater implication, namely, that most of the mutations occurring in offspring are fixed after fertilization, after the two cell stage. Therefore, the developing embryo is responsible for repairing, or at least responding to, DNA damage introduced by one of the gametes. Here, we also demonstrated that this early embryonic repair relied on the DNA damage response and repair of the capability of the oocyte (and therefore the mother). In particular, p53 was found to be important for responding to damage introduced by spermatozoa treated with the mutagen after meiosis, but not before. Furthermore, DNA damage in either gamete will induce mutations globally in the offspring. Regardless of whether the male or female germ cell was exposed to the mutagen, an increased frequency of mutations appeared in the DNA inherited from the other parent. These untargeted mutations, however, occur at a lower frequency than when the transgene is directly exposed to the mutagen.

Repeating untargeted mutation experiments in a rodent model with an identical transgene revealed that untargeted mutations occur at roughly the same frequency and with similar spectra in both the medaka and mouse, pointing to a conserved mechanism. It may be even more surprising considering the age discrepancy between the fry and pups, as mutations have been shown to accumulate over time. Pups gestated 19-21 days and were euthanized within 24 hours of birth. The fry however hatched after 7-10 days and lived up to 21days, making them older when measured from conception.

Performing *cII* mutation assays on mice and medaka side-by-side highlighted several differences between the two models. First, extracting high molecular weight DNA of sufficient quality for the λ phage to package efficiently was considerably more difficult in rodent tissue than medaka. Although large quantities of DNA were extracted from the transgenic mice, phage recovery remained comparatively lower than the medaka. This may be due to the lower copy number of the Big Blue® mice compared to medaka, or to the digestion and extraction of DNA from the entire pup, creating an untenable spool of DNA that was difficult to resuspend. Eventually, an additional Phenol:chloroform extraction, a membrane filtration, and longer resuspension steps were added to the rodent DNA extraction protocol to improve λ phage recovery.

In addition to the initial cost of the mice, there were several other reasons processing rodents was much more expensive. The total per diem husbandry costs of rodents exceeded the fish, especially when females had to have multiple litters to produce a sufficient quantity of offspring. Although the *cII* assay was identical between the two species, DNA extraction was significantly more expensive for mice. Rodent DNA extractions required more and larger centrifuge tubes, much greater volumes of phenol

(20X greater), and nitrocellulose membranes. Larger extraction volumes also created substantially more hazardous waste. Furthermore, the *cII* assay would occasionally need to be performed twice on some mouse samples to generate sufficient recoveries, increasing laboratory costs and time commitment. The similarity in untargeted responses between λ transgenic medaka and Big Blue® mice combined with the relative low cost and ease of analysis provided by the medaka indicate that for certain research questions involving germline mutagenesis, the λ transgenic medaka is a preferable animal model.

Finally, this work demonstrated that maternal p53, present in the oocyte, plays critical role in early embryo DNA damage response to certain types of DNA damage. There was no evidence that maternal p53 is required to respond to spontaneous damage, however, if the postmeiotic male germ cell is exposed to a mutagen, the embryos utilizes maternal p53 for repair. Interestingly, maternal p53 is not required if premeiotic male germ cells are damaged. This work clearly showed that the oocyte is both a transmitter and a mediator of DNA damage to offspring.

APPENDICES

Appendix A. *cII* mutant frequencies of individual medaka offspring collected prior to ENU treatment of the females. Mean *cII* mutant frequency for all progeny = 3.3×10^{-5}.

Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
A	26	1,615,000	1.6×10^{-5}
B	25	1,385,000	1.8×10^{-5}
C	35	1,835,000	1.9×10^{-5}
D	30	1,535,000	2.0×10^{-5}
E	39	1,835,000	2.1×10^{-5}
F	31	1,465,000	2.1×10^{-5}
G	31	1,405,000	2.2×10^{-5}
H	21	930,000	2.3×10^{-5}
I	45	1,990,000	2.3×10^{-5}
J	38	1,535,000	2.5×10^{-5}
K	39	1,495,000	2.6×10^{-5}
L	28	1,045,000	2.7×10^{-5}
M	38	1,395,000	2.7×10^{-5}
N	43	1,555,000	2.8×10^{-5}
O	41	1,425,000	2.9×10^{-5}
P	42	1,445,000	2.9×10^{-5}
Q	51	1,715,000	3.0×10^{-5}
R	33	1,105,000	3.0×10^{-5}
S	58	1,945,000	3.0×10^{-5}
T	39	1,265,000	3.1×10^{-5}
U	42	1,305,000	3.2×10^{-5}
V	37	1,165,000	3.2×10^{-5}
W	53	1,615,000	3.3×10^{-5}
X	49	1,430,000	3.4×10^{-5}
Y	54	1,605,000	3.4×10^{-5}

Z	36	1,060,000	3.4×10^{-5}
Aa	63	1,795,000	3.5×10^{-5}
Bb	40	1,145,000	3.5×10^{-5}
Cc	34	935,000	3.6×10^{-5}
Dd	35	960,000	3.6×10^{-5}
Ee	53	1,465,000	3.6×10^{-5}
Ff	49	1,315,000	3.7×10^{-5}
Gg	47	1,280,000	3.7×10^{-5}
Hh	47	1,235,000	3.8×10^{-5}
Ii	56	1,445,000	3.9×10^{-5}
Jj	58	1,505,000	3.9×10^{-5}
Kk	40	985,000	4.0×10^{-5}
Ll	35	855,000	4.1×10^{-5}
Mm	67	1,595,000	4.2×10^{-5}
Nn	47	1,115,000	4.2×10^{-5}
Oo	35	840,000	4.2×10^{-5}
Pp	30	700,000	4.3×10^{-5}
Qq	59	1,300,000	4.5×10^{-5}
Rr	75	1,555,000	4.8×10^{-5}
Ss	64	1,245,000	5.1×10^{-5}
Tt	33	600,000	5.5×10^{-5}
Uu	35	615,000	5.7×10^{-5}

Appendix B. *cII* mutant frequencies of individual medaka offspring derived from ENU-treated oocytes. Offspring below the single line are mutant offspring.

Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
A	1	245,000	0.4 x 10^{-5}
B	1	185,000	0.5 x 10^{-5}
C	9	1,065,000	0.9 x 10^{-5}
D	9	790,000	1.1 x 10^{-5}
E	2	150,000	1.3 x 10^{-5}
F	19	1,275,000	1.5 x 10^{-5}
G	10	665,000	1.5 x 10^{-5}
H	6	355,000	1.7 x 10^{-5}
I	7	410,000	1.7 x 10^{-5}
J	8	480,000	1.7 x 10^{-5}
K	7	380,000	1.8 x 10^{-5}
L	5	275,000	1.8 x 10^{-5}
M	7	400,000	1.8 x 10^{-5}
N	15	730,000	2.1 x 10^{-5}
O	18	865,000	2.1 x 10^{-5}
P	10	415,000	2.4 x 10^{-5}
Q	4	165,000	2.4 x 10^{-5}
R	4	165,000	2.4 x 10^{-5}
S	5	205,000	2.4 x 10^{-5}
T	25	1,020,000	2.5 x 10^{-5}
U	15	575,000	2.6 x 10^{-5}
V	24	885000	2.7 x 10^{-5}
W	6	210,000	2.9 x 10^{-5}
X	14	465,000	3.0 x 10^{-5}
Y	12	400,000	3 x 10^{-5}
Z	21	685,000	3.1 x 10^{-5}
Aa	24	785,000	3.1 x 10^{-5}
Bb	12	370,000	3.2 x 10^{-5}
Cc	23	690,000	3.3 x 10^{-5}
Dd	5	150,000	3.3 x 10^{-5}
Ee	17	500,000	3.4 x 10^{-5}

