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Isolated spermatozoa as indicators of mutations transmitted to progeny

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Abstract

Spermatozoa comprise a large and homogeneous population of cells that may serve as an alternative to resource-intensive assays of transmissible mutations based on progeny. To evaluate mutagenic responses in spermatozoa derived from germ cells exposed to a mutagen at different stages of spermatogenesis, we compared *cII* mutant frequencies (MFs) in spermatozoa collected from male λ transgenic medaka exposed to ethylnitrosourea (ENU) as either post-meiotic or pre-meiotic germ cells. *cII* MFs in spermatozoa exposed to ENU as spermatogonial stem cells were induced significantly, 9-fold, compared to controls, whereas, *cII* MFs in spermatozoa exposed as spermatozoa/late spermatids were not elevated. To directly compare responses in spermatozoa with those in progeny, we analyzed *cII* MFs directly in spermatozoa and in the offspring produced from identical sperm samples of ENU-exposed males. *cII* MFs in isolated spermatozoa exposed to ENU as post-meiotic germ cells were not significantly elevated, whereas 11–30% of the progeny derived from the identically exposed germ cells exhibited significantly elevated *cII* MFs, ~2-fold to >130-fold, compared to controls. The contradictory responses between spermatozoa and progeny analyses can be attributed to induced pre-mutational lesions that remain intact in spermatozoa but were not detected as mutations. Progeny analyses, by contrast, revealed mutant individuals with elevated *cII* mutant frequencies because persistent DNA damage in the spermatozoa was fixed as mutations in cells of the early stage embryo. Spermatozoa exposed to a mutagen as spermatogonial stem cells can provide an efficient means to detect the portion of transmissible mutations that were fixed as mutations in spermatozoa. The caveat is that direct analyses of mutations in spermatozoa excludes the contribution of mutations that arise from post-fertilization processes in cells of early stage embryos, and therefore may underestimate the actual frequency of mutant offspring.

1. Introduction

Characterization of adverse health effects in progeny of mutagen exposed parents remains a complex and challenging task. Mutations may be transmitted from the parent at exceptionally low frequencies, and require highly efficient and sensitive methods of detection. Routine analyses of transmissible mutations based almost exclusively on using traditional mouse assays such as the dominant lethal assay (DL) [1], the morphological specific locus test (STL) [2], and the heritable translocation assay (HT) [3], have provided the foundation for current

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Conflict of Interest

The authors declare there are no conflicts of interest.

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understanding of germline mutagenesis. However, limitations of these models, such as the reliance on detection of phenotypic changes among large numbers of progeny which exhibit low frequencies of spontaneous mutations and require extensive resources, contribute to making these approaches impractical for routine use [4]. Consequently, there remains a need for more efficient and sensitive approaches to evaluate genetic health risks.

Analysis of mutations directly in isolated spermatozoa has promise as an alternative to traditional progeny-based mutation assays used to assess transmissible mutations. A variety of effects are transmitted by spermatozoa, including aneuploidy, structural aberrations, epigenetic changes, pre-mutational lesions, changes in the number of trinucleotide repeats, and gene mutations [5]. Spermatozoa comprise a homogeneous and large population of cells that offer several practical benefits to the assessment of genetic health risks [6], including repeated and non-lethal sample collection from individual males, and reduced resource requirements related to production and analyses of mutations in progeny. The detection of mutations using transgenic mutation assays and expanded simple tandem repeat (ESTRs) mutation assays offer promise as efficient methods for measuring mutant frequencies and evaluating the molecular characterization of the mutations in spermatozoa and germ cells. If these analyses provide comparable results as traditional progeny-based assays, these methods may prove to be the best choice to efficiently estimate the frequencies of mutations transmitted to progeny [4].

