Mutation Research 783 (2015) 36–54

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox
Community address: www.elsevier.com/locate/mutres

Review

Approaches for identifying germ cell mutagens: Report of the 2013 IWGT workshop on germ cell assays☆☆

Carole L. Yauk[a,*], Marilyn J. Aardema[b], Jan van Benthem[c], Jack B. Bishop[d], Kerry L. Dearfield[e], David M. DeMarini[f], Yuri E. Dubrova[g], Masamitsu Honma[h], James R. Lupski[i], Francesco Marchetti[a], Marvin L. Meistrich[j], Francesca Pacchierotti[k], Jane Stewart[l], Michael D. Waters[m], George R. Douglas[a,*]

[a] *Environmental Health Science and Research Bureau, Health Canada, Ottawa, ON, Canada*
[b] *Marilyn Aardema Consulting, Fairfield OH, USA; Bioreliance, MD, USA*
[c] *National Institute for Public Health and the Environment, Bilthoven, The Netherlands*
[d] *National Institute of Environmental Health Sciences, NC, USA*
[e] *USDA, Food Safety and Inspection Service, Washington DC, USA*
[f] *University of North Carolina at Chapel Hill, NC, USA*
[g] *University of Leicester, United Kingdom*
[h] *National Institute of Health Sciences, Japan*
[i] *Department of Molecular and Human Genetics, and Department of Pediatrics, Baylor College of Medicine, USA*
[j] *University of Texas M.D. Anderson Cancer Center, USA*
[k] *ENEA, Italian National Agency for New Technologies, Energy and Sustainable Economic Development, Italy*
[l] *AstraZeneca Pharmaceuticals, United Kingdom*
[m] *Integrated Laboratory Systems, Incorporation, NC, USA*

ARTICLE INFO

Article history:
Received 22 January 2015
Accepted 23 January 2015
Available online 24 January 2015

Keywords:
Germ cell mutation
Genetic disease
Reproductive health
Copy number variants
Gene
mutation
Chromosome aberrations

ABSTRACT

This workshop reviewed the current science to inform and recommend the best evidence-based approaches on the use of germ cell genotoxicity tests. The workshop questions and key outcomes were as follows. (1) Do genotoxicity and mutagenicity assays in somatic cells predict germ cell effects? Limited data suggest that somatic cell tests detect most germ cell mutagens, but there are strong concerns that dictate caution in drawing conclusions. (2) Should germ cell tests be done, and when? If there is evidence that a chemical or its metabolite(s) will not reach target germ cells or gonadal tissue, it is not necessary to conduct germ cell tests, notwithstanding somatic outcomes. However, it was recommended that negative somatic cell mutagens with clear evidence for gonadal exposure and evidence of toxicity in germ cells could be considered for germ cell mutagenicity testing. For somatic mutagens that are known to reach the gonadal compartments and expose germ cells, the chemical could be assumed to be a germ cell mutagen without further testing. Nevertheless, germ cell mutagenicity testing would be needed for quantitative risk assessment. (3) What new assays should be implemented and how? There is an immediate need for research on the application of whole genome sequencing in heritable mutation analysis in humans and animals, and integration of germ cell assays with somatic cell genotoxicity tests. Focus should be on environmental exposures that can cause de novo mutations, particularly newly recognized types of genomic changes. Mutational events, which may occur by exposure of germ cells during embryonic development, should also be investigated. Finally, where there are indications of germ cell toxicity in repeat dose or reproductive toxicology tests, consideration should be given to leveraging those studies to inform of possible germ cell genotoxicity.

Crown Copyright © 2015 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

☆ Where applicable, this manuscript has been reviewed by the institutions of those authors and approved for publication. The opinions and conclusions expressed in this article are the views of the authors and do not necessarily reflect those of their institutions. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

* Corresponding authors. Tel.: +1 613 612 2692 (GRD)/ +1 613 314 7375(CLY); fax: +1 613 941 8530.
E-mail addresses: Carole.Yauk@hc-sc.gc.ca (C.L. Yauk), George.Douglas@hc-sc.gc.ca (G.R. Douglas).

http://dx.doi.org/10.1016/j.mrgentox.2015.01.008
1383-5718/Crown Copyright © 2015 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Contents

1. Introduction

Fifteen internationally recognized germ cell genetic and reproductive toxicology experts from government, industry, and academia, gathered in Foz do Iguacu, Brazil (October 31–November 1, 2013) for an International Workshops on Genotoxicity Testing (IWGT) meeting on advancing the science and regulatory approaches used to assess mutagenic hazards to germ cells. The overarching mandate of this workshop was the following: (1) review the current science; (2) achieve scientific consensus on issues surrounding the use of germ cell genotoxicity tests in regulatory assessments; and (3) inform and recommend the best evidence-based approaches and future prospects in this field. Discussions and presentations centered on the following topics that provided a basis for achieving consensus:

- current assays used to assess germ cell mutation;
- regulatory requirements of different countries and international organizations for germ cell tests;
- reproductive toxicology assays that can be leveraged for the assessment of heritable effects;
- assays in need of further development or validation;
- new technologies and approaches;
- the "blood-testis barrier" and pharmacokinetics in male germ cell toxicity/genotoxicity;
- endpoints most relevant to human genetic risk.