Ff	29	850,000	3.4	x 10^{-5}
Gg	30	870,000	3.5	x 10^{-5}
Hh	5	130,000	3.8	x 10^{-5}
Ii	8	205,000	3.9	x 10^{-5}
Jj	29	685,000	4.2	x 10^{-5}
Kk	76	1,825,000	4.2	x 10^{-5}
Ll	8	185,000	4.3	x 10^{-5}
Mm	16	360,000	4.4	x 10^{-5}
Nn	46	1,040,000	4.4	x 10^{-5}
Oo	35	795,000	4.4	x 10^{-5}
Pp	15	330,000	4.5	x 10^{-5}
Qq	13	280000	4.6	x 10^{-5}
Rr	28	595,000	4.7	x 10^{-5}
Ss	27	575,000	4.7	x 10^{-5}
Tt	38	775,000	4.9	x 10^{-5}
Uu	48	950,000	5.0	x 10^{-5}
Vv	28	535,000	5.2	x 10^{-5}
Ww	20	380,000	5.3	x 10^{-5}
Xx	14	240,000	5.8	x 10^{-5}
Yy	37	635,000	5.8	x 10^{-5}
Zz	7	120,000	5.8	x 10^{-5}
Aaa	18	305,000	5.9	x 10^{-5}
Bbb	71	1,200,000	5.9	x 10^{-5}
Ccc	43	690,000	6.2	x 10^{-5}
Ddd	27	435,000	6.2	x 10^{-5}
Eee	14	220,000	6.4	x 10^{-5}
Fff	14	220,000	6.4	x 10^{-5}
Ggg	17	260,000	6.5	x 10^{-5}
Hhh	17	255,000	6.7	x 10^{-5}
Iii	28	405,000	6.9	x 10^{-5}
Jjj	59	795,000	7.4	x 10^{-5}
Kkk	33	440,000	7.5	x 10^{-5}
Lll	12	160,000	7.5	x 10^{-5}
Mmm	25	330,000	7.6	x 10^{-5}

Nnn	13	155,000	8.4	x 10^{-5}
Ooo	21	240,000	8.8	x 10^{-5}
Ppp	62	705,000	8.8	x 10^{-5}
Qqq	20	215,000	9.3	x 10^{-5}
Rrr	27	290,000	9.3	x 10^{-5}
Sss	25	265,000	9.4	x 10^{-5}
Ttt	18	170,000	10.6	x 10^{-5}
Uuu	120	970,000	12.4	x 10^{-5}
Vvv	97	390,000	24.9	x 10^{-5}
www	391	910,000	43.0	x 10^{-5}
Xxx	238	455,000	52.3	x 10^{-5}
Yyy	200	295,000	67.8	x 10^{-5}
Zzz	429	445000	96.4	x 10^{-5}
Aaaa	626	325,000	193.0	x 10^{-5}
Bbbb	442	220,000	200.9	x 10^{-5}
Cccc	544	215,000	253.0	x 10^{-5}
Dddd	6260	395,000	1584.8	x 10^{-5}

Appendix C. *cII* mutant frequencies for individual medaka offspring collected prior to ENU exposure. Mean *cII* mutant frequency for all progeny = 2.2×10^{-5}.

Breeding Pair	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
2	2 a	9	650,000	1.4×10^{-5}
	2 b	15	830,000	1.5×10^{-5}
	2 c	14	775,000	1.8×10^{-5}
	2 d	20	985,000	2.0×10^{-5}
	2 e	25	1,265,000	2.0×10^{-5}
	2 f	37	1,705,000	2.2×10^{-5}
	2 g	35	1,505,000	2.3×10^{-5}
	2 h	22	785,000	2.8×10^{-5}
	2 i	43	1,445,000	3.0×10^{-5}
	2 j	42	1,255,000	3.3×10^{-5}
	2 k	27	790,000	3.4×10^{-5}
	2 l	27	775,000	3.5×10^{-5}
	2 m	38	965,000	3.9×10^{-5}
	2 n	38	870,000	4.4×10^{-5}
	2 o	29	605,000	4.8×10^{-5}

Breeding Pair	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
3	3 a	1	300,000	0.1×10^{-5}
	3 b	7	1,100,000	0.6×10^{-5}
	3 c	3	405,000	0.7×10^{-5}
	3 d	7	930,000	0.8×10^{-5}
	3 e	5	540,000	0.9×10^{-5}
	3 f	5	515,000	1.0×10^{-5}
	3 g	10	1,040,000	1.0×10^{-5}
	3 h	8	880,000	1.0×10^{-5}
	3 i	4	405,000	1.0×10^{-5}
	3 j	16	1,495,000	1.1×10^{-5}

3	k	15	1,280,000	1.2 x 10^{-5}
3	l	11	910,000	1.2 x 10^{-5}
3	m	6	450,000	1.3 x 10^{-5}
3	n	6	475,000	1.3 x 10^{-5}
3	o	15	1,130,000	1.3 x 10^{-5}
3	p	11	790,000	1.4 x 10^{-5}
3	q	8	545,000	1.5 x 10^{-5}
3	r	22	1,390,000	1.6 x 10^{-5}
3	s	11	690,000	1.6 x 10^{-5}
3	t	9	510,000	1.8 x 10^{-5}
3	u	10	530,000	1.9 x 10^{-5}
3	v	11	515,000	2.1 x 10^{-5}
3	w	12	585,000	2.1 x 10^{-5}
3	x	33	1,495,000	2.2 x 10^{-5}
3	y	27	1,235,000	2.2 x 10^{-5}
3	z	7	325,000	2.2 x 10^{-5}
3	aa	15	680,000	2.2 x 10^{-5}
3	bb	7	305,000	2.3 x 10^{-5}
3	cc	10	410,000	2.4 x 10^{-5}
3	dd	35	1,405,000	2.5 x 10^{-5}
3	ee	22	880,000	2.5 x 10^{-5}
3	ff	13	510,000	2.5 x 10^{-5}
3	gg	28	1,125,000	2.5 x 10^{-5}
3	hh	17	675,000	2.5 x 10^{-5}
3	ii	28	1,065,000	2.6 x 10^{-5}
3	jj	68	2,620,000	2.6 x 10^{-5}
3	kk	15	585,000	2.6 x 10^{-5}
3	ll	10	365,000	2.7 x 10^{-5}
3	mm	15	545,000	2.8 x 10^{-5}
3	nn	24	825,000	2.9 x 10^{-5}
3	oo	13	445,000	2.9 x 10^{-5}
3	pp	12	415,000	2.9 x 10^{-5}
3	qq	30	995,000	3.0 x 10^{-5}
3	rr	16	515,000	3.1 x 10^{-5}

3	ss	40	1,205,000	3.3	x 10^{-5}
3	tt	16	380,000	4.2	x 10^{-5}
3	uu	46	1,030,000	4.5	x 10^{-5}

Appendix D. Summary of *cII* mutant frequencies for individual offspring collected following ENU exposure. Mean *cII* mutant frequency for all progeny = 2.2 x 10^{-5}.

Male	Offspring ID		*cII* Mutants	PFU	*cII* Mutant Frequency
1	1	a	11	1,165,000	0.9 x 10^{-5}
	1	b	18	1,575,000	1.1 x 10^{-5}
	1	c	12	1,100,000	1.1 x 10^{-5}
	1	d	5	405,000	1.2 x 10^{-5}
	1	e	13	1,130,000	1.2 x 10^{-5}
	1	f	16	1,245,000	1.3 x 10^{-5}
	1	g	11	875,000	1.3 x 10^{-5}
	1	h	13	975,000	1.3 x 10^{-5}
	1	i	19	1,370,000	1.4 x 10^{-5}
	1	j	21	1,530,000	1.4 x 10^{-5}
	1	k	15	1,085,000	1.4 x 10^{-5}
	1	l	21	1,495,000	1.4 x 10^{-5}
	1	m	5	355,000	1.4 x 10^{-5}
	1	n	5	355,000	1.4 x 10^{-5}
	1	o	19	1,265,000	1.5 x 10^{-5}
	1	p	20	1,270,000	1.6 x 10^{-5}
	1	q	9	560,000	1.6 x 10^{-5}
	1	r	30	1,860,000	1.6 x 10^{-5}
	1	s	24	1,510,000	1.6 x 10^{-5}
	1	t	21	1,320,000	1.6 x 10^{-5}
	1	u	13	755,000	1.7 x 10^{-5}
	1	v	20	1,185,000	1.7 x 10^{-5}
	1	w	16	995,000	1.7 x 10^{-5}
	1	x	17	1,015,000	1.7 x 10^{-5}
	1	y	26	1,560,000	1.7 x 10^{-5}
	1	z	12	725,000	1.7 x 10^{-5}
	1	aa	25	1,415,000	1.8 x 10^{-5}
	1	bb	19	1,030,000	1.8 x 10^{-5}
	1	cc	22	1,220,000	1.8 x 10^{-5}
	1	dd	34	1,875,000	1.8 x 10^{-5}