Despite distinct practical benefits offered by using isolated spermatozoa to assess genetic health risks, increasing evidence suggests assessments based measuring genetic alterations in spermatozoa may not be equivalent to that based on progeny [4,7–9]. In particular, high levels of mutational mosaicism in the germline and somatic tissues of F1 generation individuals implicates damaged spermatozoa in mediating mutagenesis post-fertilization in cells of the early stage embryos. The mosaic mutant offspring are produced if a persistent pre-mutational lesion carried by the sperm was fixed as a mutation following fertilization in subsequent cell cleavages. [7,9–16]. Whereas such mutations are thought to manifest from a number of molecular, biochemical and cellular events that represent currently uncharacterized pathways of cellular stress response, these mutations in the progeny may represent greater health risks than previously understood [17]. Consequently, it is important to determine to what extent direct analyses of mutations in isolated spermatozoa may be comparable to the results obtained from progeny and ultimately, whether mutation analyses using spermatozoa may serve as surrogates for progeny analyses.

Recently, the λ transgenic medaka, a small fish mutation model shown previously to be a sensitive and reliable model for detecting mutations in somatic cells [18–24], was introduced as an alternative to traditional mouse models used to detect mutations in progeny derived from mutagen-exposed male germ cells [25]. In addition to providing numerous practical benefits, such as prolific reproductive capacity, breeding with multiple untreated females over consecutive days prior to and after mutagen exposure, and ability to analyze mutations using individuals or pools of offspring, the study strengthened growing evidence of common processes of DNA damage and repair shared among distantly related species. The study demonstrated that chemically induced mutations in the *cII* target gene carried by the transgenic male parents were transmitted to progeny, and that different germ cell stages contribute to different patterns of induced mutagenesis related to frequencies of mutant offspring, frequencies of mosaic and whole body mutant offspring, and the spectra of mutations carried by the offspring. This study and others using the transgenic mice and the SLT disclosed evidence of mutations carried by mutant offspring suggestive of the fundamental role of post-fertilization processes in germ cell mediated mutagenesis [7,9–11,13–16,26,27].

In this study, we evaluated the feasibility of detecting mutations directly in isolated spermatozoa of λ transgenic medaka to determine if such analyses may provide an efficient

and sensitive alternative to progeny analyses used to assess genetic health risks. We evaluated whether different germ cells differed in susceptibility to mutagen exposure, and to what extent frequencies of mutant *cII* genes in isolated spermatozoa approximated frequencies of mutant progeny produced from identical male parents. The results of these analyses add to growing evidence that implicates damaged male germ cells in mediating mutagenic processes that give rise to mutations in progeny of mutagen exposed males.

2. Materials and methods

2.1. Animals

Wild-type (CAB strain) and λ transgenic medaka (λ 310 strain) carrying the λ LIZ bacteriophage vector that harbors the *cII* mutation target gene (homozygotes, ~150 λ copies/diploid genome) [28] were obtained from in-house stocks at the Aquatic Biotechnology and Environmental Laboratory, University of Georgia. 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.

2.2. Mutagen exposure of λ transgenic males

Adult λ transgenic male medaka (4–6 month old) were immersed in water containing ethylnitrosourea (ENU, 100 mg/L, Sigma, CAS # 759-73-9) for 1 hr on 2 consecutive days. To minimize photo degradation of ENU, exposures were conducted in the dark. Fish were rinsed and maintained under standard culture conditions following mutagen treatments.

2.3. Collection of spermatozoa

To compare mutational responses of male germ cells exposed to the mutagen at different stages of spermatogenesis, we collected mature spermatozoa from males at prescribed intervals after ENU exposure (2 days after mutagen exposure represent ENU-exposed spermatozoa, 5 days, represent late spermatids; 14 days, represent spermatocytes; >30 days, represent spermatogonial stem cells) [29]. Spermatozoa collected from individual males prior to ENU treatment, or from untreated males, served as controls.

We used non-lethal procedures to collect spermatozoa by immersing individual males for ~5 min in anesthesia (150 mg/L tricaine methanesulfonate [MS-222], pH 7.0, Western Chemical Inc, Ferndale, WA, CAS # 886-86-2) and applying gentle pressure to the abdomen to release mature spermatozoa onto a Petri dish containing 160 μ l of nano-pure water or balanced salt solution (BSS) pH 7.4 [30,31]. Spermatozoa were collected using a micro-pipette, flash frozen and stored at –80°C prior to analysis, or were used immediately for in vitro fertilization of eggs collected from untreated non-transgenic females (section 2.4). Following sperm collection, fish were revived by transfer to clean water and returned to standard culture conditions.