Directed discussions were held on the following key workshop questions.

(1) Do genotoxicity and mutagenicity assays in somatic cells predict germ cell effects?

(2) Should germ cell tests be done, and when?

(3) What new assays should be implemented and how?

The workshop resulted in recommendations addressing each of these questions, with an emphasis on the need to develop improved methods for germ cell testing, including those that can be integrated with existing genetic and reproductive toxicology tests.

2. Background

Early genetic toxicology focused almost exclusively on heritable genetic effects. However, in 1973 Dr. Bruce Ames' seminal paper [1] that introduced the Salmonella bacterial mutation assay (Ames test), and other developments, changed the focus of genetic toxicology from germ cells to somatic cells and cancer. The premise that the majority of carcinogens were somatic cell mutagens and could be readily detected with short-term assays resulted in a nearly complete shift in focus from heritable genetic hazards to somatic cell effects. Though, to date, no human germ cell mutagen has definitively been identified, nearly 50 rodent germ cell mutagens are known [2], and the consequences of heritable mutations remain of concern.

BOX 1:
Unique aspects of gametogenesis

- Prolonged developmental and differentiation stages.
- Meiosis.
- Eggs are arrested in prophase 1 of meiosis from birth until puberty, and do not complete meiosis until fertilized.
- Major morphological changes occur in male germ cell including acquisition of motility.
- Haploid.
- Unique chromatin structure.
  - In sperm histones are replaced first with transition proteins and then protamines.
  - Unique epigenetic sex-specific features occur in the progenitor germ cells and in the early embryo.
- Sperm are DNA repair deficient in the final haploid stages.
- Egg DNA repair machinery in the early embryo is responsible for repairing damage incurred in the late stage non-DNA repair proficient spermatids.

Unfortunately, the field has lacked development of new, more sensitive, less animal-intensive, and higher throughput methods to detect genotoxic/mutagenic effects in the paternal and maternal germline, and accompanying heritable changes. Indeed, two of the existing OECD (Organisation for Economic Cooperation and Development) test guideline (TG) assays that are specific for germ cell mutations (dominant lethal, and heritable translocation tests, see Section 3.1 and 3.2 below) require large numbers of rodents and/or are labor-intensive; as such, they are rarely used. As a result of the lack of practical and routinely used methods for germ cell testing, some regulators assume that germline genetic integrity is protected by default through analysis of somatic cell mutagenesis, as discussed recently [3,4]. This assumption has not been rigorously tested using more recent methodologies, and exceptions have been noted that show effects occurring to a greater extent in germ cells or offspring than in somatic cells [5]. Given the unique aspects of spermatogenesis and oogenesis (see Box 1) it is possible that there are mechanistic and/or chemical-specific effects to germ cells not seen in in vivo somatic tests, or in vitro tests. Moreover, given the unique embryogenesis and development of human female (egg) versus male (sperm) germ cells, and unique milieu of each, a potential sexual dimorphism for germ cell effects is possible. In addition to the problems noted above, all the examples of induced germ cell mutagenicity observed in rodents have not been confirmed in humans. Thus, extrapolation of rodent germ cell findings to humans for risk assessment has not been applied widely. A final challenge is that the existing rodent assays capture only a portion of potential genetic effects; more recent studies applying powerful new genomics technologies in human clinical genetics are revealing critical genomic changes associated with genetic diseases that would not necessarily be identified by these traditional tests. Accordingly, there are limitations and assumptions regarding current germ cells tests that were focal points of the workshop and that must be addressed in order to move the field forward.

Despite the lack of optimised tools, regulatory agencies and expert groups around the world have acknowledged the importance of identifying germ cell mutagens, and have policies or practices that require the assessment and management of germ cell mutagenic hazards. For example, the updated International Programme on Chemical Safety (IPCS) harmonized scheme for mutagenicity testing states: "For substances that give positive results for mutagenic effects in somatic cells in vivo, their potential to affect germ cells should be considered. If there is toxicokinetic or toxicodynamic evidence that germ cells are actually exposed to the somatic mutagen or its bioactive metabolites, it is reasonable to assume that the substance may also pose a mutagenic hazard to germ cells and thus a risk to future generations" [6]. Thus, there is an urgent need to refine the appropriate germ cell tests that should be conducted and define when they should be used.