1	ee	21	1,175,000	1.8	x 10^{-5}
1	ff	27	1,450,000	1.9	x 10^{-5}
1	gg	29	1,385,000	2.1	x 10^{-5}
1	hh	27	1,315,000	2.1	x 10^{-5}
1	ii	14	745,000	2.1	x 10^{-5}
1	jj	19	875,000	2.2	x 10^{-5}
1	kk	7	320,000	2.2	x 10^{-5}
1	ll	25	1,135,000	2.2	x 10^{-5}
1	mm	38	1,715,000	2.2	x 10^{-5}
1	nn	17	745,000	2.3	x 10^{-5}
1	oo	30	1,280,000	2.3	x 10^{-5}
1	pp	34	1,455,000	2.3	x 10^{-5}
1	qq	30	1,280,000	2.3	x 10^{-5}
1	rr	21	915,000	2.3	x 10^{-5}
1	ss	24	995,000	2.4	x 10^{-5}
1	tt	27	1,125,000	2.4	x 10^{-5}
1	uu	13	530,000	2.5	x 10^{-5}
1	vv	14	690,000	2.5	x 10^{-5}
1	ww	39	1,580,000	2.5	x 10^{-5}
1	xx	17	645,000	2.6	x 10^{-5}
1	yy	29	1,135,000	2.6	x 10^{-5}
1	zz	28	1,055,000	2.7	x 10^{-5}
1	aaa	16	600,000	2.7	x 10^{-5}
1	bbb	38	1,355,000	2.8	x 10^{-5}
1	ccc	21	745,000	2.8	x 10^{-5}
1	ddd	31	1,115,000	2.8	x 10^{-5}
1	eee	26	895,000	2.9	x 10^{-5}
1	fff	32	1,120,000	2.9	x 10^{-5}
1	ggg	27	940,000	2.9	x 10^{-5}
1	hhh	29	985,000	2.9	x 10^{-5}
1	iii	45	1,550,000	2.9	x 10^{-5}
1	jjj	13	450,000	2.9	x 10^{-5}
1	kkk	15	495,000	3.0	x 10^{-5}
1	lll	17	565,000	3.0	x 10^{-5}

1	mmm	17	565,000	3.0	x 10^{-5}
1	nnn	35	1,115,000	3.1	x 10^{-5}
1	ooo	9	295,000	3.1	x 10^{-5}
1	ppp	40	1,295,000	3.1	x 10^{-5}
1	qqq	29	935,000	3.1	x 10^{-5}
1	rrr	27	865,000	3.1	x 10^{-5}
1	sss	28	915,000	3.1	x 10^{-5}
1	ttt	40	1,255,000	3.2	x 10^{-5}
1	uuu	41	1,295,000	3.2	x 10^{-5}
1	vvv	12	380,000	3.2	x 10^{-5}
1	www	12	425,000	3.2	x 10^{-5}
1	xxx	33	965,000	3.4	x 10^{-5}
1	yyy	31	925,000	3.4	x 10^{-5}
1	zzz	47	1,325,000	3.5	x 10^{-5}
1	aaaa	24	660,000	3.6	x 10^{-5}
1	bbbb	32	895,000	3.6	x 10^{-5}
1	cccc	48	1,305,000	3.7	x 10^{-5}
1	dddd	21	575,000	3.7	x 10^{-5}
1	eeee	48	1,280,000	3.8	x 10^{-5}
1	ffff	14	370,000	3.8	x 10^{-5}
1	gggg	46	1,190,000	3.9	x 10^{-5}
1	hhhh	36	925,000	3.9	x 10^{-5}
1	iiii	13	330,000	3.9	x 10^{-5}
1	jjjj	10	250,000	4.0	x 10^{-5}
1	kkkk	22	545,000	4.0	x 10^{-5}
1	llll	63	1,530,000	4.1	x 10^{-5}
1	mmmm	39	940,000	4.1	x 10^{-5}
1	nnnn	13	305,000	4.3	x 10^{-5}
1	oooo	38	880,000	4.3	x 10^{-5}
1	pppp	16	370,000	4.3	x 10^{-5}
1	qqqq	17	400,000	4.3	x 10^{-5}
1	rrrr	29	62,000	4.7	x 10^{-5}
1	ssss	52	1,085,000	4.8	x 10^{-5}
1	tttt	47	915,000	5.1	x 10^{-5}

	1	uuuu	19	350,000	5.4	x 10^{-5}
	1	vvvv	20	355,000	5.6	x 10^{-5}
	1	wwww	20	325,000	6.2	x 10^{-5}
	1	xxxx	19	265,000	7.2	x 10^{-5}
	1	yyyy	258	2,085,000	12.4	x 10^{-5}

Male	**Offspring ID**		***cII* Mutants**	**PFU**	***cII* Mutant Frequency**	
2	2	a	13	1,925,000	0.7	x 10^{-5}
	2	b	16	1,840,000	0.9	x 10^{-5}
	2	c	9	920,000	1.0	x 10^{-5}
	2	d	9	770,000	1.2	x 10^{-5}
	2	e	7	535,000	1.3	x 10^{-5}
	2	f	18	1,365,000	1.3	x 10^{-5}
	2	g	28	1,965,000	1.4	x 10^{-5}
	2	h	6	420,000	1.4	x 10^{-5}
	2	i	16	1,050,000	1.5	x 10^{-5}
	2	j	19	1,275,000	1.5	x 10^{-5}
	2	k	23	1,530,000	1.5	x 10^{-5}
	2	l	20	1,330,000	1.5	x 10^{-5}
	2	m	11	675,000	1.6	x 10^{-5}
	2	n	7	445,000	1.6	x 10^{-5}
	2	o	24	1,485,000	1.6	x 10^{-5}
	2	p	22	1,340,000	1.6	x 10^{-5}
	2	q	12	750,000	1.6	x 10^{-5}
	2	r	16	975,000	1.6	x 10^{-5}
	2	s	20	1,185,000	1.7	x 10^{-5}
	2	t	27	1,505,000	1.8	x 10^{-5}
	2	u	14	785,000	1.8	x 10^{-5}
	2	v	7	385,000	1.8	x 10^{-5}
	2	w	6	330,000	1.8	x 10^{-5}
	2	x	16	885,000	1.8	x 10^{-5}
	2	y	7	380,000	1.8	x 10^{-5}
	2	z	16	855,000	1.9	x 10^{-5}
	2	aa	18	935,000	1.9	x 10^{-5}

2	bb	37	1,880,000	2.0	x 10^{-5}
2	cc	27	1,320,000	2.0	x 10^{-5}
2	dd	20	1,015,000	2.0	x 10^{-5}
2	ee	30	1,465,000	2.0	x 10^{-5}
2	ff	26	1,250,000	2.1	x 10^{-5}
2	gg	30	1,455,000	2.1	x 10^{-5}
2	hh	34	1,515,000	2.2	x 10^{-5}
2	ii	17	760,000	2.2	x 10^{-5}
2	jj	20	895,000	2.2	x 10^{-5}
2	kk	42	1,845,000	2.3	x 10^{-5}
2	ll	18	785,000	2.3	x 10^{-5}
2	mm	19	810,000	2.4	x 10^{-5}
2	nn	32	1,315,000	2.4	x 10^{-5}
2	oo	24	1,020,000	2.4	x 10^{-5}
2	pp	32	1,315,000	2.4	x 10^{-5}
2	qq	29	1,175,000	2.5	x 10^{-5}
2	rr	34	1,380,000	2.5	x 10^{-5}
2	ss	22	890,000	2.5	x 10^{-5}
2	tt	19	735,000	2.5	x 10^{-5}
2	uu	30	1,165,000	2.6	x 10^{-5}
2	vv	14	545,000	2.6	x 10^{-5}
2	ww	16	625,000	2.6	x 10^{-5}
2	xx	16	605,000	2.6	x 10^{-5}
2	yy	37	1,405,000	2.6	x 10^{-5}
2	zz	14	535,000	2.6	x 10^{-5}
2	aaa	35	1,305,000	2.7	x 10^{-5}
2	bbb	32	1,165,000	2.7	x 10^{-5}
2	ccc	38	1,390,000	2.7	x 10^{-5}
2	ddd	32	1,125,000	2.8	x 10^{-5}
2	eee	24	850,000	2.8	x 10^{-5}
2	fff	16	580,000	2.8	x 10^{-5}
2	ggg	36	1,275,000	2.8	x 10^{-5}
2	hhh	40	1,400,000	2.9	x 10^{-5}
2	iii	46	1,705,000	2.9	x 10^{-5}

