

ISOLATED SPERMATOZOA AS INDICATORS OF MUTATIONS TRANSMITTED TO
PROGENY

by

MICHELLE BETH NORRIS

(Under the Direction of Richard N. Winn)

ABSTRACT

We used λ transgenic medaka to evaluate whether analysis of mutations directly in isolated spermatozoa is a reliable alternative to using progeny to assess genetic health risks. Mutant frequencies of *c//* targets (MF) in spermatozoa exposed to ethylnitrosurea (ENU) at either post-meiotic or pre-meiotic germ-cell stages of spermatogenesis were compared. The MFs of exposed pre-meiotic stem cell spermatogonia showed a significant 9-fold induction in MF, consistent with progeny analyses. By contrast, significantly elevated MFs were detected in progeny derived from ENU-treated post-meiotic germ cells, but not in isolated spermatozoa. DNA damage not fixed as a *c//* mutation in spermatozoa will not be detected. However, damage in spermatozoa that persists to or after fertilization can be fixed as a mutation and is detected in the embryo. Consequently, using isolated spermatozoa can provide a reliable alternative to using progeny providing the spermatozoa sampled correspond to pre-meiotic germ-cell stages exposed to the mutagen.

INDEX WORDS: Germ cells, Spermatozoa, *c//* mutation target gene, Mutant frequency

ISOLATED SPERMATOZOA AS INDICATORS OF MUTATIONS TRANSMITTED TO
PROGENY

by

MICHELLE BETH NORRIS

B. S., The University of Georgia, 1998

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2008

© 2008

Michelle Beth Norris

All Rights Reserved

ISOLATED SPERMATOZOA AS INDICATORS OF MUTATIONS TRANSMITTED TO
PROGENY

by

MICHELLE BETH NORRIS

Major Professor: Richard N. Winn

Committee: Travis C. Glenn
Don G. Ennis

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2008

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor and mentor, Dr. Richard Winn. The wisdom and proficiency I have gained over the last thirteen years will stay with me for life, and will influence all that I do. I would also like to thank my committee members, Dr. Travis Glenn and Dr. Don Ennis, for their guidance and support. I would also like to thank all of my fellow labmates and students, past and present. I have gained something from all of them. Finally, I want to thank Stephen and Leah for their never ending patience and support throughout this process.

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS	iv
INTRODUCTION	1
MATERIALS AND METHODS	7
RESULTS	16
DISCUSSION	29
REFERENCES.....	34

INTRODUCTION

Characterization of adverse effects in progeny of mutagen exposed parents continues to be a complex and challenging undertaking due to the large numbers of animals and extensive resources necessary for these studies. Gene mutations passed from an exposed parent are difficult to measure because they occur at low frequencies and require an efficient method for detection. The traditional assays for measuring genetic health risk are based on using mouse models and include the specific locus test (SLT) (Russell et al. 1981), dominant lethal assay (DL) (Russell and Matter 1980), and heritable translocation assay (HLT) (Generoso et al. 1980). Studies utilizing these assays have built the foundation for our understanding of germline mutagenesis. These tests utilize direct analysis progeny and have not become routine because they are expensive and require extensive resources such as thousands to ten thousands of animals (Singer et al. 2006). Further, in the case of the DL, death is the endpoint, and therefore only offers limited information necessary for predicting genetic health risk (Adler 1996). The analysis of progeny from transgenic mutation models is proving to be a sensitive and unequivocal method for identifying mutations transmitted to progeny, with dramatically improved efficiency, compared to these other methods, in the number of animals required (Barnett et al. 2002; Winn et al. 2008). However, despite these improvements in efficiency, there remains a need for a more efficient method to evaluate genetic health risk.

Analysis of mutations directly in spermatozoa has shown promise for predicting the detrimental genetic health risks in progeny of exposed male parents. The analysis of spermatozoa allows identification of mutations from a population of gametes without requiring the resources of producing and screening many offspring. Spermatozoa provide a homogeneous cell population with large numbers of cells available from an individual (Adler 1996). There is growing evidence that a number of genetic defects are transmitted by spermatozoa: including aneuploidy, structural aberrations, epigenetic changes, premutational lesions, changes in the number of trinucleotide repeats, and gene mutations (Marchetti and Wyrobeck 2005). The transgenic mutation assays in rodents and expanded simple tandem repeats (ESTR) assays are proving to be very useful for the evaluation of mutations in germ cells and spermatozoa (Singer et al. 2006), and appear to be the best choice to efficiently estimate the frequency of mutations transmitted to progeny. However, measuring the genetic alterations in spermatozoa is not equivalent to characterization of mutant progeny (Adler 1996). There are many complex biological processes occurring at fertilization, and in the early stages of development that may cause differences between endpoints in gametes and those measured in progeny. However, if the differences between using isolated spermatozoa compared to using progeny can be defined and characterized, the analysis of isolated spermatozoa offers a practical and cost-effective solution for evaluating genetic health risk.

Transgenic mutation assays allow for the characterization of mutations induced in progeny (Barnett et al. 2002; Winn et al. 2008) and germ cells (Ashby et al. 1997; Douglas et al. 1997; Singer et al. 2006), and offer significant promise as a method for

the evaluation of germline mutations. Transgenic mutation assays utilize prokaryotic vectors, integrated into the genome, that harbor mutation reporter genes that can in turn, be efficiently recovered from tissues and analyzed for mutations. Transgenic mutation assays have been developed in rodents (Boerrigter et al. 1995; Burkhart et al. 1993; Gossen et al. 1989; Kohler et al. 1991; Manjanatha et al. 1996) and in fish (Amanuma et al. 2000; Winn et al. 2001; Winn et al. 2000; Winn et al. 1995) and allow for *in vivo* mutation detection through efficient screening of large numbers of genetically neutral mutational targets. Both quantification and molecular characterization of mutations can be determined using transgenic mutation assays. Transgenic mutation assays are cost-effective because they require far fewer animals than the traditional assays such as the SLT. The λ transgenic medaka (*Oryzias latipes*), a small fish model, carries multiple copies (75 copies/haploid genome) of the *lacI* and *cII* mutation target genes. The λ transgenic medaka has been used to characterize chemically induced mutations in various somatic tissues including liver, kidney, skin, testes, and whole body (Winn and Norris 2005; Winn et al. 2000; Winn et al. 2005). The induced MFs and mutational spectra from the well characterized mutagens ethylnitrosurea (ENU), benzo(a)pyrene (BaP), dimethylnitrosamine (DMN), and diethylnitrosamine (DEN), evaluated in the λ transgenic medaka, have shown similar mechanisms of action as in transgenic rodents (Winn and Norris 2005; Winn et al. 2000). The λ transgenic medaka model has demonstrated comparable sensitivities to mutagens as the rodent models, and has proven to be a versatile model, with studies ranging from assessment of contaminants in aquatic environments (Cachot et al. 2007; McElroy et al. 2006) to characterization of mutations transmitted to progeny from mutagen-exposed parents

(Winn et al. 2008). The λ transgenic medaka has significant practical benefits that make it ideal for evaluating germline mutations in both progeny and germ-cells. Medaka are relatively inexpensive to culture, reach sexual maturity in 6 weeks, a single male is capable of producing large numbers of offspring (200–300 per day), and spermatozoa are easily collected in a non-lethal manner.

To determine if transgenic mutation assays using isolated spermatozoa may serve as a possible replacement for assays of genetic health risk using progeny, it would be necessary to compare the differences between genetic alterations occurring in germ cells and those occurring in progeny. Extensive analyses of male germ cells have been carried out in transgenic rodents and have determined the most appropriate experimental conditions, including the timing necessary to evaluate relevant developmental stages, the most appropriate germ cell tissues to be analyzed, and the optimal sampling time necessary for correct interpretation (Ashby et al. 1997; Douglas et al. 1997; Douglas et al. 1995; Singer et al. 2006). Recent progeny analyses using λ transgenic medaka have built upon traditional germline studies and provided further insights into mutant offspring generated from mutagen exposed male parents (Winn et al. 2008). Used together, this new generation of mutation assays will provide further clarification and understanding of the differences and similarities between the spermatozoa and progeny studies, and will allow researchers to identify when the use of germ cells may serve as an appropriate surrogate, or more importantly, if these assays are not acceptable surrogates to predict genetic health risk.

In this study we sought to develop an efficient and sensitive alternative approach to progeny analyses to assess mutations transmitted to progeny from mutagen exposed

male parents. We sought to determine if measuring mutations in spermatozoa can be used as reliable indicators of mutations transmitted to progeny. The first aim of this study was to utilize λ transgenic medaka to evaluate if detecting mutations in isolated spermatozoa provides an efficient and sensitive means for detecting mutations transmitted to progeny. Next, we wanted to determine whether direct analysis of mutations in isolated spermatozoa exposed to ENU at different stages of spermatogenesis vary in susceptibility. Finally, we wanted to compare mutations measured in isolated spermatozoa directly to frequencies of mutant progeny generated from the same exposed male parents. Based on prior studies with germ cells and progeny, we tested several hypotheses:

1. Induced mutations will not be detected in mutagen-exposed post-meiotic spermatozoa and late spermatids, due likely to a lack of DNA replication and repair that does not allow the DNA damage to become fixed as a mutation.
2. A significant increase in the frequency of mutant progeny derived from mutagen-exposed post-meiotic spermatozoa and late spermatids will be detected, likely resulting from DNA damage carried by the spermatozoa that is recognized and fixed as mutations by processes post-fertilization in the embryo.
3. Mutations measured in mutagen-exposed pre-meiotic germ cells will likely be more representative of those measured in progeny due to the presence of DNA repair processes that contribute to DNA damage fixed as a mutation in the resulting spermatozoa.