Several practical assays have emerged more recently that address some of the gaps in testing described above: (1) the transgenic rodent mutation assays OECD test guideline (TG488) [41] that includes recommendations for male germ cell mutation analysis; (2) sperm and pedigree tandem repeat mutation analysis [7]; (3) improved methods to quantify sperm DNA damage and chromatin effects [8]; and (4) high-throughput screening for aneuploidy in *Caenorhabditis elegans* eggs [9]. Most importantly, the rapid technological evolution of genomics tools, including DNA microarrays and next generation sequencing, is poised to revolutionize the field dramatically. Indeed, whole genome sequencing has recently been applied to establish that increasing paternal age in humans is strongly associated with increased transmission of de novo mutations to offspring [10]; this conclusion is supported by an increased prevalence of various diseases in the offspring of older fathers [11]. Findings on paternal age effects extend to global analysis of microsatellite mutations [12] as well as specific types of copy number variants (CNV) [13] in humans. Overall, advances in technologies are a primary reason for the refocus in attention on germ cell mutagenicity.

The development of new methods to measure germ cell mutagenicity and the increasing number of human epidemiological studies that assess markers of germ cell mutagenesis has resulted in a growing weight of evidence supporting the existence of human germ cell mutagens (e.g. paternal age, ionizing radiation, cigarette smoke, chemotherapeutic agents) [10,14–19]. As opposed to somatic cell mutagenesis, which is generally associated with carcinogenesis after sufficient functional mutations have accumulated, a single germ cell mutation can potentially lead to an array of disease phenotypes in addition to being a primary cause of embryonic and fetal death. Indeed, de novo mutations are now recognized as contributing to human diseases including neurological disorders, cancer, and a multitude of other disorders [20–24]. It is estimated that each human genome contains approximately 100 loss-of-function variants, with as many as 20 of these exhibiting complete loss of gene function [25]; all of which would have originated as de novo mutations. Indeed age associated mutations in sperm are predicted to be of relatively equivalent importance to the population burden of genetic disease caused by the maternal age effect on aneuploidy [26]. However, despite such new knowledge, the contribution of environmental effects to the incidence of de novo mutations is currently unknown. Nevertheless, de novo germ cell mutations do contribute to the population burden of genetic disease, and present a major psychological, emotional, and economic burden on societies. Moreover, this analysis provides strong support for the use of new genomics approaches in identifying the causes and consequences of germ cell mutations.

Clearly, identification of potential hazards to germ cell genomic integrity is important in regulatory efforts to protect population health. These assays must be able to detect chemical agents that induce the broad spectrum of DNA and chromosome damage that is documented to occur in germ cells and to be transmitted to offspring. Premutational lesions are transmitted by sperm and may result in de novo mutations if unrepaired or misrepaired by egg DNA repair machinery. Additional endpoints include chromosomal aneuploidies, chromosomal structural aberrations, CNV, tandem repeat mutations, single nucleotide variants and insertions/deletions, gene mutations, and mutations in non-coding sequences. The latter is a particularly important gap as emerging evidence indicates the importance of non-coding DNA to normal

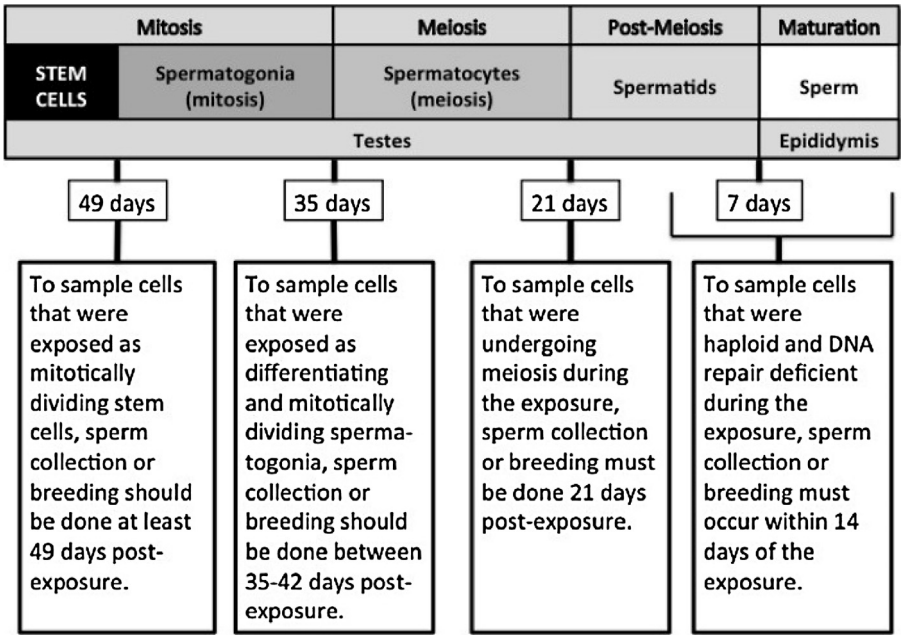

Fig. 1. Depiction of the phases of mouse spermatogenesis, the length of time required from that phase until the sperm is fully mature, and experimental design aimed to capture exposures during specific cellular phases.
Please note, only male gametogenesis is shown, as the assays described focus virtually exclusively on male germ cells. Female gametogenesis is much more difficult to study as it occurs once over a prolonged period of time beginning in utero and finishing at fertilization of the mature egg. In addition, there are very few eggs in a mature female relative to sperm in a mature male, which makes analysis of mutations occurring in eggs much more difficult. Understanding the effects of chemicals on female germ cells is an important gap in this field.

biological function [27,28]. While epigenetic change in the germline is an important, rapidly developing area of investigation, it was not within the scope of this workshop.