2.4. Comparison of *cII* MFs in spermatozoa and individual progeny

To compare the *cII* MFs in isolated spermatozoa with the frequencies of mutant offspring derived from individual male parents, we collected spermatozoa from individual male λ transgenic medaka (2 males) prior to, and 5 days after ENU exposure. We divided each sample of spermatozoa into 2 aliquots, with one aliquot flash frozen and stored at –80°C for subsequent analyses, and the other used immediately to fertilize oocytes of non-transgenic, untreated females (9–10 females/male, Fig. 1) [30,31]. All progeny, hemizygous for the λ LIZ vector contributed by the male parent, were maintained separately for each breeding group, allowed to hatch, flash frozen (10–14 days post-hatch) and stored at –80°C for mutation analysis.

2.5. DNA extraction

Genomic DNA was isolated from spermatozoa or from individual progeny using procedures described previously [22,25,28]. Briefly, spermatozoa or whole animals were digested with proteinase (1 × 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).

2.6. *cII* mutation assay

Frequencies of mutant λcII genes in spermatozoa or progeny were determined using a positive-selection assay to detect the *cII* null-mutations [32]. 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 and contributes to the failure of λ^+ bacteriophage to form plaques. Following similar procedures [28], genomic 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 lyse the E. coli host. To select λcII^- mutants, the packaged phage were 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 λcII 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 *cII* MFs were previously established was used in each assay to serve as an internal standard. Samples with vector recoveries less than 200,000 PFU, were excluded from further analyses.

2.7. Statistical analysis

The mean *cII* 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 [33]. A sample, either DNA isolated from spermatozoa, or from individual offspring was designated as mutant if the *cII* MFs was significantly elevated compared to untreated controls and/or historical spontaneous *cII* MFs ($p < 0.05$).

3. Results

3.1. *cII* MFs in spermatozoa exposed to ENU at different stages of spermatogenesis

To compare *cII* MFs in isolated spermatozoa exposed at different stages of spermatogenesis, spermatozoa were collected from males at four prescribed times: prior to ENU-exposure, and 2 days (spermatozoa), 5 days (spermatozoa/late spermatids), and 32 days (spermatogonial stem cells) after ENU exposure. The mean *cII* MFs ($\text{MF} \pm \text{standard error of mean} \times 10^{-5}$) in spermatozoa isolated from males exposed to ENU as spermatozoa ($1.7 \pm 0.3 \times 10^{-5}$, $p=0.1$, $n=7$) or late spermatids ($2.4 \pm 0.6 \times 10^{-5}$, $p=0.07$, $n=7$) were not significantly different from *cII* MFs in spermatozoa collected from males prior to ENU-exposure ($1.5 \pm 0.2 \times 10^{-5}$, $n=14$, Fig. 2).

By contrast, the mean *cII* MF in isolated spermatozoa exposed to ENU as spermatogonial stem cells was significantly elevated, 9-fold ($13.6 \pm 2.6 \times 10^{-5}$, $p=0.02$, $n=6$ Fig. 2), compared to that of controls ($1.5 \pm 0.2 \times 10^{-5}$, $n=14$).

3.2. Comparison of *cII* MFs in isolated spermatozoa and in progeny

To examine mutagenic responses based on direct analysis of mutations in spermatozoa compared to that based on mutations in progeny, we analyzed *cII* MFs in isolated spermatozoa and offspring obtained from two ENU-exposed male parents (Fig. 1). The *cII* MFs in isolated spermatozoa collected from each male prior to ENU treatment (male A, 1.2×10^{-5} , and male B, 0.9×10^{-5}), and in spermatozoa collected 5 days following mutagen treatment were not significantly different from one another (male A, 1.7×10^{-5} , and male B, 1.1×10^{-5}), or from historical spontaneous *cII* MFs (2.5×10^{-5}) [25]. Similarly, *cII* MFs in progeny derived from spermatozoa collected from each male prior to ENU exposure (0.9 to 4.8×10^{-5}) were not significantly elevated (Fig. 3), with the mean *cII* MF of $2.5 \pm 0.2 \times 10^{-5}$ (n=18) for progeny from male A and $2.2 \pm 0.3 \times 10^{-5}$ (n= 14) for male B progeny.