We utilized the *c//* mutation target genes recovered from λ transgenic medaka to provide a direct comparison of the mutations generated in spermatozoa and progeny and provide further support to these hypotheses.

MATERIALS AND METHODS

SPERMATOGENESIS

The induced response at each germ-cell stage can be evaluated by sampling mature spermatozoa, or mating animals, at the appropriate sampling interval following mutagen exposure (Figure 1). The earliest germ-cell stages (spermatogonial stem cells) require the longest post-treatment period before sampling (Ashby et al. 1997; Douglas et al. 1997; Favor and Neuhauser-Klaus 1994; Russell 2004; Shima and Shimada 1994; Singer et al. 2006). In this study we measured the MFs of spermatogonial stem cells, spermatocytes, late spermatids, and spermatozoa by collecting mature spermatozoa at the appropriate number of days following exposure to represent the exposed germ-cell type. To simplify the remaining text, we will refer to each simply by the stage at exposure: i.e. spermatogonial stem cells, rather than mature spermatozoa corresponding to exposed spermatogonial stem cells.

Spermatogenesis in medaka has been shown to be comparable to mammalian spermatogenesis, but with a faster progression from stem cell spermatogonia to mature spermatozoa than in rodents and humans (Egami and Hyodo-Taguchi 1967; Saiki et al. 1997). The duration of spermatogenesis is approximately 30 days in the medaka, compared to 42 days in the mouse and 100 days in humans (Figure 1) (Marchetti and Wyrobeck 2005; Shima and Shimada 1994). Spermatogenesis involves a series of mitotic and reductive meiotic cell divisions in the progression of diploid stem cell spermatogonia to haploid spermatozoa. DNA replication takes place in the mitotic

divisions carried out by spermatogonia and the meiosis occurring to form primary spermatocytes. DNA repair has been shown to occur from spermatogonia to the mid-spermatid stages (Singer et al. 2006). Consequently, DNA lesions introduced in the later stages of spermatogenesis that lack replication and repair processes will not be fixed as mutations until after fertilization (Douglas et al. 1995; Favor 1999; Favor and Neuhauser-Klaus 1994; Marchetti and Wyrobeck 2005; Singer et al. 2006).

INDUCED MF IN GERM-CELLS

The λ transgenic medaka has attributes well suited for the evaluation of induced mutation in germ-cells. The short duration of spermatogenesis (30 days) allows for rapid evaluation of the germ-cell stages, and mature spermatozoa can be easily collected by anesthetizing the fish and applying gentle pressure to the abdomen to release the sperm. In this test, we sought to provide a precise examination of ENU induced MFs in post-meiotic spermatozoa and late spermatids, and pre-meiotic spermatogonial stem cells. We collected mature spermatozoa from control or ENU exposed, 4-6 month old homozygous adult transgenic males, at days 2, 5, or 32 following treatment, corresponding to spermatozoa, late spermatids, and spermatogonial stem cells, respectively, exposed to the mutagen (Shima and Shimada 1994).

MF IN GERM CELLS FROM INDIVIDUAL MALES

The ability to collect mature spermatozoa in medaka without sacrificing the male donor has provided a unique opportunity to compare MFs through various stages of spermatogenesis. In this test, we sought to track the changes in MF through multiple germ-cell stages (late spermatids, spermatocytes, and spermatogonial stem cells) of

individual exposed males to repeat the comparison of MFs at post-meiotic and pre-meiotic germ-cell stages, while gaining insight into the biological response of individuals to mutagen insult. Adult transgenic male medaka (homozygous λ/λ) were exposed to ENU. Spermatozoa were collected prior to, and 5, 14, or 32 days following exposure. We also collected spermatozoa from two unexposed males at each time point to serve as controls. The fish were anesthetized for each collection, and were revived, to allow for collection of spermatozoa at multiple stages of spermatogenesis.

COMPARISON OF SPERMATOZOA AND PROGENY

To provide a direct comparison of ENU induced mutations measured in isolated spermatozoa and mutant progeny generated from exposure, we measured both endpoints from the same male parents. Two male medaka were exposed to ENU and spermatozoa was collected and split into aliquots. One aliquot of the spermatozoa was used to perform *in vitro* fertilization to generate progeny for analysis, and the other aliquot was used for direct analysis of mutations in spermatozoa (Figure 2). We collected spermatozoa from two adult male λ transgenic medaka prior to, and 5 days after ENU exposure. At each collection, the spermatozoa were split into 2 aliquots, one flash frozen and stored at -80°C for nucleic acid purification and direct analysis of mutations, and the other aliquot was used to *in vitro* fertilize oocytes from non-transgenic females (Ando and Wakamatsu 1995; Iwamatsu 1983). Progeny from all crosses were hemizygous for the λ bacteriophage containing the *cII* target gene contributed only by the male parent. Progeny from each breeding group were maintained separately, and allowed to hatch, cultured under standard conditions for ~

10 days, at which they were flash frozen and stored at -80°C prior to processing for mutation analysis.

ANIMALS

Wild-type (CAB strain) and λ transgenic medaka (strain λ 310) containing the λ LIZ 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 (Winn et al. 2000). Fish were maintained in dechlorinated municipal water at 24°C on a 12-hr light-dark cycle, except for breeding groups maintained on a 16:8 light-dark cycle, and fed 3 times daily with either brine shrimp nauplii (*Artemia*, Great Salt Lake, UT), or flake food. The Institutional Animal Care and Use Committee approved the protocol used in this study.

MUTAGEN TREATMENT

All medaka were exposed to 100 mg/L ENU (Sigma) for 1 hr for 2 consecutive days in beakers under static renewal conditions. To minimize the degradation of ENU, exposures were carried out in darkness. The exposed fish were rinsed and held in standard culture conditions following exposure.

COLLECTION OF SPERMATOOZOA

To collect spermatozoa for MF analysis, medaka males were anesthetized for ~ 5 min in 150 mg/L tricaine methanesulfonate pH 7.0 (Western Chemical Inc, Ferndale, WA) and gentle pressure was applied to the abdomen, examined using a dissection (compound) microscope, to release mature spermatozoa onto a petri dish containing 160 μ l of nano-pure water or balanced salt solution (BSS) pH 7.4 (Ando and Wakamatsu 1995; Iwamatsu 1983). The spermatozoa were collected with a micro-

pipette, and either flash frozen, and stored at -80°C prior to analysis, or used for *in vitro* fertilization to produce progeny.

DNA EXTRACTION

DNA was isolated from spermatozoa or from individual progeny using procedures described previously (Winn et al. 2008; Winn and Norris 2005; Winn et al. 2000). Briefly, spermatozoa or tissues from whole animals were digested with proteinase (1 X SSC, 20% SDS, 20 mg/ml proteinase K) and extracted with equal volumes of phenol:chloroform. DNA was precipitated using ethanol and resuspended in Tris-EDTA buffer (pH 7.5).

cII MUTATION ASSAY

Mutations in spermatozoa or progeny were analyzed using a positive-selection assay for the *cII* null-mutations (Jakubczak et al. 1996). The assay is based on the role of the *cII* protein in the commitment of bacteriophage λ^{+} to the lysogenic cycle in *hfl* mutant *E. coli*. Selection of mutant λcII is facilitated by using a *hfl* mutant *E. coli* strain (G1250) that extends the longevity of the *cII* product so that λ^{+} fail to form plaques. Following the similar approach used in Winn et al. 2008, DNA was mixed with *in vitro* packaging extracts to simultaneously excise and package the λ LIZ vector into viable bacteriophage. The packaged phage particles were then allowed to infect and lyses the *E. coli* host. To select λcII mutants, the packaged phage was mixed with *E. coli* cells, plated, and incubated at 24°C for 40 hr. The phage with wild-type *cII* remain indistinguishable in the *E. coli* lawn, whereas phage 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) per packaging mix on the selective screening plates by the titer of total λ^+ and *cII* phage on a permissive host. Analyses were statistically “blocked” by treatment for each round of *cII* mutation assays to account for any variability in assay conditions between separate analyses. DNA from untreated fish for which packaging efficiency and MFs was previously established was used in each assay to serve as an internal standard.