3. Current assays used to assess germ cell mutation

The currently available assays for detecting mutations in germ cells focus primarily on effects in male germ cells because sperm are more readily accessible and available. Nevertheless, assays for female germ cells are recognized as an important gap in regulatory toxicology testing. Male germ cell assays differ in many aspects from each other, e.g. in their degree of standardization, the endpoints detected, and the sensitivity and specificity for detecting mutagenic chemicals. Male germ cell tests require careful experimental design to ensure that the appropriate phases of spermatogenesis are tested by waiting specific periods of time post-exposure prior to sample collection or breeding (Fig. 1). We briefly discuss regulatory guideline tests that are conducted less frequently today than in the past, and then we review the more commonly conducted current assays.

3.1. Heritable translocation and specific locus tests

Until the advent of molecular cytogenetics and genomics technologies, the gold standards for germ cell testing in the offspring of exposed parents were the mouse heritable translocation test (HTT, OECD Test Guideline 485) [29], and the mouse specific locus test (SLT) [30,31]. The HTT detects genome-wide, chromosomal rearrangements that result in sterility or semi-sterility of F_1 offspring of treated males. The SLT detects viable, null mutations at a few specific loci, which, on a molecular basis, range from base substitutions to deletions spanning beyond the locus itself. The main benefit of these tests is that they detect genetic changes similar to those associated with human genetic diseases that are identified in the offspring of treated parents, demonstrating the actual transmission of germ cell mutations to the next generation. However, these assays are extremely time consuming and require very large numbers of animals. In addition, the SLT requires the use of a mutant mouse strain that is homozygous for 7 recessive mutations; this mouse strain is no longer maintained on a routine basis in any laboratory in the world. For these reasons, the HTT and SLT assays are no longer performed and are not considered further in this report.

3.2. Dominant lethal test

The dominant lethal test (DLT) measures genetic changes in germ cells that lead to subsequent embryonic or fetal death (OECD TG 478) [32,33], and has been the most extensively used germ cell mutagenicity test. The assay is conducted in either rats or mice, usually in males. Following paternal exposure, the effects of the toxicant on the various spermatogenic cell types is tested using sequential mating intervals with virgin females (e.g. Fig. 1, but usually mating is done every week for a total of 8 weeks in mice and 10 weeks in rats). Alternatively, males can be treated throughout an entire cycle of spermatogenesis, with one mating at the end of the treatment [34]. After an appropriate period of time (e.g. at mid-gestation or beyond), the ovaries and contents of the uteri of females exhibiting evidence of mating (i.e. mating plug or presence of sperm in their vagina) are examined to determine the numbers of eggs ovulated (via counting corpora lutea) plus the number of implants and live and dead embryos. Numbers of these events per female in the treated and control groups are compared to calculate the dominant lethal effect.

In the DLT, pre- and post-implantation embryonic losses, which are ostensibly caused by severe structural or numerical chromosome changes inherited from exposed fathers, are scored. Several studies support the chromosomal origin of the embryonic death [35,36]. While many of the embryonic losses are likely due to chromosome aberration, gene mutation, and teratogenic effects, the involvement of cytotoxicity cannot be excluded. Although the type and location of these lethal genetic changes are not identified, the DLT is well standardized and, over the years, a considerable

number of chemicals have been tested using this procedure. Indeed, the methodology for the test has not changed significantly since 1984 and it is still in use (e.g. [37]). The workshop participants acknowledged that although the mutational effects are manifest in the offspring, the endpoint cannot be considered heritable because the measured outcome is embryonic death; however, the great majority of chemicals that are positive in the DLT are also positive in the HTT, which does measure an inherited effect [32,33].