2	jjj	18	625,000	2.9	x 10^{-5}
2	kkk	13	435,000	3.0	x 10^{-5}
2	lll	10	335,000	3.0	x 10^{-5}
2	mmm	54	1,815,000	3.0	x 10^{-5}
2	nnn	48	1,610,000	3.0	x 10^{-5}
2	ooo	38	1,240,000	3.1	x 10^{-5}
2	ppp	28	875,000	3.2	x 10^{-5}
2	qqq	21	620,000	3.4	x 10^{-5}
2	rrr	11	315,000	3.5	x 10^{-5}
2	sss	37	1,040,000	3.6	x 10^{-5}
2	ttt	30	845,000	3.6	x 10^{-5}
2	uuu	54	1,505,000	3.6	x 10^{-5}
2	vvv	26	725,000	3.6	x 10^{-5}
2	www	44	605,000	3.7	x 10^{-5}
2	xxx	25	665,000	3.8	x 10^{-5}
2	yyy	48	1,260,000	3.8	x 10^{-5}
2	zzz	13	345,000	3.8	x 10^{-5}
2	aaaa	22	565,000	3.9	x 10^{-5}
2	bbbb	19	490,000	3.9	x 10^{-5}
2	cccc	36	895,000	4.0	x 10^{-5}
2	dddd	18	430,000	4.2	x 10^{-5}
2	eeee	28	650,000	4.3	x 10^{-5}
2	ffff	33	755,000	4.4	x 10^{-5}
2	gggg	18	400,000	4.5	x 10^{-5}
2	hhhh	50	1,085,000	4.6	x 10^{-5}
2	iiii	29	615,000	4.7	x 10^{-5}
2	jjjj	36	770,000	4.7	x 10^{-5}
2	kkkk	31	630,000	4.9	x 10^{-5}
2	llll	69	1,360,000	5.1	x 10^{-5}
2	mmmm	58	1,100,000	5.3	x 10^{-5}
2	nnnn	29	465,000	6.2	x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
3	3 a	1	1,045,000	0.1 x 10^{-5}
	3 b	4	1,064,000	0.4 x 10^{-5}
	3 c	6	1,140,000	0.5 x 10^{-5}
	3 d	12	2,275,000	0.5 x 10^{-5}
	3 e	8	1,615,000	0.5 x 10^{-5}
	3 f	10	650,000	0.6 x 10^{-5}
	3 g	5	765,000	0.7 x 10^{-5}
	3 h	11	1,530,000	0.7 x 10^{-5}
	3 i	10	1,445,000	0.7 x 10^{-5}
	3 j	13	1,375,000	0.9 x 10^{-5}
	3 k	16	1,845,000	0.9 x 10^{-5}
	3 l	7	777,000	0.9 x 10^{-5}
	3 m	19	2,235,000	0.9 x 10^{-5}
	3 n	17	1,940,000	0.9 x 10^{-5}
	3 o	11	1,065,000	1.0 x 10^{-5}
	3 p	16	1,620,000	1.0 x 10^{-5}
	3 q	14	1,390,000	1.0 x 10^{-5}
	3 r	20	2,095,000	1.0 x 10^{-5}
	3 s	23	2,115,000	1.1 x 10^{-5}
	3 t	16	1,460,000	1.1 x 10^{-5}
	3 u	23	2,155,000	1.1 x 10^{-5}
	3 v	20	1,888,000	1.1 x 10^{-5}
	3 w	21	2,155,000	1.1 x 10^{-5}
	3 x	13	1,235,000	1.1 x 10^{-5}
	3 y	10	910,000	1.1 x 10^{-5}
	3 z	13	1,150,000	1.1 x 10^{-5}
	3 aa	15	1,115,000	1.3 x 10^{-5}
	3 bb	18	1,380,000	1.3 x 10^{-5}
	3 cc	17	1,290,000	1.3 x 10^{-5}
	3 dd	22	1,755,000	1.3 x 10^{-5}
	3 ee	26	1,815,000	1.4 x 10^{-5}
	3 ff	33	2,210,000	1.5 x 10^{-5}

3	gg	32	2,065,000	1.5	x 10^{-5}
3	hh	25	1,560,000	1.6	x 10^{-5}
3	ii	28	1,755,000	1.6	x 10^{-5}
3	jj	38	2,355,000	1.6	x 10^{-5}
3	kk	32	2,005,000	1.6	x 10^{-5}
3	ll	39	2,385,000	1.6	x 10^{-5}
3	mm	27	1,690,000	1.6	x 10^{-5}
3	nn	7	410,000	1.7	x 10^{-5}
3	oo	22	1,320,000	1.7	x 10^{-5}
3	pp	15	1,130,000	1.7	x 10^{-5}
3	qq	15	895,000	1.7	x 10^{-5}
3	rr	17	975,000	1.7	x 10^{-5}
3	ss	17	1,010,000	1.7	x 10^{-5}
3	tt	21	1,225,000	1.7	x 10^{-5}
3	uu	55	30,800,000	1.8	x 10^{-5}
3	vv	18	1,020,000	1.8	x 10^{-5}
3	ww	15	820,000	1.8	x 10^{-5}
3	xx	4	215,000	1.9	x 10^{-5}
3	yy	9	485,000	1.9	x 10^{-5}
3	zz	24	1,280,000	1.9	x 10^{-5}
3	aaa	11	575,000	1.9	x 10^{-5}
3	bbb	21	1,090,000	1.9	x 10^{-5}
3	ccc	29	1,535,000	1.9	x 10^{-5}
3	ddd	30	1,570,000	1.9	x 10^{-5}
3	eee	30	1,405,000	2.1	x 10^{-5}
3	fff	29	1,295,000	2.2	x 10^{-5}
3	ggg	21	960,000	2.2	x 10^{-5}
3	hhh	38	1,755,000	2.2	x 10^{-5}
3	iii	23	1,025,000	2.2	x 10^{-5}
3	jjj	20	925,000	2.2	x 10^{-5}
3	kkk	21	895,000	2.3	x 10^{-5}
3	lll	20	855,000	2.3	x 10^{-5}
3	mmm	33	1,450,000	2.3	x 10^{-5}
3	nnn	34	1,410,000	2.4	x 10^{-5}

3	ooo	27	1,125,000	2.4	x 10^{-5}
3	ppp	10	425,000	2.4	x 10^{-5}
3	qqq	30	1,265,000	2.4	x 10^{-5}
3	rrr	39	1,630,000	2.4	x 10^{-5}
3	sss	18	720,000	2.5	x 10^{-5}
3	ttt	45	1,785,000	2.5	x 10^{-5}
3	uuu	43	1,700,000	2.5	x 10^{-5}
3	vvv	20	780,000	2.6	x 10^{-5}
3	www	36	1,385,000	2.6	x 10^{-5}
3	xxx	19	705,000	2.7	x 10^{-5}
3	yyy	7	255,000	2.7	x 10^{-5}
3	zzz	33	1,185,000	2.8	x 10^{-5}
3	aaaa	0	205,000	2.9	x 10^{-5}
3	bbbb	14	475,000	2.9	x 10^{-5}
3	cccc	59	1,970,000	3.0	x 10^{-5}
3	dddd	43	1,440,000	3.0	x 10^{-5}
3	eeee	27	890,000	3.2	x 10^{-5}
3	ffff	7	215,000	3.3	x 10^{-5}
3	gggg	10	350,000	3.3	x 10^{-5}
3	hhhh	28	850,000	3.3	x 10^{-5}
3	iiii	46	1,345,000	3.4	x 10^{-5}
3	jjjj	17	495,000	3.4	x 10^{-5}
3	kkkk	26	775,000	3.4	x 10^{-5}
3	llll	35	1,040,000	3.4	x 10^{-5}
3	mmmm	49	1,455,000	3.4	x 10^{-5}
3	nnnn	42	1,085,000	3.9	x 10^{-5}
3	oooo	8	205,000	3.9	x 10^{-5}
3	pppp	21	495,000	4.2	x 10^{-5}
3	qqqq	47	1,095,000	4.3	x 10^{-5}
3	rrrr	28	558,000	5.0	x 10^{-5}
3	ssss	70	1,365,000	5.1	x 10^{-5}

Appendix E. Summary of *cII* mutant frequencies for individual mouse offspring collected prior to ENU exposure. Mean *cII* mutant frequency for all progeny= 3.8×10^{-5}.