In sharp contrast to direct analyses of mutations in spermatozoa, progeny derived ENU-exposed post-meiotic germ cells (5 days post treatment) exhibited *cII* MFs that varied widely in magnitude, ranging from 0.5 to 90.8×10^{-5} for progeny of male A (n=19), and 1.1 to 325.6×10^{-5} for progeny of male B (n=20, Fig. 3). Whereas the majority of male A progeny (89%, 17/19) exhibited *cII* MFs comparable to progeny collected prior to ENU exposure, two offspring exhibited significantly elevated *cII* MFs, 1.5-fold (7.3×10^{-5}) and 18-fold (90.8×10^{-5}), compared to controls (2.5×10^{-5}). Similarly, the majority of Male B progeny (70%, 14/20 offspring) exhibited *cII* MFs comparable to progeny collected prior to ENU exposure. However, male B produced a higher frequency of mutant offspring (30%), including six offspring with *cII* MFs elevated from ~2-fold to more than 130-fold (5.1 to 325.6×10^{-5}) compared to offspring of untreated males. The frequencies of mutant offspring (11–30%) generated from these male parents are comparable to those observed previously (3.5–20%) derived from ENU-exposed post-meiotic germ cells (spermatozoa/late spermatids) [25].

4. Discussion

The timing of mutagen exposure relative to the stage of spermatogenesis, the differences in susceptibility to damage among germ cell stages, and the various processes related to DNA repair/attempted repair at or following fertilization all play important roles in contributing to the mutations in mutant offspring of mutagen-exposed parents [4,5,9,25,34]. These factors contribute complexity to the analysis of induced genetic damage, and ultimately, affect the reliability of using spermatozoa as an alternative to progeny analyses to assess transmission of mutations. Analyses of spermatozoa in the λ transgenic medaka measures fixed DNA changes as base substitutions, small insertions and deletions, and frameshift mutations. DNA damage and large chromosomal rearrangements are not measured by this model.

We showed direct assessment of induced mutations in spermatozoa isolated from λ transgenic medaka males provides numerous practical benefits, including non-lethal and repeated sample collection from individual males, as well as, the reduction in extensive breeding and collection of large numbers of animals typically required for progeny analyses. These results suggest that analyses based on isolated spermatozoa may have particular promise as a valuable high-throughput screening approach to estimate genetic health risks. However, we also showed that direct analyses of mutations in spermatozoa provided different responses depending upon the stage of spermatogenesis at which the germ cell was exposed to the mutagen. Spermatozoa exposed as pre-meiotic germ cells (spermatogonial stem cells) to ENU exhibited significantly elevated frequencies of mutant *cII* genes, whereas spermatozoa treated as post-meiotic germ cells (spermatozoa, late spermatids) did not show significant induction. Further, in the direct comparison of differential responsiveness between analyses, we showed no significant mutational response in isolated spermatozoa, but showed from 11% to 30% of the offspring derived from identical spermatozoa exposed to ENU as post-meiotic germ cells exhibited significantly elevated *cII* MFs. Whereas no previous studies have compared the mutational

responses in spermatozoa and progeny collected from the same male, evidence supports analyses of mutations in spermatozoa as being less sensitive compared to those based on progeny [9,26].

The lack of mutation induction of *cII* MFs in λ transgenic medaka spermatozoa exposed to ENU as post-meiotic germ cells suggests the damage carried by the spermatozoa was retained as persistent pre-mutational lesions. Considering that spermatozoa and late spermatids are non-dividing, DNA repair-deficient cells [15], and that fixation of a mutation requires DNA synthesis or error-prone DNA repair [35], it follows that lesions induced by ENU persist in the germ cells at least until after fertilization. Additionally, because the *cII* mutation assay detects permanent sequence changes in the *cII* gene, but not lesions or other damage to the DNA, persistent damage carried by spermatozoa will not be detected as fixed *cII* mutations in isolated spermatozoa. Further the elevated *cII* MFs exhibited by progeny, in contrast to the lack of mutation induction of *cII* MFs in isolated spermatozoa, is consistent with studies that suggest damage retained by spermatozoa can trigger maternal DNA repair pathways using stored mRNAs and proteins in the oocyte [4,5,7,25,34]. It follows that early stage embryos utilized these processes to fix the damage carried by the spermatozoa resulting in measurable *cII* mutations in progeny.