3.3. Cytogenetic analysis of spermatogonia or embryos

The cytogenetic analysis of spermatogonial metaphases [38] (OECD TG 483) is a standardized method to detect chromosomal aberrations in male germ cells of mice and rats. The main limitation of this approach is that possible mutagenic effects are observed at the beginning of germ cell differentiation; thus, their transmission to mature gametes and the offspring has not been demonstrated. The transmission of germline chromosome aberrations to the fertilized egg is detectable by the cytogenetic analysis of first cleavage zygote metaphases [39]. The application of chromosome painting has greatly improved the quality and amount of information that can be obtained by such a test [40], allowing for a distinction between stable balanced aberrations (e.g. reciprocal translocations) and unstable aberrations (e.g. acentric fragments, dicentric chromosomes). Comparison of chromosome aberrations in the zygote with DLT and HTT data following various chemical treatments has provided evidence to support assumptions on the fate of different types of chromosome aberrations [36]. However, only a limited number of embryos can be collected from each animal, and the technique for preparing good quality metaphase spreads in the zygote requires a significant amount of skill, thus hampering wide dissemination of the approach.

3.4. Transgenic rodent somatic and germ cell mutation assay

The transgenic rodent mutation assay (TGR; OECD TG 488) [41] is based on mutation detection in a transgenic sequence that can be rescued from most rodent tissues and expressed in a bacterial system [42–44]. The TGR assay is amenable to the analysis of testicular cells and epididymal sperm providing a tool to detect gene mutations in male germ cells. In addition, mutation spectrum (base substitutions, insertions/deletions, frameshifts) following chemical exposure can be determined. Analysis of testicular tissues would enable the integration of this assay with the standard somatic cell transgenic mutation assay protocol, significantly reducing cost, animals and time. However, the sensitivity of the test applied to cells retrieved from testicular tissues (i.e. cells from a variety of spermatogenic phases) has not been rigorously tested. This is an important avenue for future research (discussed in more detail in Section 10.3), and the test can be improved further by enrichment for specific germ cell subpopulations from testes prior to mutant frequency analysis. Although the chemical database for this test in germ cells is still limited, the approach holds promise for male germ cell mutagenicity studies because it allows a quantitative comparison of the same mutagenic endpoints between somatic and germ cell tissues [42,44]. However, the potential for using variations of the transgenic rodent mutation assay to detect female germ cell mutations is hampered by the vastly insufficient number of oocytes available per female, which precludes the conduct of the assay. Therefore, there are no quantitative comparisons of the same mutagenic endpoints in somatic versus germ cells of females.

3.5. Genotoxicity tests in sperm

Genotoxicity tests in sperm are especially relevant because they can be applied in both laboratory rodents and humans, thus providing bridging biomarkers between experimental and biomonitoring studies. Different types of pre-mutational and mutational changes can be detected in sperm, including DNA breaks and abasic sites detectable by the comet assay [45], unscheduled DNA synthesis (UDS) [46], chromatin packaging alterations detectable by the sperm chromatin structure assay (SCSA) [47], and numerical and structural chromosome changes detectable by fluorescent in situ hybridization (FISH) [48]. These tests could offer potentially quick, higher throughput pre-screening tools for detecting germ cell mutagens, even though they do not assess heritable effects. However, in many cases, protocols have yet to undergo standardization and harmonization processes, and basic science is needed to identify the mechanisms of induction and the molecular nature of the detected endpoints. Some of these methods, such as the comet assay and the SCSA, are applied in the clinical diagnosis and management of male infertility, which might expedite their standardization. The most relevant methods are discussed in Section 6 in more detail.

4. Reproductive toxicology and general toxicity assays capable of providing signals of potential germ cell genotoxicity

Both reproductive toxicology and repeat dose toxicity studies may provide signals pertinent to germ cell genotoxicity, and are an important source of information relating to potential germ cell hazards that has been overlooked in most cases. These studies provide a wealth of information on reproductive endpoints that can indicate both delivery of the agent to male and female germ cells and gonadal tissues, as well cytotoxic effects that may occur following exposure to genotoxicants. A very brief synopsis of the repeat dose toxicity tests and relevant standard reproductive toxicity tests is given below.

4.1. Segmented reproductive toxicology designs

Segmented studies expose and assess particular time periods of development rather than considering the entire life cycle of an organism all at once. For example, the in utero development of the fetus may be examined separately from post-natal stages, and other critical developmental periods, using different exposure and assessment windows. The International Conference on Harmonization (ICH) guideline S5(R2) [49] for the testing of pharmaceuticals, describes three different segmented designs. The first of these, the fertility and early embryonic development study, typically begins exposure 4 weeks prior to mating in males, or 2 weeks in females, and continues from fertilization through to implantation. The OECD test guideline 421: reproduction/developmental toxicity screening test [50] also specifies dosing females for 2 weeks prior to pairing with males that have been dosed for a minimum of 2 weeks. Unlike the ICH design, this OECD screening study continues dosing of the females throughout gestation and for 4 days postnatally; therefore, it provides an initial assessment of effects on fertility and developmental toxicity. Other segmented study designs specify dosing exclusively during pregnancy. For example, the OECD prenatal developmental toxicity study (OECD TG 414 [51]) involves exposure from implantation through to parturition. The next ICH segment involves exposure of the pregnant dam from implantation through fetal development (assessing organogenesis), i.e. the ICH embryo-fetal development study. In the final segmented ICH test, the pre- and post-natal developmental (PPND) study, administration to the dam occurs from implantation and through lactation until pup weaning. These segmented studies are generally not multi-generational studies.