MUTATIONAL SPECTRA

To characterize the spectra of *cII* mutations carried by spermatozoa, a fraction of *cII*⁻ plaques were randomly chosen from the mutant screening plates from the selected treatments. Plaques were cored, purified individually on *E. coli* cells, followed by PCR sequencing using DNA sequencing methods previously described (Winn et al. 2000). Multiple mutations occurring at the same nucleotide in one animal were considered to be representative of clonal mutant cells from a mutant spermatogonial stem cell. Individual λ *cII*⁻ mutant plaques were cored at random from mutant screening plates, purified on G1250 *E. coli* cells, excised and amplified by PCR. PCR products were electrophoresed on a 1% agarose gel, purified (QIAquick PCR Purification kit; Qiagen, Valencia, CA), quantified, and diluted to 0.1 $\mu\text{g}/\mu\text{L}$ using Tris-EDTA. Diluted PCR 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 ANALYSIS

The average MFs from each treatment were compared using the COCHARM program (Troy Johnson, Proctor and Gamble, Cincinnati, OH), a modification of the generalized Cochran-Armitage test (Carr and Gorelick 1995). The spectra of mutations were compared among nine classes of mutations, using a program developed specifically for comparisons of mutation spectra (Cariello et al. 1994) based on the Monte Carlo method of Adams and Skopek (Adams and Skopek 1987). A value of $p < 0.05$ was considered significant in both types of analyses.

		Medaka	Mouse	Human	
DNA Repair Proficient	Stem cells	>30 days	>42 days	>100 days	Spermatozoa collected 32 days after treatment are derived from (pre-meiotic) stem cell spermatogonia exposed to the mutagen.
	Differentiating Spermatogonia	16-29	36-41	65-99	
	Spermatocytes	10-15	22-35	39-64	
	Round (early spermatids)	7-9	15-21	26-38	Spermatozoa collected 2 and 5 days after treatment are derived from (post-meiotic) sperm or spermatids exposed to the mutagen.
	Spermatids				
	Elongated (late spermatids)	4-6	8-14	16-25	
Spermatozoa	0-3	0-7	0-15		

Figure 1. Comparison of the post-treatment sampling intervals required for sampling mature spermatozoa exposed at earlier stages of development in medaka, rodents, and humans. The earlier in development the cells were during exposure, the longer the post-treatment period (adapted from (Favor and Neuhauser-Klaus 1994; Marchetti and Wyrobeck 2005; Shima and Shimada 1994; Singer et al. 2006).

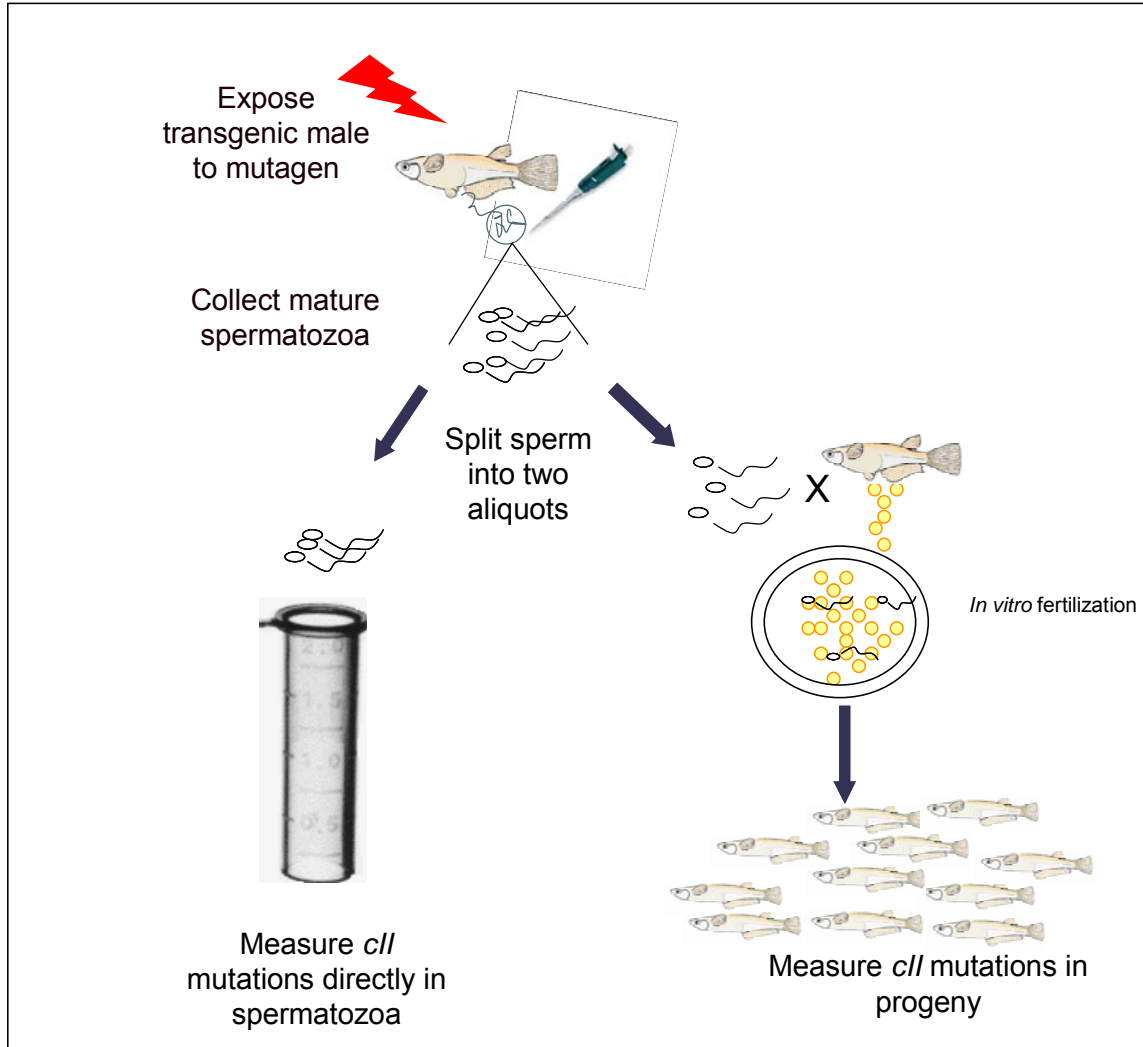


Figure 2. Schematic diagram outlining the method using λ transgenic medaka to compare the use of spermatozoa and progeny to characterize mutations transmitted from an ENU-exposed male transgenic parent.

RESULTS

INDUCED MF IN GERM-CELLS

Spermatozoa collected from 14 unexposed male medaka served as controls and displayed a mean MF of $1.5 (\pm 0.2) \times 10^{-5}$ (Table 1, Figure 3). Only samples with recoveries greater than 200,000 plaque forming units (PFU) were reported, resulting in unequal sample size at each treatment. We have shown that different germ-cell types have different susceptibilities. MFs in ENU exposed post-meiotic spermatozoa and late spermatids were not significantly different from control spermatozoa and showed average MFs of $1.7 (\pm 0.3) \times 10^{-5}$ (n=7) (p=0.1) and $2.4 (\pm 0.6) \times 10^{-5}$ (n=7) (p=0.07), respectively (Table 1, Figure 3). These results are consistent with transgenic rodent studies analyzing epididymal and vas deferential spermatozoa and late spermatids (Douglas et al. 1997; Douglas et al. 1995; Katoh et al. 1997; Provost et al. 1997; Singer et al. 2006; Suzuki et al. 1997; Van Delft and Baan 1995). The DNA replication and repair processes necessary for premutational ENU-induced DNA lesions fixed into mutations do not occur in these post-meiotic stages, and these results provide further support to the hypothesis that an induction of mutations require pre-meiotic processing of ENU-damage (Douglas et al. 1997; Favor 1999). A possible subtle induction of MF from 2 days to 5 days was observed, however these differences were not statistically significant. The timing of our collection of spermatozoa in this experiment (day 5 following two days of exposure) may allow some overlap with the next stage of

spermatogenesis, resulting in measurement of mutations in some early spermatids that undergo attempted DNA repair and accumulate mutations.

By comparison, ENU treated spermatogonial stem cells showed a significant 9-fold increase over controls with an average MF of $13.6 (\pm 2.6) \times 10^{-5}$ ($p=0.02$, $n=6$) (Table 1, Figure 3). Analysis of ENU exposed spermatogonial stem cells in transgenic rodent studies showed comparable results with significant increases in MFs ranging from 5-fold (Douglas et al. 1995) to 10-fold (Kato et al. 1997). The progression of spermatogonial stem cells to mature spermatozoa entails mitotic and meiotic divisions, providing the DNA replication and repair processes necessary for fixation of a DNA lesion into a mutation. Our data provide further support that the induction of mutations in spermatogonial stem cells is dependent upon processing of DNA damage in these rapidly dividing germ-cells.

MF IN GERM CELLS FROM INDIVIDUAL MALES

The MFs were evaluated in late spermatids, spermatocytes, and spermatogonial stem cells in 4 individual male medaka exposed to ENU and 2 unexposed males (Figure 4). Consistent with our first test, we did not measure a significant increase ($p=0.3$) in MF in mutagen exposed late spermatids ($2.3 (\pm 0.3) \times 10^{-5}$, $n=4$) when compared to spermatozoa collected prior to exposure and controls ($1.8 (\pm 0.3) \times 10^{-5}$, $n=11$). Spermatozoa analyzed 14 days post exposure, corresponding to exposed spermatocytes, showed a significant, 6-fold increase ($p=0.03$) in MF ($11.5 (\pm 3.1) \times 10^{-5}$, $n=4$), and spermatogonial stem cells show a significant, 10-fold increase ($p=0.02$) in MF ($17.2(\pm 3.6) \times 10^{-5}$, $n=4$). The MFs in spermatogonial stem cells and late spermatids are comparable to those measured in the first test. The high variability seen in ENU

exposed germ cells is likely due to biological variation in individuals, because it is not seen in the spermatozoa analyzed from the unexposed control males at each time point following exposure. With the exception of male 4, all of the exposed males showed the same relative rank in the increase in magnitudes of induced MFs at times post exposure corresponding to ENU-treated spermatocytes and spermatogonial stem cells.