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
1	1a	2	145,000	1.4×10^{-5}
	1b	10	410,000	2.4×10^{-5}
	1c	12	385,000	3.1×10^{-5}
	1d	7	205,000	3.4×10^{-5}
	1e	17	290,000	5.9×10^{-5}
Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
2	2a	6	240,000	2.5×10^{-5}
	2b	5	195,000	2.6×10^{-5}
	2c	17	205,000	8.3×10^{-5}
Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
3	3a	6	665,000	0.9×10^{-5}
	3b	2	170,000	1.2×10^{-5}
	3c	2	165,000	1.2×10^{-5}
	3d	4	180,000	2.2×10^{-5}
	3e	3	130,000	2.3×10^{-5}
	3f	6	265,000	2.3×10^{-5}
	3g	5	205,000	2.4×10^{-5}
	3h	3	125,000	2.4×10^{-5}
	3i	7	255,000	2.7×10^{-5}
	3j	6	205,000	2.9×10^{-5}
	3k	15	405,000	3.7×10^{-5}
	3l	9	250,000	3.9×10^{-5}
	3m	9	185,000	4.9×10^{-5}
	3n	9	180,000	5.0×10^{-5}
	3o	41	660,000	6.2×10^{-5}
	3p	10	150,000	6.7×10^{-5}
	3q	38	525,000	7.2×10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
4	4a	3	270,000	1.1 x 10^{-5}
	4b	5	270,000	1.9 x 10^{-5}
	4c	8	385,000	2.1 x 10^{-5}
	4d	10	390,000	2.6 x 10^{-5}
	4e	12	440,000	2.7 x 10^{-5}
	4f	7	215,000	3.3 x 10^{-5}
	4g	7	200,000	3.5 x 10^{-5}
	4h	11	310,000	3.6 x 10^{-5}
	4i	10	250,000	4.0 x 10^{-5}
	4j	20	450,000	4.4 x 10^{-5}
	4k	13	295,000	4.4 x 10^{-5}
	4l	8	180,000	4.4 x 10^{-5}
	4m	8	180,000	4.4 x 10^{-5}
	4n	13	280,000	4.6 x 10^{-5}
	4o	9	170,000	5.3 x 10^{-5}
	4p	7	125,000	5.6 x 10^{-5}
	4q	9	155,000	5.8 x 10^{-5}
	4r	10	145,000	6.9 x 10^{-5}
	4s	23	335,000	6.9 x 10^{-5}
	4t	13	175,000	7.4 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
5	5a	2	205,000	1.0 x 10^{-5}
	5b	4	345,000	1.2 x 10^{-5}
	5c	4	220,000	1.8 x 10^{-5}
	5d	6	245,000	2.4 x 10^{-5}
	5e	9	310,000	2.9 x 10^{-5}
	5f	5	175,000	2.9 x 10^{-5}
	5g	10	315,000	3.2 x 10^{-5}
	5h	7	195,000	3.6 x 10^{-5}
	5i	7	185,000	3.8 x 10^{-5}
	5j	7	175,000	4.0 x 10^{-5}

	5k	9	205,000	4.4 x 10^{-5}
	5l	7	160,000	4.4 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
6	6a	4	140,000	2.9 x 10^{-5}
	6b	14	420,000	3.3 x 10^{-5}
	6c	11	300,000	3.7 x 10^{-5}
	6d	7	190,000	3.7 x 10^{-5}
	6e	9	240,000	3.8 x 10^{-5}
	6f	7	180,000	3.9 x 10^{-5}
	6g	8	200,000	4.0 x 10^{-5}
	6h	7	160,000	4.4 x 10^{-5}
	6i	10	195,000	5.1 x 10^{-5}
	6j	13	220,000	5.9 x 10^{-5}
	6k	10	165,000	6.0 x 10^{-5}
	6l	7	105,000	6.7 x 10^{-5}
	6m	13	180,000	7.2 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
7	7a	4	215,000	1.9 x 10^{-5}
	7b	4	200,000	2.0 x 10^{-5}
	7c	7	270,000	2.6 x 10^{-5}
	7d	10	235,000	4.3 x 10^{-5}
	7e	11	235,000	4.7 x 10^{-5}
	7f	10	185,000	5.4 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
8	8a	1	205,000	0.5 x 10^{-5}
	8b	9	360,000	2.5 x 10^{-5}
	8c	10	365,000	2.7 x 10^{-5}
	8d	20	390,000	5.1 x 10^{-5}
	8e	12	170,000	7.1 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
9	9a	4	225,000	1.8 x 10^{-5}
	9b	4	165,000	2.4 x 10^{-5}
	9c	6	220,000	2.7 x 10^{-5}
	9d	6	225,000	2.7 x 10^{-5}
	9e	7	225,000	3.1 x 10^{-5}
	9f	10	310,000	3.2 x 10^{-5}
	9g	9	230,000	3.9 x 10^{-5}
	9h	9	225,000	4.0 x 10^{-5}
	9i	5	115,000	4.3 x 10^{-5}
	9j	13	265,000	4.9 x 10^{-5}
	9k	10	190,000	5.3 x 10^{-5}
	9l	8	150,000	5.3 x 10^{-5}
Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
10	10a	3	150,000	2.0 x 10^{-5}
	10b	6	230,000	2.6 x 10^{-5}
	10c	8	265,000	3.0 x 10^{-5}
	10d	10	320,000	3.1 x 10^{-5}
	10e	15	350,000	4.3 x 10^{-5}
	10f	15	325,000	4.6 x 10^{-5}
	10g	16	315,000	5.1 x 10^{-5}
	10h	15	285,000	5.3 x 10^{-5}

Appendix F. Summary of *cII* mutant frequencies for individual mouse offspring derived from ENU-exposed postmeiotic germ cells. Offspring that exhibited *cII* MFs > 8.8×10^{-5} were scored as mutant offspring.

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
1	1a	2	105,000	1.9×10^{-5}
	1b	3	130,000	2.3×10^{-5}
	1c	5	195,000	2.6×10^{-5}
	1d	5	175,000	2.9×10^{-5}
	1e	4	117,000	3.4×10^{-5}
	1f	8	160,000	5.0×10^{-5}
	1g	13	244,000	5.3×10^{-5}
	1h	18	350,000	5.4×10^{-5}
	1i	8	135,000	5.9×10^{-5}
	1j	15	225,000	6.7×10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
2	2a	2	160,000	1.3×10^{-5}
	2b	3	205,000	1.5×10^{-5}
	2c	10	260,000	3.8×10^{-5}
	2d	10	180,000	5.6×10^{-5}
	2e	10	130,000	7.7×10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
3	3a	3	185,000	1.6×10^{-5}
	3b	4	185,000	2.2×10^{-5}
	3c	3	120,000	2.5×10^{-5}
	3d	4	140,000	2.9×10^{-5}
	3e	9	270,000	3.3×10^{-5}
	3f	7	195,000	3.6×10^{-5}
	3g	19	520,000	3.7×10^{-5}
	3h	13	345,000	3.8×10^{-5}
	3i	6	155,000	3.9×10^{-5}
	3j	10	255,000	3.9×10^{-5}
	3k	7	175,000	4.0×10^{-5}

	3l	17	425,000	4.0	x 10^{-5}
	3m	13	310,000	4.2	x 10^{-5}
	3n	5	115,000	4.3	x 10^{-5}
	3o	8	180,000	4.4	x 10^{-5}
	3p	5	105,000	4.8	x 10^{-5}
	3q	36	715,000	5.0	x 10^{-5}
	3r	6	115,000	5.2	x 10^{-5}
	3s	8	155,000	5.2	x 10^{-5}
	3t	7	125,000	5.6	x 10^{-5}
	3u	10	160,000	6.3	x 10^{-5}
	3v	11	170,000	6.5	x 10^{-5}
	3w	18	260,000	6.9	x 10^{-5}
	3x	15	205,000	7.3	x 10^{-5}
	3y	24	310,000	7.7	x 10^{-5}
	3z	10	130,000	7.7	x 10^{-5}
	3aa	14	180,000	7.8	x 10^{-5}
	3bb	39	485,000	8.0	x 10^{-5}
	3cc	51	570,000	9.0	x 10^{-5}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
4	4a	1	170,000	0.6 x 10^{-5}
	4b	1	125,000	0.8 x 10^{-5}
	4c	2	110,000	1.8 x 10^{-5}
	4d	3	160,000	1.9 x 10^{-5}
	4e	9	330,000	2.7 x 10^{-5}
	4f	8	300,000	2.7 x 10^{-5}
	4g	5	175,000	2.9 x 10^{-5}
	4h	4	120,000	3.3 x 10^{-5}
	4i	4	120,000	3.3 x 10^{-5}
	4j	8	185,000	4.3 x 10^{-5}
	4k	5	105,000	4.8 x 10^{-5}
	4l	31	570,000	5.4 x 10^{-5}
	4m	12	220,000	5.5 x 10^{-5}
	4n	11	185,000	5.9 x 10^{-5}