Various sampling times are used to assess different developmental outcomes in the fetuses or pups from the above studies. For example, fetal tissues can be examined to assess morphologic changes and functional tests, including reproductive performance testing that can be carried out in the offspring of the exposed dams. Typically, in utero and lactationally exposed male and female pups are raised to maturity and mated. The pregnant female is usually euthanized in mid- gestation to assess effects on fertility and litter size. In pharmaceutical designs, assessment of the gonads of the in utero and lactationally exposed F1 is not mandated, nor are any specific observations made on the F2 fetuses at the mid-gestation termination.

4.2. Continuous cycle designs

In contrast to segmented designs, continuous cycle designs cover all of the different stages from germ cell through fetal development, to adulthood (also known as 'womb to tomb'). Most continuous cycle designs evaluate multiple generations, and exposure spans these generations. The two main approaches for continuous study designs include the National Toxicology Program's (NTP) reproductive assessment by continuous breeding (RACB) [52] and the OECD multigeneration study (OECD TG 416 [53]). Various effects are assessed in the F0 that may be relevant to germ cell mutagenicity, including histopathology on all parts of the reproductive and endocrine systems. The F0 rodents are mated at maturity to produce an F1 generation. This mating provides information relating to fertility and fecundity in the F0. Effects arising in the F1 generation, which is also exposed in utero, may be relevant to potential germ cell effects arising in the F0, but we caution that effects of exposure in utero cannot be excluded.

4.3. One generation

Various modifications to the multigenerational studies described above have been developed, including the one-generation reproduction toxicity study (OECD TG 415 [54]) and the extended one-generation study design (enhanced pre and postnatal studies) (OECD TG 443 [55]). In the modified one-generation study rodents are dosed before mating through gestation. However, the exposure is stopped at various times, and the rodents are either necropsied for assessment or mated to produce an F1 generation. The F1 are handled similarly to the F0, and mating is done to produce F2 pups. Considerations for when to assess the second generation are discussed in OECD Guidance Document 117 [56].

4.4. Repeat dose toxicity studies

Repeat dose toxicology study designs can provide information pertaining to germ cell effects. These include both short-term and long-term study designs, such as 90 day studies, which can be combined with reproduction/development toxicity screening tests (e.g. OECD TG 408 and 422 [57,58]). For pharmaceuticals, it is also easy to combine assessment of male reproductive performance into 3 or 6 month toxicity tests [59]. Various tissues are assessed in these studies, including germ cells. Ovarian and testicular histopathology, sperm count, motility and morphology, can be used to indicate potential germ cell effects. It is noteworthy that rodent sperm morphology is not generally a sensitive indicator of male reproductive toxicants and does not correlate with genetic toxicity [60,61]. As previously described, in general, in repeat dose toxicity tests, it is easier to use measures assessing the male gonad as indicators for potential germ cell effects than it is on the female gonad. The endpoints most likely to be affected by genetic damage are fertility and fecundity, testicular histopathology, testicular weight and sperm count.

4.5. Opportunities for adapting reproductive and repeat dose toxicity designs to assess germ cell genetic toxicity

Based on the review of the above toxicology assays, the workshop participants recommended specific endpoints from these assays that should be considered indicative of potential genotoxic effects to germ cells. The details of this discussion and the recommendations are summarized in Section 10.3.

Overall, it was noted that these assays capture important developmental stages (e.g. in utero exposure, most of spermatogenesis) that are not assessed using standard genetic toxicology approaches. In addition to assessing potential effects that are aligned with toxicity to germ cells across various developmental stages, in both males and females, the above studies could provide a valuable repository of exposed germ cells and pedigrees that can be used as a source for future genome studies on germ cell mutations and de novo mutations arising in offspring.

5. Assays in need of further development or validation

A number of additional assays have been developed over the past decade or more. Many of these assays have been used extensively to measure the effects of germ cell mutagens, but they are in need of further development and/or validation. A summary of the advantages and disadvantages of these methods is given in Table 1 and they are described in more detail below.

5.1. Transgenic rodent gene (TGR) mutation reporter assay

As discussed above, the transgenic rodent assay (OECD TG 488) shows great promise in enabling more efficient and effective screening for chemically induced germ cell mutations. Indeed, numerous studies have demonstrated the ability of the TGR assay to detect male germ cell mutagens [42,44]. These studies suggest a good correlation between mutagens detected with the TGR assay and the SLT [43]. Moreover, prototypical mutagens exhibit the expected dose-response in male germ cells for transgene mutations suggesting that the TGR loci respond appropriately and are representative of effects in other gene regions.