COMPARISON OF PROGENY AND SPERMATOZOA

In studies using DL and SLT mutation assays and transgenic progeny, late spermatids and spermatozoa have been shown to be the most sensitive stages for the induction of mutations (Favor 1999; Winn et al. 2008). This contradicts our results from mutation analyses of spermatozoa and late spermatids. We measured MFs in directly in isolated spermatozoa from Males A and B (Figure 5). In Male A, spermatozoa collected prior to exposure showed MF of 1.2×10^{-5} and those collected 5 days following ENU exposure showed a MF of 1.7×10^{-5} . In Male B, spermatozoa collected prior to exposure showed MF of 0.9×10^{-5} and those collected 5 days following ENU exposure showed a MF of 1.1×10^{-5} . Although analysis of isolated spermatozoa from two individuals does not provide sufficient power to test for significant differences, these values are comparable to previous analyses using isolated spermatozoa from larger numbers of ENU exposed individuals, in which MFs of controls and spermatozoa collected 5-days post-exposure were not significantly different.

The number of progeny generated from each male parent varied from 14 to 20 individuals per treatment, and was dependent upon *in vitro* fertilization success. The average MF for 18 control offspring from Male A (Figure 6) was $2.5 (\pm 0.2) \times 10^{-5}$, and control offspring for Male B (Figure 7) demonstrated an average MF of $2.2 (\pm 0.3) \times 10^{-5}$

(n= 14). The threshold MF used to score offspring as a mutant was 5.0×10^{-5} , representing a 2-fold increase in the average MF in the control offspring. MFs from control progeny ranged from 0.9 to 4.8×10^{-5} , and none of the control progeny had MFs above the threshold.

In an evaluation of 19 progeny collected 5 days after ENU exposure, Male A (Figure 6) exhibited a MOF of 11% (2/19). The MF values in the 19 progeny ranged from 0.5 to 90.8×10^{-5} . The majority of progeny (89%, 17/19 offspring) exhibited MFs comparable to those collected prior to ENU exposure. The two progeny scored as mutants exhibited MFs from 1.5-fold to 18-fold (7.3 and 90.8×10^{-5}) above the threshold. Twenty progeny, collected 5 days after ENU exposure, from Male B (Figure 7) showed MF values 1.1 to 325.6×10^{-5} . The majority of progeny (70%, 14/20 offspring) exhibited MFs comparable to progeny collected prior to ENU exposure. Male B exhibited a MOF of 30%, with six progeny exhibiting MFs (ranging from 5.1 to 325.6×10^{-5}) above the threshold. Winn et. al (2008) measured MOFs ranging from 3.5 to 20%, consistent with results here.

Table 1. Isolated spermatozoa collected 2 days, 5 days, or 32 days following ENU treatment of males corresponding to spermatozoa, late spermatids, and stem cell spermatogonia.

Germ-cell Stage (days post-exposure)	<i>cII</i> Mutants	PFU	MF x 10 ⁻⁵	AVG MF x 10 ⁻⁵	+/- SEM
Controls (0)	7	355,000	2.0		
	21	1,260,000	1.7		
	4	305,000	1.3		
	2	445,000	0.4		
	13	535,000	2.4		
	14	660,000	2.1		
	5	805,500	0.6		
	34	920,000	3.7		
	18	1,680,000	1.1		
	10	1,055,000	0.9		
	13	1,815,000	0.7		
	9	1,145,000	0.8		
	35	1,705,000	2.1		
20	1,880,000	1.1	1.5	0.2	
ENU Exposed Spermatozoa (2)	3	295,000	1.0		
	13	1,065,000	1.2		
	30	1,360,000	2.2		
	20	785,000	2.5		
	22	1,280,000	1.7		
	2	255,000	0.8		
14	690,000	2.0	1.7	0.3	
ENU Exposed Late Spermatids (5)	11	745,000	1.5		
	38	1,285,000	3.0		
	18	320,000	5.6		
	2	350,000	0.6		
	43	1,605,000	2.7		
	9	315,000	2.9		
14	1,515,000	0.9	2.4	0.6	
ENU Exposed Stem Cell Spermatogonia (32)	107	530,000	20.2		
	333	1,630,000	20.4		
	39	440,000	8.9		
	28	468,000	6.0		
	28	300,000	9.3		
58	340,000	17.1	13.6*	2.6	

* Significant p=0.03

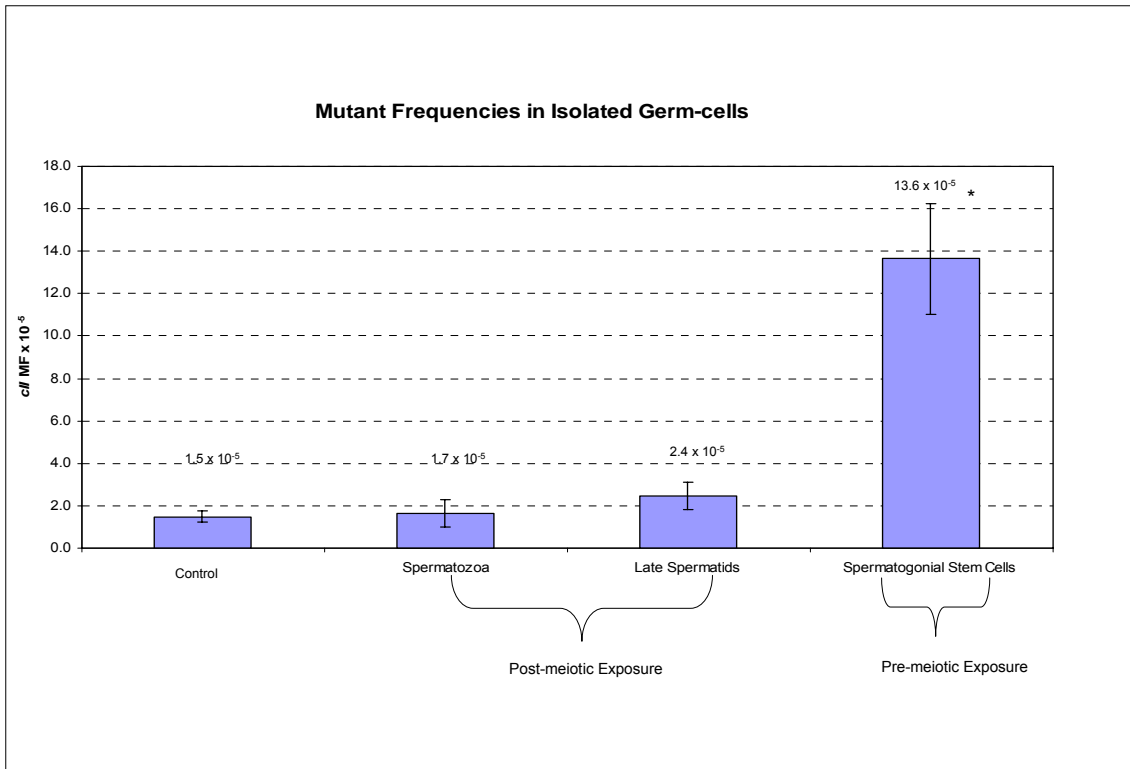


Figure 3. MFs in ENU exposed post-meiotic spermatozoa and late spermatids, and pre-meiotic stem cell spermatogonia. MFs in spermatozoa and late spermatids were not significantly different from controls ($p=0.11$ and 0.07 , respectively). MFs in stem cell spermatogonia showed a significant ($p=0.02$), 9-fold increase over untreated samples.