	4o	13	180,000	7.2 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
5	5a	2	140,000	1.4 x 10^{-5}
	5b	5	250,000	2.0 x 10^{-5}
	5c	4	145,000	2.8 x 10^{-5}
	5d	13	460,000	2.8 x 10^{-5}
	5e	6	210,000	2.9 x 10^{-5}
	5f	4	125,000	3.2 x 10^{-5}
	5g	21	625,000	3.4 x 10^{-5}
	5h	33	905,000	3.6 x 10^{-5}
	5i	7	175,000	4.0 x 10^{-5}
	5j	12	270,000	4.4 x 10^{-5}
	5k	9	200,000	4.5 x 10^{-5}
	5l	15	325,000	4.6 x 10^{-5}
	5m	8	140,000	5.7 x 10^{-5}
	5n	15	255,000	5.9 x 10^{-5}
	5o	26	410,000	6.3 x 10^{-5}
	5p	8	115,000	7.0 x 10^{-5}
	5q	23	295,000	7.8 x 10^{-5}
	5r	19	180,000	10.6 x 10^{-5}
	5s	29	210,000	13.8 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
6	6a	3	165,000	1.8 x 10^{-5}
	6b	5	230,000	2.2 x 10^{-5}
	6c	3	120,000	2.5 x 10^{-5}
	6d	5	195,000	2.6 x 10^{-5}
	6e	6	185,000	3.2 x 10^{-5}
	6f	5	125,000	4.0 x 10^{-5}
	6g	7	155,000	4.5 x 10^{-5}
	6h	8	175,000	4.6 x 10^{-5}
	6i	5	105,000	4.8 x 10^{-5}
	6j	5	105,000	4.8 x 10^{-5}

	6k	6	120,000	5.0 x 10^{-5}
	6l	8	150,000	5.3 x 10^{-5}
	6m	11	195,000	5.6 x 10^{-5}
	6n	11	180,000	6.1 x 10^{-5}
	6o	9	145,000	6.2 x 10^{-5}
	6p	24	370,000	6.5 x 10^{-5}
	6q	9	135,000	6.7 x 10^{-5}
	6r	20	300,000	6.7 x 10^{-5}
	6s	14	200,000	7.0 x 10^{-5}
	6t	24	345,000	7.0 x 10^{-5}
	6u	8	110,000	7.3 x 10^{-5}
	6v	10	135,000	7.4 x 10^{-5}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
7	7a	4	220,000	1.8 x 10^{-5}
	7b	3	170,000	1.8 x 10^{-5}
	7c	4	210,000	2.3 x 10^{-5}
	7d	9	355,000	2.5 x 10^{-5}
	7e	5	200,000	2.5 x 10^{-5}
	7f	8	310,000	2.6 x 10^{-5}
	7g	6	180,000	3.3 x 10^{-5}
	7h	17	415,000	4.1 x 10^{-5}
	7i	14	265,000	5.3 x 10^{-5}
	7j	13	210,000	6.2 x 10^{-5}
	7k	12	190,000	6.3 x 10^{-5}
	7l	36	450,000	8.0 x 10^{-5}
	7m	37	435,000	8.5 x 10^{-5}
	7n	22	250,000	8.8 x 10^{-5}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
8	8a	1	135,000	0.7 x 10^{-5}
	8b	6	225,000	2.7 x 10^{-5}
	8c	6	225,000	2.7 x 10^{-5}
	8d	4	110,000	3.6 x 10^{-5}

	8e	7	185,000	3.8 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
9	9a	2	290,000	0.7 x 10^{-5}
	9b	2	255,000	0.8 x 10^{-5}
	9c	4	265,000	1.5 x 10^{-5}
	9d	6	280,000	2.1 x 10^{-5}
	9e	8	280,000	2.9 x 10^{-5}
	9f	4	135,000	3.0 x 10^{-5}
	9g	4	125,000	3.2 x 10^{-5}
	9h	12	380,000	3.2 x 10^{-5}
	9i	7	205,000	3.4 x 10^{-5}
	9j	13	325,000	4.0 x 10^{-5}
	9k	7	166,500	4.2 x 10^{-5}
	9l	20	465,000	4.3 x 10^{-5}
	9m	17	350,000	4.9 x 10^{-5}
	9n	5	145,000	5.5 x 10^{-5}
	9o	11	200,000	5.5 x 10^{-5}
	9p	14	250,000	5.6 x 10^{-5}
	9q	13	210,000	6.2 x 10^{-5}
	9r	15	230,000	6.5 x 10^{-5}
	9s	14	180,000	7.8 x 10^{-5}
	9t	11	140,000	7.9 x 10^{-5}
	9u	18	225,000	8.0 x 10^{-5}
	9v	24	270,000	8.9 x 10^{-5}
	9w	26	185,000	14.1 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
10	10a	4	275,000	1.5 x 10^{-5}
	10b	4	230,000	1.7 x 10^{-5}
	10c	6	295,000	2.0 x 10^{-5}
	10d	4	195,000	2.1 x 10^{-5}
	10e	6	255,000	2.4 x 10^{-5}
	10f	7	220,000	3.2 x 10^{-5}

10g	6	180,000	3.3	x 10^{-5}
10h	4	120,000	3.3	x 10^{-5}
10i	10	280,000	3.6	x 10^{-5}
10j	10	230,000	4.4	x 10^{-5}
10k	5	110,000	4.5	x 10^{-5}
10l	8	165,000	4.8	x 10^{-5}
10m	26	490,000	4.9	x 10^{-5}
10n	9	175,000	5.1	x 10^{-5}
10o	28	495,000	5.7	x 10^{-5}
10p	18	295,000	6.1	x 10^{-5}
10q	10	160,000	6.3	x 10^{-5}
10r	27	430,000	6.3	x 10^{-5}
10s	9	140,000	6.4	x 10^{-5}
10t	11	165,000	6.7	x 10^{-5}
10u	19	240,000	7.9	x 10^{-5}
10v	43	480,000	9.0	x 10^{-5}

Appendix G. Summary of *cII* mutant frequencies for individual mouse offspring derived from ENU-exposed premeiotic germ cells. Offspring that exhibited *cII* MFs >8.8 x 10^{-5} were scored as mutant offspring.

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
3	3a	1	255,000	0.4 x 10^{-5}
	3b	1	145,000	0.7 x 10^{-5}
	3c	3	320,000	0.9 x 10^{-5}
	3d	2	170,000	1.2 x 10^{-5}
	3e	4	305,000	1.3 x 10^{-5}
	3f	7	260,000	2.7 x 10^{-5}
	3g	8	275,000	2.9 x 10^{-5}
	3h	8	220,000	3.6 x 10^{-5}
	3i	9	225,000	4.0 x 10^{-5}
	3j	8	190,000	4.2 x 10^{-5}
	3k	18	335,000	5.3 x 10^{-5}
	3l	10	185,000	5.4 x 10^{-5}
	3m	20	355,000	5.6 x 10^{-5}
	3n	22	275,000	8.0 x 10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
6	6a	9	320,000	2.8 x 10^{-5}
	6b	5	135,000	3.7 x 10^{-5}
	6c	9	225,000	4.0 x 10^{-5}
	6d	12	290,000	4.1 x 10^{-5}
	6e	22	480,000	4.5 x 10^{-5}
	6f	17	335,000	5.1 x 10^{-5}
	6g	6	115,000	5.2 x 10^{-5}
	6h	17	315,000	5.4 x 10^{-5}
	6i	11	205,000	5.4 x 10^{-5}
	6j	20	360,000	5.6 x 10^{-5}
	6k	26	455,000	5.7 x 10^{-5}
	6l	14	245,000	5.7 x 10^{-5}
	6m	21	355,000	5.9 x 10^{-5}
	6n	22	350,000	6.3 x 10^{-5}