Limitations: There are uncertainties about the optimal experimental protocol for assessing mutagenic effects in germ cells when integrating germ cell and somatic cell testing. Currently, TG 488 recommends mutation analysis of somatic cells in mice treated for 28 days and euthanized three days after the final exposure (i.e., 28 + 3d protocol). Also, TG 488 indicates that, for optimal results, mutations should be evaluated in germ cells sampled from: (a) the seminiferous tubules in mice from a 28 + 3d protocol; and (b) the cauda epididymis (i.e. mature sperm) in mice treated for 28 days followed by a 49 day sampling time (i.e. 28 + 49d). The latter is the minimum time required for stem cells to produce sperm and is widely acknowledged to be the standard for accurately evaluating mutagenic effects in male germline stem cells [39]. Unfortunately, sampling at two time points effectively doubles the number of animals and the cost of a study, and restricts opportunities to combine this assay with other repeat dose studies, leading to a reluctance among some users to adopt this protocol. Accordingly, it would be desirable to have only one sampling time for male germ cells (i.e. the 28 + 3d used for somatic cells).

Sampling at a single time point may be feasible if cells from the seminiferous tubule at the 28 + 3d time point can be used to represent various germ cell stages. However, it should be noted that these cells represent a mixed population that may not be adequately exposed as stem cells at the recommended time point for

Table 1
Summary of the advantages and disadvantages of existing assays in development or validation stages.

Endpoint	Advantages	Disadvantages
Transgenic rodent mutation	Can be done on most tissues enabling a comparison of somatic and germ cell sensitivity/specificity, neutral gene, scores gene mutation, OECD guideline, relatively simple (integrated into multiple test strategies).	Need transgenic rodents, scores mutations in a non-transcribed exogenous gene, performed on germ cells not pedigrees thus inheritance is unclear, may miss some types of mutations.
Tandem repeat assays	Endogenous loci, high spontaneous mutation rate, can be adapted to any species, some markers linked to diseases, sensitive at low doses, should be able to be integrated into other tests but validation has not been done.	Unclear indirect mechanism of mutation, non-coding markers, unclear relevance of tandem repeat mutation to gene mutations, small dynamic range, some technical challenges.
Spermatid micronucleus (MN)	Easily integrated into transgene mutation reporter assay and other toxicity tests, any species, can be directly compared to somatic MN to study germ cell specificity/sensitivity.	Currently laborious (but potential for flow cytometry), small database, not inherited.
Sperm comet assays	Can be done in any species, relatively simple, can be compared with most somatic cell types, can detect a variety of DNA damage.	Difficult to integrate with other tests, high variability across laboratories and studies, biological relevance of endpoint unclear, technical issues, premutational damage only.
Sperm chromatin structure	Fast (flow cytometry approach), can be done in any species including humans, major validation exercises underway.	Germ cells only, premutagenic lesion (thus implications unclear), mechanisms causing changes in chromatin unclear, biological and technical variability results in differences across studies/laboratories.

somatic tissue analysis (i.e. 28 + 3d); they may not provide the same sensitivity to detect germ stem cell mutagens as the analysis of sperm derived from exposed stem cells (i.e. 28 + 49d). Efforts under the auspices of the Health and Environmental Sciences Institute's Genetic Toxicology Technical Committee (germ cell work group) are generating data to determine whether the analysis of seminiferous tubules at 28 + 3d represent a reasonable compromise. Preliminary data suggest that analysis of mutations in cells from seminiferous tubules at 28 + 3d provides an acceptable estimate of mutant frequencies in stem cells but may greatly underestimate effects in dividing spermatogonia (Marchetti et al., unpublished data). Further investigation is needed to determine whether the 28 + 3d protocol provides adequate sensitivity for germ cells, or if another single sampling time would be more suitable.

TG 488 requires the use of a transgenic rodent model carrying a mutation reporter gene. Such models are limited in use and availability. The assay is restricted to scoring mutations in a non-transcribed exogenous gene that is heavily methylated. Although studies have suggested that care must be taken in extrapolating to other genomic regions [62], extensive empirical evidence indicates a high degree of concordance between endogenous and reporter gene mutation spectra and frequencies [63]. The assay may miss some types of mutations, including large deletions/insertions for some TGR loci, and rearrangements or CNVs. While this assay is performed on germ cells per se, not offspring, potential inheritance of mutations is inferred. Moreover, the heritability of transgenic mutations (i.e., the transmission of transgenic lacZ mutations to offspring) has been demonstrated in one study [64]. Furthermore, in support of the heritability of TGR mutations, it should be noted that the TGR germ cell assay detects chemicals that are also positive in the SLT [43], which supports the inference of a high probability for the identification of heritable mutations.