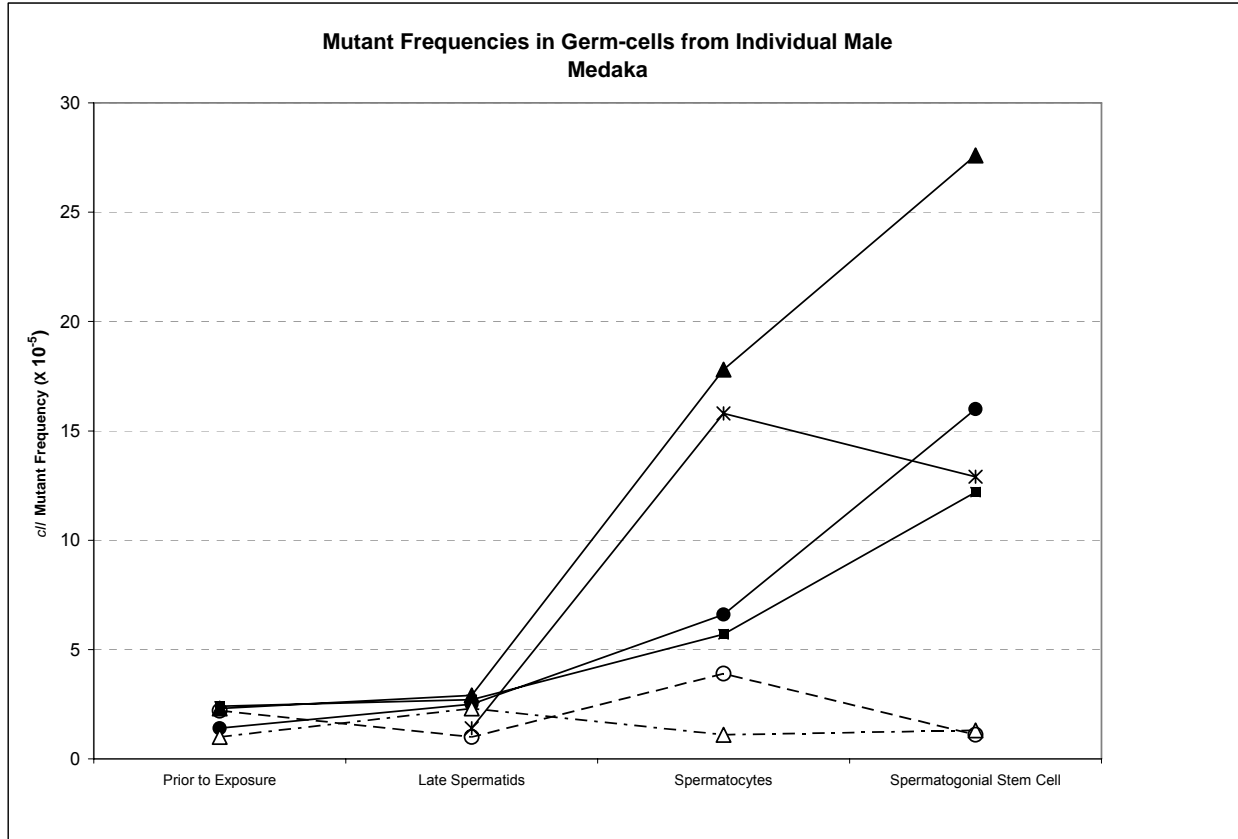


Figure 4. Comparison of the relative responsiveness of different germ cells in individual males. Males 1 -▲-, 2 -■-, 3 -●-, and 4 -*- were exposed to ENU, Males 5 -○- and 6 -△- were unexposed. MF in late spermatids did not show a significant increase ($p=0.32$). Each of the pre-meiotic germ-cell stages showed significant inductions of mutations with a measured a 6-fold increase ($p=0.03$) in spermatocytes and a 10-fold increase ($p=0.02$) in spermatogonial stem cells exposed to ENU.

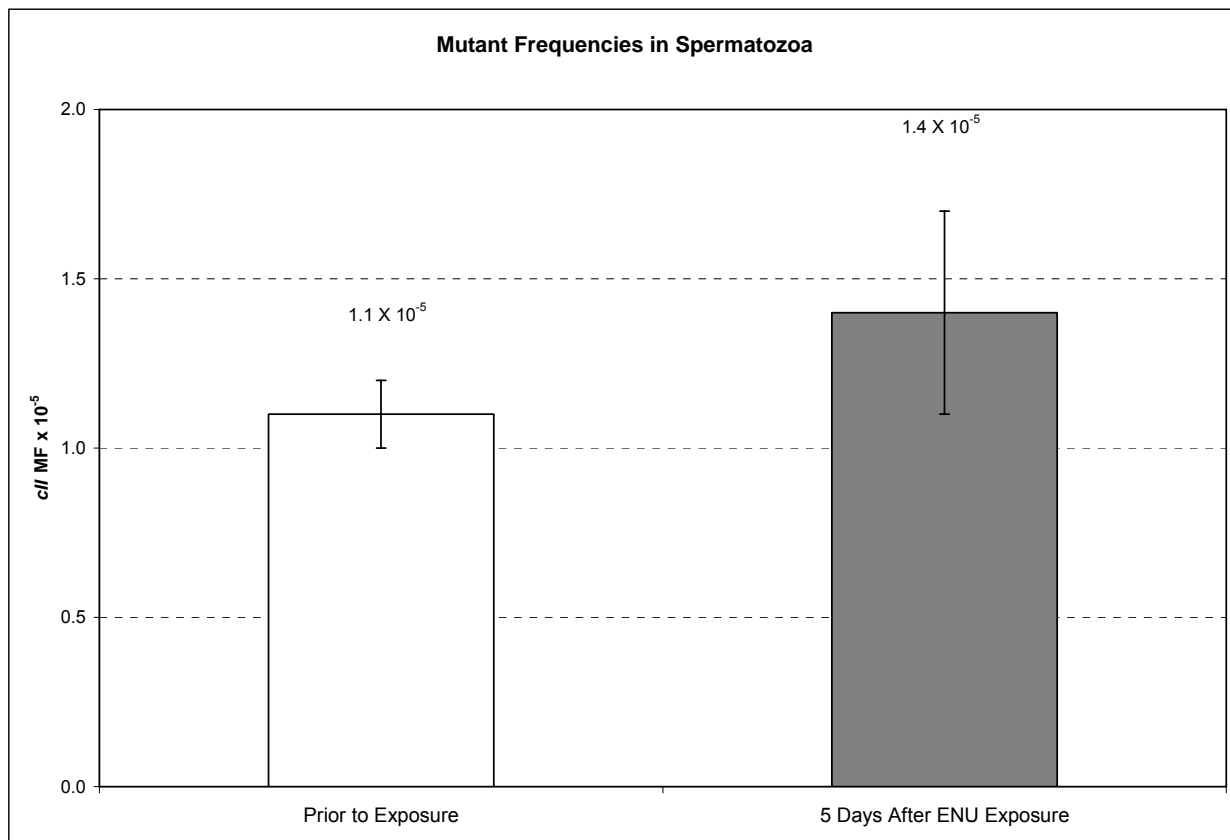


Figure 5. Average MFs measured directly in isolated spermatozoa from males A and B in the spermatozoa and progeny comparison. Spermatozoa collected prior to exposure showed an average MF of $1.1 (\pm 0.1) \times 10^{-5}$, and those collected 5 days following ENU exposure showed an average MF of $1.4 (\pm 0.3) \times 10^{-5}$.

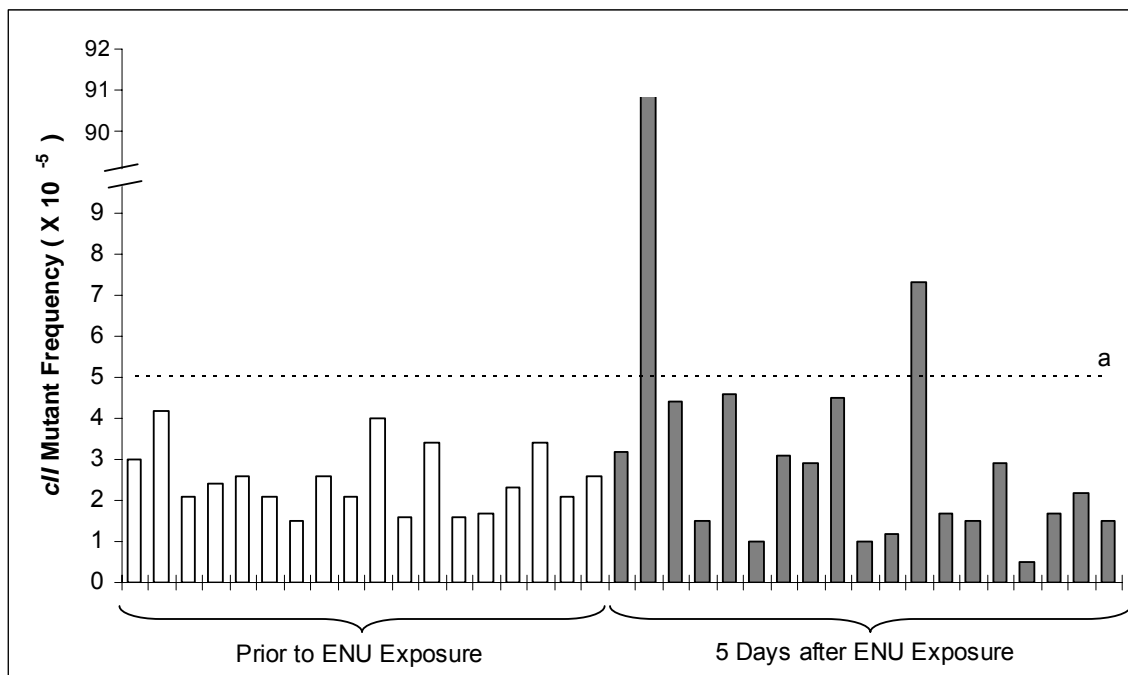


Figure 6. MFs in progeny from Male A collected prior to and 5 days after ENU exposure. Offspring collected prior to exposure had an average MF= $2.5 (\pm 0.2) \times 10^{-5}$ (n= 18). The threshold MF (a) used to determine a mutant offspring was 5.0×10^{-5} and corresponded to a significant two-fold MF induction compared to controls. Eleven percent (2/19) of the offspring collected following mutagen exposure were mutants (MOF= 11%).