In previous progeny studies using medaka we showed frequencies of mutant offspring differed according to germ cell stage exposed to the mutagen, with higher frequencies of mutant offspring derived from ENU-treated spermatozoa/late spermatids compared to those derived from mutagen exposed spermatogonial stem cells [25]. Consistent with studies from the SLT in medaka [13], this study also revealed, independent of the germ cell stage exposed to the mutagen, that the majority (>70%) of mutant offspring carried mutant *cII* genes in a portion of cells (i.e. mosaic mutant offspring), rather than in all the cells of the individual (i.e. whole body mutant offspring). Mosaic mutant offspring are generated from the fixation of DNA damage as mutations at or after the second cell cleavage, whereas whole body mutants are generated from mutations fixed either in the gametes, or at the one-cell stage. Therefore, the predominance of mosaic mutant offspring contributes to growing evidence that reveals the importance of persistent DNA damage in germ cells, and the post-fertilization processes that fix this damage as mutations in the early stage embryo. These data highlight the role of the spermatozoa not only as direct transmitters of genetic damage (e.g. fixed mutations), but also as mediators of indirect mutagenesis in the cells of early stage embryos.

The predominance of mosaic mutant individual offspring and the post-fertilization processes that contribute to the mutations the mutant offspring carry have distinct implications for assessment of transmissible mutations. Mosaicism is widely recognized as an important and common mechanism responsible for the origin of genetic disorders [17]. However, the presence of mosaic mutant offspring can significantly confound the screening and confirmation of mutant offspring depending upon the mutant offspring [26], and as a consequence, mosaic mutant offspring are commonly excluded from assessments of transmissible mutations [4]. The mutations carried by mutant offspring, whether generated from direct or indirect mechanisms of mutagenesis, may contribute to a variety of health consequences such as cancer, genetic diseases, infertility, or death [34].

Mutation assays, including analyses based on progeny or isolated spermatozoa that do not account for mosaic mutant offspring, will likely underestimate the actual frequency of mutant offspring, and ultimately the genetic health risks. The comparison of mutagenic responses in isolated spermatozoa and progeny illustrates that some damage introduced in germ cells at various stages of spermatogenesis can be retained but will not be revealed from direct analysis of isolated spermatozoa using mutation assays. By contrast, progeny-based analyses detect this damage carried by the male germ cells because the persistent damage is subjected to processes

at fertilization or in subsequent cell cleavages that fix the mutations in the embryo. As shown here, significant induction of mutations will not be revealed from direct analyses of spermatozoa exposed to the mutagen at the post-meiotic germ cell stage. Whereas analysis of mutations in spermatozoa exposed as pre-meiotic germ cells can provide an estimate of the frequencies of mutations transmitted to progeny, this indirect approach will estimate a subset of the damage manifested in the genomes of offspring of mutagen-exposed males.

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Fig. 1.

Approach used to compare mutational response in isolated spermatozoa and progeny derived from male germ cells exposed to ENU at the post-meiotic stage of spermatogenesis. *cII* MFs were analyzed directly in isolated spermatozoa or in progeny collected from each male.

Fig. 2.

cII MFs in isolated spermatozoa exposed to ENU at different stages of spermatogenesis. Spermatozoa were collected from males at intervals after mutagen exposure corresponding to ENU-treated spermatozoa (2 days), late spermatids (5 days), or spermatogonial stem cells (30 days). *cII* MFs in spermatozoa (n=7) and late spermatids (n=7) were not significantly different from those collected prior to exposure (n=14). *cII* MFs in spermatogonial stem cells showed a significant 9-fold increase (n=6, p=0.02).

Fig. 3. *cII* MFs in offspring collected prior to and after ENU exposure (5 days) of λ transgenic medaka males. Eleven percent of the offspring from Male A (n=19), and 30% of Male B offspring (n=20) exhibited *cII* MFs >2-fold that detected in progeny collected prior to ENU exposure (Male A n=18, Male B n=14).