	6o	8	125,000	6.4 x 10^{-5}
	6p	19	295,000	6.4 x 10^{-5}
	6q	19	290,000	6.6 x 10^{-5}
	6r	15	220,000	6.8 x 10^{-5}
	6s	15	210,000	7.1 x 10^{-5}
	6t	32	375,000	8.8 x 10^{-5}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
9	9a	6	270,000	2.2 x 10^{-5}
	9b	8	250,000	3.2 x 10^{-5}
	9c	5	120,000	4.2 x 10^{-5}
	9d	10	210,000	4.8 x 10^{-5}
	9e	10	180,000	5.6 x 10^{-5}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
10	10a	14	490,000	2.9 x 10^{-5}
	10b	16	420,000	3.8 x 10^{-5}
	10c	12	250,000	4.8 x 10^{-5}
	10d	7	140,000	5.0 x 10^{-5}
	10e	14	260,000	5.4 x 10^{-5}
	10f	11	200,000	5.5 x 10^{-5}
	10g	17	255,000	6.7 x 10^{-5}
	10h	23	270,000	8.5 x 10^{-5}
	10i	25	285,000	8.8 x 10^{-5}
	10j	15	150,000	10.0 x 10^{-5}

Appendix H. Mutational spectra of Big Blue mouse mutant offspring derived from ENU-treated postmeiotic germ cells.

Individual	*cII* MF (x 10^{-5})	Base	Mutation	Sequences
9w	14.1	179	G Insertion	6
		179	G Deletion	4
		64	G→A	2
		52	C→T	1
		89	C→T	1
		124	A→T	1
		149	A→C	1
		180	G→A	1
		196	G→A	1
			Total	**18**
5s	13.8	179	G Insertion	6
		179	G Deletion	6
		20	G→A	1
		25	G→A	1
		28	G→A	1
		35	G→A	1
		57	C→A	1
		86	C→T	1
		89	C→T	1
		127	T→C	1
		152	T→C	1
		168	T Insertion	1
		220	G→A	1
		274	C→A	1
			Total	**24**
5r	10.6	179	G Insertion	5
		28	G→A	1
		29	C→T	1
		50	T→C	1
		64	G→A	1
		124	A→T	1
		141	G→T	1
		179	G Deletion	1
		242	A Deletion	1

			Total	**13**
3cc	9.0	179	G Insertion	8
		179	G Deletion	8
		28	G → T	1
		28	G → A	1
		35	G → A	1
		38	T → C	1
		51	G → T	1
		89	C → A	1
		113	C → T	1
		118	A → T	1
		161	T → C	1
		180	G → A	1
		196	G → A	1
		221	T → G	1
			Total	**28**
10v	9.0	141	G → T	12
		179	G Insertion	12
		25	G → T	1
		29	C → G	1
		64	G → A	1
		113	C → T	1
		118	A → T	1
		127	T → A	1
		146	C → A	1
		161	T → C	1
		179	G Deletion	1
		181	G → T	1
		186	T → G	1
		203	G → A	1
		211	G → A	1
		215	C → T	1
		220	A → T	1
			Total	**39**
9v	8.9	179	G Insertion	6
		180	G→A	2
		28	G→A	1
		68	T→C	1

89	C→G	1
103	G→A	1
163	C→A	1
179	G Deletion	1
185	T→G	1
223	G→A	1
	Total	**16**

Appendix I. Mutational spectra of Big Blue mouse mutant offspring derived from ENU-treated premeiotic germ cells.

Individual	*cII* MF (x 10^{-5})	Base	Mutation	Sequences
10j	10.0	141	G → A	3
		179	G Deletion	3
		179	G Insertion	2
		15	C → A	1
		94	G → A	1
		121	A → G	1
		130	A → G	1
		212	C → T	1
		292	T → C	1
			Total	**14**
10i	8.8	179	G Insertion	8
		179	G Deletion	4
		50	T → A	1
		52	C → A	1
		62	T → C	1
		64	G → T	1
		94	G → A	1
		113	C → T	1
		136	G → T	1
		164	T → C	1
		179-180	2 G Deletion	1
		205	C → T	1
		212	C → T	1
		218	A → G	1
		241	A Deletion	1
			Total	**25**
6t	8.8	179	G Insertion	8
		179	G Deletion	3
		230	T → A	2
		1	A → G	1
		3	G → A	1
		29	C → A	1
		64	G → A	1

85	A → G	1
95	C → T	1
121	A → G	1
167	C → T	1
175	G → T	1
212	C → T	1
223	G → A	1
233	T → C	1
	Total	**25**

Appendix J. Summary of *cII* mutant frequencies for individual offspring derived from $p53^-$ females, collected prior to ENU exposure. Mean *cII* mutant frequency for all progeny = 1.7×10^{-5}. Offspring that exhibited *cII* MFs > 5.1×10^{-5} were scored as mutant offspring and are tabulated below a dividing line for each male.

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
1	1a	4	423,000	1.0×10^{-5}
	1b	10	955,000	1.0×10^{-5}
	1c	9	570,000	1.6×10^{-5}
	1d	24	1,355,000	1.8×10^{-5}
	1e	9	500,000	1.8×10^{-5}
	1f	19	905,000	2.1×10^{-5}
	1g	23	1,110,000	2.1×10^{-5}
	1h	9	360,000	2.5×10^{-5}
	1j	8	292,500	2.7×10^{-5}
	1k	8	290,000	2.8×10^{-5}
	1l	15	485,000	3.1×10^{-5}
	1m	31	920,000	3.4×10^{-5}
	1n	25	520,000	4.8×10^{-5}
	1o	23	415,000	5.5×10^{-5}

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
2	2a	2	1,445,000	0.1×10^{-5}
	2b	1	630,000	0.2×10^{-5}
	2c	3	1,000,000	0.3×10^{-5}
	2d	7	1,915,000	0.4×10^{-5}
	2e	3	620,000	0.5×10^{-5}
	2f	5	970,000	0.5×10^{-5}
	2g	2	395,000	0.5×10^{-5}
	2h	9	1,655,000	0.5×10^{-5}
	2i	8	1,250,000	0.6×10^{-5}
	2j	1	585,000	0.7×10^{-5}
	2k	9	1,155,000	0.8×10^{-5}
	2l	18	1,905,000	0.9×10^{-5}
	2m	5	505,000	1.0×10^{-5}

	2n	9	685,000	1.3 x 10^{-5}
	2o	20	1,315,000	1.5 x 10^{-5}
	2p	18	1,125,000	1.6 x 10^{-5}
	2q	11	420,000	2.6 x 10^{-5}
	2r	36	1,290,000	3.1 x 10^{-5}
	2s	30	690,000	4.3 x 10^{-5}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
3	3a	5	1,535,000	0.3 x 10^{-5}
	3b	8	1,500,000	0.5 x 10^{-5}
	3c	6	1,085,000	0.6 x 10^{-5}
	3d	7	1,115,000	0.6 x 10^{-5}
	3e	9	1,210,000	0.7 x 10^{-5}
	3f	15	1,550,000	1.0 x 10^{-5}
	3g	11	795,000	1.4 x 10^{-5}
	3h	6	365,000	1.6 x 10^{-5}
	3i	12	780,000	1.6 x 10^{-5}
	3j	17	870,000	2.0 x 10^{-5}
	3k	6	290,000	2.1 x 10^{-5}
	3l	24	1,075,000	2.2 x 10^{-5}
	3m	20	575,000	3.5 x 10^{-5}
	3n	37	1,040,000	3.6 x 10^{-5}
	3o	24	590,000	4.1 x 10^{-5}

Appendix K. Summary of *cII* mutant frequencies for individual offspring derived from ENU-exposed postmeiotic male germ cells and $p53^{-}$ female medaka. Offspring that exhibited *cII* MFs > 5.1 x 10^{-5} were scored as mutant offspring and are tabulated below a dividing line for each male.