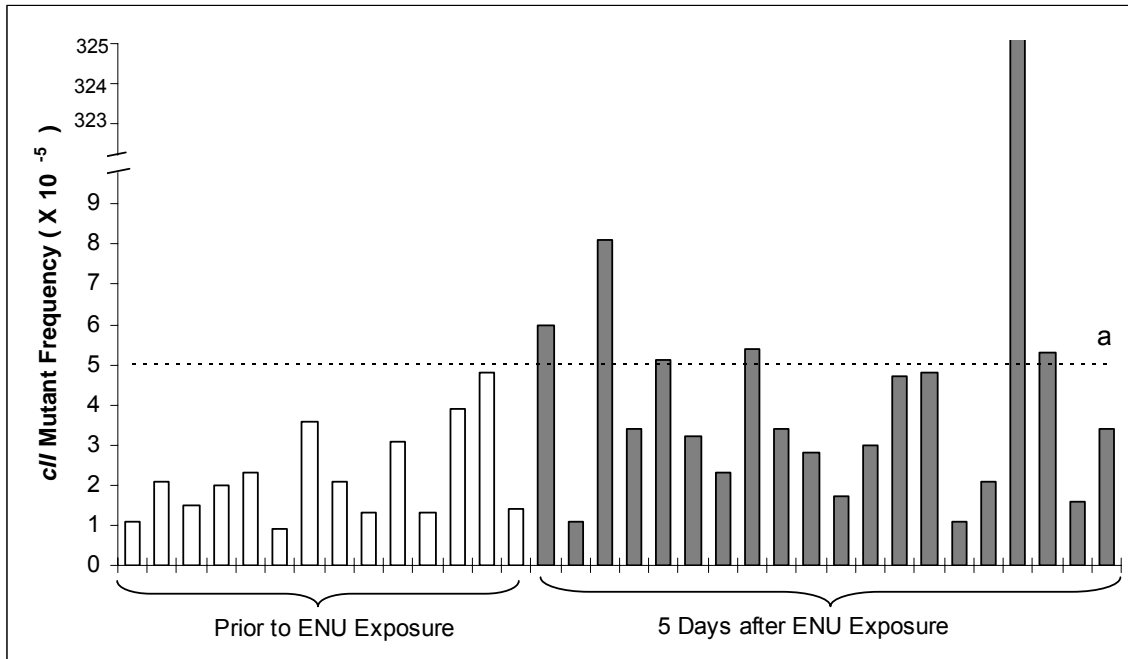


Figure 7. MFs in progeny from Male B collected prior to and 5 days after ENU exposure. Offspring collected prior to exposure had an average MF= $2.2 (\pm 0.3) \times 10^{-5}$ (n= 14). The threshold MF (a) used to determine a mutant offspring was 5.0×10^{-5} and corresponded to a significant two-fold MF induction compared to controls. Thirty percent (6/20) of the offspring collected following mutagen exposure were mutants (MOF= 30%).

MUTATIONAL SPECTRA

We performed sequence analyses to identify mutations from representative mutant λ *cII* plaques from unexposed and ENU exposed spermatogonial stem cells. Twelve plaques were analyzed from controls. Three identical mutations suggestive of clonal mutant cells from a mutant spermatogonial stem cell were identified in untreated spermatozoa. These were +1 insertions within the homonucleotide run of guanosines (sense strand nucleotides 179-184), a known mutation hotspot (Harbach et al. 1999; Shane et al. 1997; Watson et al. 1998; Winn et al. 2000). Of the remaining 9 independent mutations, one sequence contained a complex triple deletion of TCT at nucleotides 169-171. Single base substitutions were the most numerous mutations (78%), with 5 G:C-A:T transitions comprising the majority (56%) of the mutations, all of which occurred at CpG dinucleotides. Cytosine methylation at CpG sites presumably leads to G:C-A:T transitions during replication (Douglas et al. 1995; Hayward et al. 1995; Piergorsch et al. 1995; Provost et al. 1993; Provost and Short 1994; Shane et al. 1997). The high percentage of transitions at CpG sites coupled with a predominance of single base substitutions measured spontaneously in spermatozoa are consistent with those seen in somatic tissues of *lacI* and *lacZ* transgenic mice, and λ transgenic medaka (de Boer et al. 1997; Douglas et al. 1994; Hayward et al. 1995; Piergorsch et al. 1995; Provost et al. 1993; Shane et al. 1997; Winn et al. 2000), and in germ cells of *lacI* and *lacZ* transgenic mice (Douglas et al. 1995; Provost and Short 1994).

Twenty-six mutations from mutant λ *cII* plaques were characterized from spermatogonial stem cells exposed to ENU. One mutation found outside the *cII* protein-coding region and 2 duplicate mutations suggestive of clonal mutant cells from a mutant

spermatogonial stem cell were excluded from analysis. As observed in the controls, these identical mutations were +1 insertions within the homonucleotide run of guanosines (sense strand nucleotides 179-184). Of the remaining 19 independent mutations, 37% (7 mutants) were A:T-C:G transitions, followed by 21% (4 mutants) A:T-T:A transversions. Fifty-eight percent of the ENU induced mutations occurred at A:T base pairs, in contrast to controls, in which only 11% of the mutations were at A:T base pairs. The increase in the proportion of mutations at A:T base pairs is highly characteristic of ENU exposure, and has been demonstrated in both somatic and germ cells in *lacI* and *lacZ* transgenic mice, and λ transgenic medaka (Douglas et al. 1995; Provost and Short 1994; Walker et al. 1996; Watson et al. 1998; Winn et al. 2000). The accumulation of mutations at A:T base pairs is likely a result of ENU induced O⁴-ethylthymine and O²-ethylthymine adducts, respectively (Douglas et al. 1995; Provost and Short 1994). Four of the mutants (21%) were G:C-A:T transitions, of which 75% occurred at CpG dinucleotides. Of the remaining mutations, 3 (16%) were G:C-T:A transversions, and 1 (5%) was a frameshift.

Table 2. *cII* mutational spectra in isolated spermatozoa from control and ENU treated λ transgenic medaka.

	Spermatozoa Collected Prior to Exposure			Spermatozoa Collected 32 Days After ENU Exposure*		
	Mutants	%	<i>cII</i> mutation frequency ($\times 10^{-5}$)	Mutants	%	<i>cII</i> mutation frequency ($\times 10^{-5}$)
Transitions						
G/C \rightarrow A/T	5	55.6	0.8	4	21.0	2.9
% at CpG sites		100%			75%	
A/T \rightarrow G/C	1	11.1	0.2	7	36.8	5.0
Transversions						
G/C \rightarrow T/A	1	11.1	0.2	3	15.8	2.2
G/C \rightarrow C/G	0	0	0	0	0	0
A/T \rightarrow T/A	0	0	0	4	21.0	2.9
A/T \rightarrow C/G	0	0	0	0	0	0
Other mutations						
frameshift (+1)	1	11.1	0.2	1	5.3	0.7
frameshift (-1)	0	0	0	0	0	0
complex	1	11.1	0.2	0	0	0
Total	9	100	1.5	19	100	13.6

*Significant $p=0.00001$

DISCUSSION

This study illustrates the complexity in detecting mutations transmitted to progeny from mutagen exposed parents. Spermatogenesis and the timing of exposure, the varying susceptibilities of germ-cells, and the processes occurring at or immediately following fertilization, all play important roles in contributing to the frequencies and types of mutations transmitted from a mutagen exposed parent (Aitken and Bennetts 2006; Favor 1999; Marchetti and Wyrobeck 2005; Singer et al. 2006; Winn et al. 2008). As seen in rodent studies, analysis of mutations directly in isolated spermatozoa showed different stages of germ cells in medaka vary in susceptibility to mutagen exposure (Douglas et al. 1997; Douglas et al. 1995; Provost et al. 1990; Provost and Short 1994; Singer et al. 2006). When measuring mutations directly in isolated spermatozoa, we did not measure significant induction of mutations in exposed spermatozoa and late spermatids. However, we did measure a significant induction of mutations in exposed spermatocytes and spermatogonial stem cells. By contrast, when measuring mutations in progeny, we measured significant increases in mutant offspring derived from both ENU-exposed late spermatids and stem cell spermatogonia.

An explanation for why significantly elevated MFs were detected in progeny derived from ENU-treated post-meiotic germ cells, but not in isolated spermatozoa is related to whether the DNA damage persists or whether it is fixed as a mutation that is detected by the *cII* mutation assay. In considering that spermatozoa are non-dividing cells, and are deficient in DNA repair, it follows that any lesions carried by these cells

will persist until acted upon by processes contributed by the maternal genome of the zygote. Further, because the *cII* mutation assay detects only mutations, (i.e. changes in the *cII* sequences rather than pre-mutagenic lesions or other damage), DNA damage other than mutations in spermatozoa would not be detected using this assay. In progeny analyses by contrast, the explanation for why elevated frequencies of *cII* mutations in progeny derived from ENU-exposed post-meiotic germ cells are detected is related to these same DNA repair or attempted repair processes available in the fertilized egg. The DNA damage carried by spermatozoa has been suggested to trigger the oocyte to repair the damage and induce mutations in the developing embryo (Aitken and Bennetts 2006; Douglas et al. 1995; Marchetti and Wyrobeck 2005; Singer et al. 2006; Winn et al. 2008). Consistent with the results seen here, Douglas *et al.* (1995) also noted a lack of significant increase in post-meiotic spermatozoa in *lacZ* transgenic mice in contrast to the high proportion of mosaic-specific locus mutations observed in the progeny of male mice derived from ENU exposed post-meiotic germ cells (Douglas et al. 1995; Favor et al. 1990). The specific locus test has been shown to detect peak mutagenic activity in the post-spermatogonial stages (Singer et al. 2006). Here, we showed ENU-exposed spermatozoa and late spermatids did not exhibit significant induction of mutations, indicating that the DNA damage was not fixed as mutations. By comparison, analysis of progeny derived from those germ cells exposed to ENU showed a significant 21% increase in mutant offspring.

Although analyses of isolated spermatozoa from ENU exposed stem cell spermatogonia and spermatocytes showed significantly induced MFs, nevertheless, the actual magnitude of the mutations transmitted to progeny will be underestimated using

this approach. Analysis of *c//* MF directly in isolated spermatozoa does not provide an estimate of DNA damage in spermatozoa that will contribute to the production of mosaic mutant offspring. Mosaic mutant offspring carry mutations in a portion of their cells, and are produced by fixation of DNA damage as a mutation in cell divisions subsequent to fertilization. A number of studies have shown mosaic mutant offspring have been produced from mutagen-exposed post-meiotic germ cells (Favor 1999; Russell et al. 1991; Shima and Shimada 1994). In particular, mosaic mutant offspring have recently been shown to make up the predominant proportion (80%) of mutant offspring from exposure of both pre-meiotic and post-meiotic germ cells in λ transgenic medaka (Winn et al. 2008).

Sequence analyses suggest that spontaneous and ENU exposed mutational spectra in isolated spermatozoa corresponding to mutagen exposed stem cell spermatogonia may be similar to those measured in both somatic tissues and germ-cells in rodents and fish. However, the data presented here is limited, and more analyses will be needed to confirm these results. More importantly, analyses of mutational spectra in isolated spermatozoa may have limited utility for estimating the genetic risk to progeny from mutagen exposed parents. Sequence analysis in isolated spermatozoa may provide insights into possible mechanisms of ENU, but it does not help to predict the numbers or types of mutations transmitted to offspring. Mutational spectral analysis of ENU induced mutations in isolated spermatozoa represents only the portion of mutations that would be manifested in whole body mutant offspring. The mutations in mutant offspring generated from aberrant repair of DNA damage carried by spermatozoa are not likely represented by sequence analysis of isolated spermatozoa.

Because these mutations are likely formed by DNA repair processes contributed by the maternal genome in the zygote, the mutations characterized in isolated spermatozoa are not likely to be similar to those in mutant progeny. Since mosaic mutant offspring comprise a significant portion of the mutant offspring from ENU exposed male parents, mutational spectra obtained from isolated spermatozoa will not likely represent the mutations transmitted to mutant offspring.

Based on the results from this study and those obtained from using other models (Aitken and Bennetts 2006; Douglas et al. 1995; Marchetti and Wyrobeck 2005; Winn et al. 2008), we can identify two mechanisms by which male germ cells contribute mutations in offspring. These mechanisms differ based on whether the mutagen exposed male germ cells give rise to spermatozoa carrying DNA damage that has been fixed as a mutation **prior to/at** fertilization or **after** fertilization. Analysis of mutations using isolated spermatozoa will only provide an estimate of that proportion of the total mutant offspring generated from DNA damage that was already fixed as a mutation. Mutant offspring generated from DNA damage carried by spermatozoa that was fixed as a mutation after fertilization will not be detected. Analysis of mutations using isolated spermatozoa is an indirect approach to estimate the frequency of mutations transmitted to progeny, and provides only a subset of the potential genetic damage that can be manifested in the progeny. As a consequence, studies using spermatozoa must be designed and conducted taking into account these mechanisms, to ensure that isolated spermatozoa provide a reliable alternative to progeny studies.

Mutant offspring, whether generated from direct or indirect induction of mutations, carry mutational loads which may result in consequences such as cancer,

genetic diseases, infertility, or death (Aitken and Bennetts 2006). Transgenic animal mutation models have provided a better understanding of the mechanisms by which mutant offspring are generated and have allowed a more detailed scrutiny of the efficacy of using isolated spermatozoa to determine the risks of mutations transmitted to progeny from mutagen exposed male parents. Analysis of mutations directly in isolated spermatozoa is an extremely practical and efficient tool, and, if the caveats related to differences in sensitivity are understood, provides a high-throughput method for estimating genetic risk to progeny of mutagen exposed male parents. To use isolated spermatozoa to measure mutations transmitted from mutagen exposed parents to progeny, mature spermatozoa must be sampled at the appropriate sampling interval following mutagen exposure representing the early pre-meiotic germ cell stages (spermatocytes and spermatogonial stem cells) exposed to the mutagen. In medaka that would correspond to a period of at least 14 days following exposure before the sampling of spermatozoa. Analysis of mutations in spermatozoa collected at earlier time points will underestimate the frequency of mutant offspring. Depending upon the research application, the use of spermatozoa offers speed and practical benefits at the expense of precision in estimating the magnitude of genetic health risks to progeny of mutagen exposed male parents.

REFERENCES

- Adams WT, Skopek TR. 1987. Statistical test for the comparison of samples from mutational spectra. *J. Mol. Biol.* 194:391-396.
- Adler ID. 1996. Future research directions to study genetic damage in germ cells and estimate genetic risk. *Environ. Health Perspect.* 104(Suppl 3):619-624.
- Aitken JR, Bennetts L. 2006. Reactive oxygen species: friend or foe. In: De Jonge C, Barratt C, editors. *The Sperm Cell*. New York: Cambridge University Press. p 170-193.
- Amanuma K, Takeda H, Amanuma H, Aoki Y. 2000. Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. *Nat. Biotechnol.* 18:62-65.
- Ando s, Wakamatsu Y. 1995. Production of chimeric medaka (*Oryzias latipes*). *Fish Biol. J. Medaka* 7:65-68.
- Ashby J, Gorelick NJ, Shelby MD. 1997. Mutation assays in male germ cells from transgenic mice: overview of study and conclusions. *Mutation Research* 388:111-122.
- Barnett LB, Tyl RW, Shane BS, Shelby MD, Lewis SE. 2002. Transmission of Mutations in the *lacI* Transgene to the Offspring of ENU-Treated Big Blue® Mice. *Environmental and Molecular Mutagenesis*(40):251-257.

- Boerrigter M, Dolle M, Martus H, Gossen JA, Vijg J. 1995. Plasmid based transgenic mouse model for studying *in vivo* mutations. *Nature* 377:657-659.
- Burkhart JG, Winn RN, Van Beneden RJ, Malling HV. 1993. Spontaneous and induced mutagenesis in transgenic animals containing FX174. *Environ. Mol. Mutant.* 21 (Suppl. 12):9.
- Cachot J, Law M, Pottier D, Peluhet L, Norris M, Budzinski H, Winn R. 2007. Characterization of toxic effects of sediment-associated organic pollutants using λ transgenic medaka. *Environ. Sci. Tech.* 41(22).
- Cariello NF, Piergorsch WW, Adams WT, Skopek TR. 1994. Computer program for the analysis of mutational spectra: application to p53 mutations. *Carcinogenesis* 15(10):2281-2285.
- Carr GJ, Gorelick NJ. 1995. Statistical design and analysis of mutation studies in transgenic mice. *Environ. Mol. Mutagen.* 25:246-255.
- de Boer JG, Erfke H, Walsh D, Holcroft J, Provost JS, Rogers B, Tindall KR, Glickman BW. 1997. Spectrum of spontaneous mutations in liver tissue of *lacI* transgenic mice. *Environmental and Molecular Mutagenesis* 30:273-286.
- Douglas GR, Gingerich JD, Gossen JA, Bartlett SA. 1994. Sequence spectra of spontaneous *lacZ* gene mutations in transgenic mouse somatic and germline tissues. *Mutagenesis* 9:451-458.
- Douglas GR, Gingerich JD, Soper LM, Jiao J. 1997. Toward an understanding of the use of transgenic mice for the detection of gene mutations in germ cells. *Mutation Research* 388:197-212.