Male	Offspring ID	PFU	*cII* Mutants	*cII* Mutant Frequency
1	1a	370,000	2	0.5 x 10^{-5}
	1b	290,000	2	0.7 x 10^{-5}
	1c	395,000	3	0.8 x 10^{-5}
	1d	1410000	19	1.3 x 10^{-5}
	1e	1855000	28	1.5 x 10^{-5}
	1f	855000	15	1.8 x 10^{-5}
	1g	450,000	8	1.8 x 10^{-5}
	1h	455000	10	2.2 x 10^{-5}
	1i	760,000	17	2.2 x 10^{-5}
	1j	920,000	22	2.4 x 10^{-5}
	1k	290,000	7	2.4 x 10^{-5}
	1l	165,000	4	2.4 x 10^{-5}
	1m	1,160,000	29	2.5 x 10^{-5}
	1n	140,000	4	2.9 x 10^{-5}
	1o	510000	15	2.9 x 10^{-5}
	1p	360,000	11	3.1 x 10^{-5}
	1q	115,000	4	3.5 x 10^{-5}
	1r	485,000	20	4.1 x 10^{-5}
	1s	500,000	21	4.2 x 10^{-5}
	1t	185,000	9	4.9 x 10^{-5}
	1u	705000	35	5.0 x 10^{-5}
	1v	580,000	31	5.3 x 10^{-5}
	1w	135,000	8	5.9 x 10^{-5}
	1x	135,000	8	5.9 x 10^{-5}
	1y	530000	38	7.2 x 10^{-5}
	1z	430,000	31	7.2 x 10^{-5}
	1aa	465,000	35	7.5 x 10^{-5}
	1bb	355,000	27	7.6 x 10^{-5}
	1cc	240,000	24	10.0 x 10^{-4}
	1dd	180,000	20	11.1 x 10^{-4}

	1ee	210,000	66	31.4	x 10^{-4}
	1ff	510,000	249	48.8	x 10^{-4}
	1gg	165,000	181	109.7	x 10^{-3}
	1hh	895000	1744	194.9	x 10^{-3}
	1ii	1765000	5760	326.3	x 10^{-3}
	1jj	310,000	1456	469.7	x 10^{-3}

Male	**Offspring ID**	**PFU**	***cII* Mutants**	***cII* Mutant Frequency**	
2	2a	1155000	4	0.3	x 10^{-5}
	2b	1275000	12	0.9	x 10^{-5}
	2c	690000	11	1.6	x 10^{-5}
	2d	960000	17	1.8	x 10^{-5}
	2e	395000	157	39.8	x 10^{-4}
	2f	985000	1210	122.8	x 10^{-3}

Male	**Offspring ID**	**PFU**	***cII* Mutants**	***cII* Mutant Frequency**	
3	4a	1470000	20	1.4	x 10^{-5}
	4b	935000	16	1.7	x 10^{-5}
	4c	1815000	33	1.8	x 10^{-5}
	4d	1590000	35	2.2	x 10^{-5}
	4e	690000	21	3.0	x 10^{-5}
	4f	730000	23	3.2	x 10^{-5}
	4g	1215000	23	3.8	x 10^{-5}
	4h	640000	33	5.2	x 10^{-5}

Appendix L. Summary of *cII* mutant frequencies for individual offspring derived from ENU-exposed premeiotic male germ cells and $p53^{-}$ female medaka. Offspring that exhibited *cII* MFs >5.1 x 10^{-5} were scored as mutant offspring and are tabulated below a dividing line for each male.

Male	Offspring ID	*cII* Mutants	PFU	*cII* Mutant Frequency
1	1	5	760,000	0.7 x 10^{-5}
	2	19	1970000	1.0 x 10^{-5}
	3	8	825000	1.0 x 10^{-5}
	4	8	750,000	1.1 x 10^{-5}
	5	6	550,000	1.1 x 10^{-5}
	6	11	970000	1.1 x 10^{-5}
	7	24	2085000	1.2 x 10^{-5}
	8	8	620000	1.3 x 10^{-5}
	9	26	2060000	1.3 x 10^{-5}
	10	15	1125000	1.3 x 10^{-5}
	11	9	620,000	1.5 x 10^{-5}
	12	13	895,000	1.5 x 10^{-5}
	13	23	1395000	1.6 x 10^{-5}
	14	10	605,000	1.7 x 10^{-5}
	15	22	1300000	1.7 x 10^{-5}
	16	25	1470000	1.7 x 10^{-5}
	17	11	645000	1.7 x 10^{-5}
	18	15	855,000	1.8 x 10^{-5}
	19	23	1275000	1.8 x 10^{-5}
	20	24	1235000	1.9 x 10^{-5}
	21	30	1545000	1.9 x 10^{-5}
	22	11	565000	1.9 x 10^{-5}
	23	15	765,000	2.0 x 10^{-5}
	24	19	930000	2.0 x 10^{-5}
	25	6	285000	2.1 x 10^{-5}
	26	30	1405000	2.1 x 10^{-5}
	27	32	1480000	2.2 x 10^{-5}
	28	11	500,000	2.2 x 10^{-5}
	29	5	225000	2.2 x 10^{-5}
	30	22	985000	2.2 x 10^{-5}

31	15	655,000	2.3	x 10^{-5}
32	27	1150000	2.3	x 10^{-5}
33	15	635,000	2.4	x 10^{-5}
34	22	915000	2.4	x 10^{-5}
35	11	455,000	2.4	x 10^{-5}
36	12	475,000	2.5	x 10^{-5}
37	12	470,000	2.6	x 10^{-5}
38	11	415,000	2.7	x 10^{-5}
39	11	415,000	2.7	x 10^{-5}
40	17	635000	2.7	x 10^{-5}
41	29	1075000	2.7	x 10^{-5}
42	30	1060000	2.8	x 10^{-5}
43	13	385,000	3.4	x 10^{-5}
44	26	750000	3.5	x 10^{-5}
45	14	395000	3.5	x 10^{-5}
46	20	450,000	4.4	x 10^{-5}
47	25	530,000	4.7	x 10^{-5}
48	7668	875000	8.8	x 10^{-3}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
2	1	8	1270000	0.6 x 10^{-5}
	2	12	1420000	0.8 x 10^{-5}
	3	14	1705000	0.8 x 10^{-5}
	4	12	1405000	0.9 x 10^{-5}
	5	15	1735000	0.9 x 10^{-5}
	6	11	1225000	0.9 x 10^{-5}
	7	13	1215000	1.1 x 10^{-5}
	8	23	1730000	1.3 x 10^{-5}
	9	16	1120000	1.4 x 10^{-5}
	10	21	1335000	1.6 x 10^{-5}
	11	17	1085000	1.6 x 10^{-5}
	12	20	1275000	1.6 x 10^{-5}
	13	10	630000	1.6 x 10^{-5}
	14	29	1585000	1.8 x 10^{-5}

	15	12	625000	1.9	x 10^{-5}
	16	32	1495000	2.1	x 10^{-5}
	17	37	1655000	2.2	x 10^{-5}
	18	19	820000	2.3	x 10^{-5}
	19	15	590000	2.5	x 10^{-5}
	20	23	760000	3.0	x 10^{-5}
	21	43	1395000	3.1	x 10^{-5}
	22	14	415000	3.4	x 10^{-5}
	23	31	865000	3.6	x 10^{-5}
	24	56	1575000	3.6	x 10^{-5}
	25	32	860000	3.7	x 10^{-5}
	26	20	390000	5.7	x 10^{-5}
	27	24	375000	6.4	x 10^{-5}
	28	12320	1830000	6.7	x 10^{-3}

Male	**Offspring ID**	***cII* Mutants**	**PFU**	***cII* Mutant Frequency**
3	1	4	945000	0.4 x 10^{-5}
	2	4	695000	0.6 x 10^{-5}
	3	6	815000	0.7 x 10^{-5}
	4	8	905000	0.9 x 10^{-5}
	5	6	705000	0.9 x 10^{-5}
	6	8	785000	1.0 x 10^{-5}
	7	10	890000	1.1 x 10^{-5}
	8	13	1220000	1.1 x 10^{-5}
	9	10	785000	1.3 x 10^{-5}
	10	16	1205000	1.3 x 10^{-5}
	11	4	310000	1.3 x 10^{-5}
	12	18	1295000	1.4 x 10^{-5}
	13	3	215000	1.4 x 10^{-5}
	14	13	825000	1.6 x 10^{-5}
	15	49	1875000	2.6 x 10^{-5}
	16	25	960000	2.6 x 10^{-5}
	17	16	615000	2.6 x 10^{-5}
	18	29	1085000	2.7 x 10^{-5}
	19	34	1130000	3.0 x 10^{-5}

20	35	1125000	3.1	x 10^{-5}
21	25	725000	3.4	x 10^{-5}
22	65	1835000	3.5	x 10^{-5}
23	40	1070000	3.7	x 10^{-5}
24	494	320000	1.5	x 10^{-3}
25	800	430000	1.9	x 10^{-3}