- Douglas GR, Jiao J, Gingerich JD, Gossen JA, Soper LM. 1995. Temporal and molecular characteristics of mutations induced by ethylnitrosourea in germ cells isolated from seminiferous tubules and in spermatozoa of *lacZ* transgenic mice. Proc. Natl. Acad. Sci. USA 92:7485-7489.
- Egami N, Hyodo-Taguchi Y. 1967. An autoradiographic examination of rate of spermatogenesis at different temperatures in the fish *Oryzias latipes*. Exp. Cell Res. 47(665-667).
- Favor J. 1999. Mechanisms of mutation induction in germ cells of the mouse as assessed by the specific locus test. Mutation Research 428:227-236.
- Favor J, Neuhauser-Klaus A. 1994. Genetic mosaicism in the house mouse. Annu. Rev. Genet. 28:27-47.
- Favor J, Neuhauser-Klaus A, Ehling UH. 1990. The frequency of dominant cataract and recessive specific-locus mutations and mutation mosaics in F1 mice derived from post-spermatogonial treatment with ethylnitrosourea. Mutation Research 229:105-114.
- Generoso WM, Bishop JB, Gosslee DG, Newell GW, Sheu CJ, Halle Ev. 1980. Heritable translocation test in mice. Mutat. Res. 76:191-215.
- Gossen JA, De Leeuw WJF, Tan CHT, Zwarthoff EC, Berends F, Lohman PHM, Knook DL, Vijg J. 1989. Efficient rescue of integrated shuttle vectors from transgenic mice: a model for studying mutations *in vivo*. Proc. Natl. Acad. Sci. USA 86:7971-7975.

- Harbach PR, Zimmer DM, Filipunas AL, Mattes WB, Aaron CS. 1999. Spontaneous mutation spectrum at the lambda *cII* locus in liver, lung, and spleen tissue of Big Blue® transgenic mice. *Environ. Mol. Mutagen.* 33:132-143.
- Hayward JJ, Shane BS, Tindall KR, Cunningham ML. 1995. *In vivo* mutant frequency of *lacI* in liver DNA of transgenic mice following dietary exposure to the carcinogen-non-carcinogen pair 2,4,- and 2,6-diaminotoluene. *Carcinogenesis* 16:2429-2433.
- Iwamatsu T. 1983. A new technique for dechoriation and observations on the development of the naked egg in *Oryzias latipes*. *Zool. Sci.* 11:825-839.
- Jakubczak JL, Merlina G, French JE, Muller WJ, Paul B, Adhya S, Garges S. 1996. Analysis of genetic instability during mammary tumor progression using a novel selection-based assay for *in vivo* mutations in a bacteriophage I transgene target. *Proc. Natl. Acad. Sci. USA* 93:9073-9078.
- Katoh M, Horiya N, Valdivia RPA. 1997. Mutations induced in male germ cells after treatment of transgenic mice with ethylnitrosourea. *Mutat. Res.* 388:229-237.
- Kohler SW, Provost GS, Fieck A, Kretz PL, Bullock WO, Sorge JA, Putman DL, Short JM. 1991. Spectra of spontaneous and mutagen-induced mutations in the *lacI* gene in transgenic mice. *Proc. Natl. Acad. Sci. USA* 88:7958-7962.
- Manjanatha MG, Chen JB, Shaddock JGJ, Harris AJ, Shelton SD, Casciano DA. 1996. Molecular analysis of *lacI* mutations in Rat2™ cells exposed to 7, 12-dimethylbenz[a]anthracene: evidence for DNA sequence and DNA strand biases for mutation. *Mutat. Res.* 372:53-64.

- Marchetti F, Wyrobeck AJ. 2005. Mechanisms and consequences of paternally transmitted chromosomal abnormalities. *Birth Defects Res. C. Embryo Today* 75:112-129.
- McElroy A, Bogler A, Weisbaum D, Norris M, Mendelman L, Setlow R, Winn R. 2006. Uptake, metabolism, mutant frequencies and mutational spectra in lambda transgenic medaka embryos exposed to benzo(a)pyrene dosed sediments. *Marine Environmental Research* 62:S273-S277
- Piergorsch WW, Margolin BH, Shelby NM, Johnson A, French JE, Tennant RW, Tindall KR. 1995. Study design and sample sizes for *lacI* transgenic mouse mutation assay. *Environ. Mol. Mutant.* 25:231-245.
- Provost GS, Kohler SW, Fieck A, Kretz PL, Molina T, Short JM. 1990. Short-term germ line and somatic cell mutagenesis testing with *LacI* lambda phage shuttle vectors in transgenic mice. *Strategies in Molecular Biology* 4(4):55-56.
- Provost GS, Kretz PL, Hammer RT, Matthews CD, Rogers BJ, Lundberg KS, Dyaico MJ, Short JM. 1993. Transgenic systems for *in vivo* mutation analysis. *Mutat. Res.* 288:133-149.
- Provost GS, Rogers BJ, Dyaico MJ, Carr G. 1997. Evaluation of the transgenic Lambda/*LacI* mouse model as a short-term predictor of heritable risk. *Mutat. Res.* 388:129-136.
- Provost GS, Short JM. 1994. Characterization of mutations induced by ethylnitrosourea in seminiferous tubule germ cells of transgenic B6C3F₁ mice. *Proc. Natl. Acad. Sci. USA* 91:6564-6568.

- Russell LB. 2004. Effects of male germ-cell stage on the frequency, nature, and spectrum of induced specific-locus mutations in the mouse. *Genetica* 122:25-36.
- Russell LB, Hunsicker PR, Cacheiro NLA, Generoso WM. 1991. Induction of specific-locus mutations in male germ cells of the mouse by acrylamide monomer. *Mutation Research* 262:101-107.
- Russell LB, Matter B. 1980. Whole-mammal mutagenicity tests: evaluation of five methods. *Mutat. Res.* 75:279-302.
- Russell LB, Selby PB, Halle Ev, Sheridan W, Valcovic L. 1981. The mouse specific-locus test with agents other than radiations: interpretation of data and recommendations for future work. *Mutat. Res.* 86:329-354.
- Saiki A, Tamura M, Matsumoto M, Katowgi J, Watanabe A, Onitake K. 1997. Establishment of *in vitro* spermatogenesis from spermatocytes in the medaka, *oryzias latipes*. *Develop. Growth Differ.* 39:337-344.
- Shane BS, Lockhart A-MC, Winston GW, Tindall KR. 1997. Mutant frequency of lacI in transgenic mice following benzo[a]pyrene treatment and partial hepatectomy. *Mutat. Res.* 377:1-11.
- Shima A, Shimada A. 1994. The japanese medaka, *Oryzias latipes*, as a new model organism for studying environmental germ-cell mutagenesis. *Environ. Health Perspect.* 102 (Suppl. 12):33-35.
- Singer TM, Lambert IB, Williams A, Douglas GR, Yauk CL. 2006. Detection of induced male germline mutation: Correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays. *Mutation Research* 598:164-193.

- Suzuki T, Itoh S, Takemoto N, Yajima N, Miura M, Hayashi M, Shimada H, Sofuni T. 1997. Ethyl nitrosourea and methyl methasulfonate mutagenicity in sperm and testicular germ cells of *lacZ* transgenic mice (MutaTMMouse). *Mutat. Res.* 388:155-163.
- Van Delft JHM, Baan RA. 1995. Germ cell mutagenesis in *lacZ* transgenic mice treated with ethylnitrosourea; comparison with specific-locus test. *Mutagenesis* 10(3):209-214.
- Walker VE, Gorelick NJ, Andrews JL, Craft TR, De Boer JG, Glickman BW, Skopek TR. 1996. Frequency spectrum of ethylnitrosourea-induced mutation fixation and molecular spectra at the *hprt* and *lacI* loci in splenic lymphocytes of exposed *lacI* transgenic mice. *Cancer Research* 56:4654-4661.
- Watson DE, Cunningham ML, Tindall KR. 1998. Spontaneous and ENU-induced mutation spectra at the *cII* locus in Big Blue Rat2 embryonic fibroblasts. *Mutagenesis* 13(5):487-497.
- Winn RN, Majeske AJ, Jagoe CH, Glenn TC, Smith MH, Norris MB. 2008. Transgenic λ Medaka as a New Model for Germ Cell Mutagenesis. *Environmental and Molecular Mutagenesis* 49:173-184.
- Winn RN, Norris M, Muller S, Torres C, Brayer K. 2001. Bacteriophage λ and plasmid pUR288 transgenic fish models for detecting *in vivo* mutations. *Mar. Biotech.* 3:S185-S195.
- Winn RN, Norris MB. 2005. Analysis of mutations in λ transgenic medaka using the *cII* mutation assay. In: Ostrander GK, editor. *Techniques in Aquatic Toxicology* 2nd edition. 2nd ed. ed. New York: CRC Press. p 705-734.

- Winn RN, Norris MB, Brayer KJ, Torres C, Muller SL. 2000. Detection of mutations in transgenic fish carrying a bacteriophage λ *cII* transgene target. *Proc. Natl. Acad. Sci.* 97(23):12655-12660.
- Winn RN, Norris MB, Turner J, Kimlin M. 2005. Assessment of lambda transgenic fish as a new model of UV mutagenesis. *Transactions on Ecology and the Environment* 85:205-213.
- Winn RN, Van Beneden RJ, Burkhart JG. 1995. Transfer, methylation and spontaneous mutation frequency of fX174*am3cs70* sequences in Medaka (*Oryzias latipes*) and Mummichog (*Fundulus heteroclitus*): implications for gene transfer and environmental mutagenesis in aquatic species. *Mar. Environ. Res.* 40(3):247-265.