5.2. Expanded simple tandem repeat (ESTR) assays

ESTRs consist of long homogenous arrays of relatively short repeats (4–9 bp) that show a very high spontaneous mutation rate of length changes both in germline and somatic cells [65]. ESTR loci may be regarded as a class of expanded microsatellites, where the mechanisms underlying spontaneous mutation are replication-driven [66,67]. The very high spontaneous mutation rate potentially makes the analysis of length change mutations occurring at ESTR loci an attractive approach for monitoring germline mutation induction in mice. Since 1993, these loci have extensively been used for the analysis of mutation induction in the germline of male mice exposed to ionizing radiation, chemical mutagens, and anticancer drugs [68–74], in addition to environmental air pollutants [75–79]. In the early studies, ESTR mutations were detected using a pedigree-based approach by profiling DNA samples extracted from all parents and their offspring. Later, a more sensitive technique using single-molecule PCR was developed [80] . This approach involves diluting sperm genomic DNA, and amplifying multiple samples of this DNA (each of which contains approximately one ESTR molecule). This procedure permits the detection of an indefinitely large number of de novo mutants in DNA samples extracted from sperm or other cell types. Single-molecule sperm DNA analysis dramatically reduces the numbers of mice needed for the measurement of germline mutation frequencies, and the experimental time, by bypassing the need to wait for mating and birth. Moreover, this approach may be directly applicable to human studies [81–83].

The dose-response of ESTR mutation induction is very close to that previously obtained using traditional mutation scoring systems in mice, including the SLT. Statistically significant evidence for mutation induction is obtained by analyzing hundreds of mice using ESTRs; whereas, other systems require thousands or even hundreds of thousands of mice. ESTR mutation rate in the germline or offspring of male mice exposed to X-rays of fission neutrons increases linearly with radiation dose [70,71]. An increase in ESTR mutation rate is detectable at doses substantially lower than can be monitored by standard genetic techniques in mice. The alkylating agents ENU and iPMS cause a statistically significant increase in ESTR mutation rate in the offspring of exposed male mice [74], and increases in sperm ESTR mutation frequencies occur following exposure of male mice to commonly used anticancer drugs [73]. Importantly, mutation induction can be measured within the range of the clinically-relevant doses for humans for anticancer drugs. Thus, the assay is sensitive and shows great promise for assessing potential germ cell hazards.

Limitations: The assay scores mutations occurring in a very specific genomic context: tandem repeats. However, it should be noted that a growing number of repeat mutations are associated with (or causative of) human genetic disorders [84].

The mechanisms underlying ESTR mutation induction following exposure to ionizing radiation and chemical mutagens cannot be explained by direct targeting of these small loci by mutagens. Specifically, the observed increases in ESTR mutation rate in the offspring and germ cells of exposed male mice are too high to be attributed to the total number of DNA damaged sites within these loci. It has been suggested that ESTR mutation induction may

reflect non-targeted events, where the initial mutagen-related DNA damage occurring elsewhere in the genome somehow increases mutation rate at these loci [85]. So, whereas the mechanisms of this non-targeted process remain unknown, ESTR loci can currently be regarded as a useful biomarker of exposure to mutagens.

Because all mouse strains carry ESTR loci, the assay can be integrated with standard genetic toxicology tests in mice. However, mutations occur in replicating cells; thus, the relevant phase of spermatogenesis must be sampled, which would mean including an additional set of mice for the ESTR assay for appropriate timing of sample collection in standard genetic toxicology testing (see Fig. 1). The ability to score ESTR mutations in testicular cells sampled during standard genetic toxicity testing has not yet been investigated but should be a subject of future research.

The assay requires the PCR amplification of GC-rich repetitive regions of DNA from very low concentrations, which can be technically challenging for rodent strains with large alleles. Finally, ESTR mutation detection requires scoring band length shifts on autoradiographs, which can be subjective and vary across individuals and laboratories. This is generally resolved by requiring a generous shift in size (at least 1 mm), by blinded analyses of samples during mutation scoring, and by having two individuals score. However, capillary electrophoresis of smaller repeat loci will permit the analysis of smaller ESTRs/microsatellites and thus should eliminate any subjectivity [81]. This should be a focus of future research.

5.3. Spermatid micronucleus (MN) assay

The analysis of induced MN in somatic cells is one of the most widely used assays in regulatory genetic toxicology testing, and is the predominant in vivo assay implemented as a follow-up to positive results in vitro. MN are the product of chromosome damage and/or spindle malfunction, and there are existing OECD test guidelines for both in vitro (TG 487) and in vivo somatic cell testing (TG 474). The use of the assay has been greatly expanded by the development of flow cytometry-based methods that allow the interrogation of thousands of cells, thus providing a high sensitivity to detect small effects both in vivo [86] and in vitro [87]. There is a need to have an equivalent assay in germ cells.

An assay for detecting MN in spermatids of rats was developed in the 1990's [88]. The assay was subsequently adapted to detect MN in mouse spermatids and used to investigate the genotoxicity of several chemicals. MN detected by this assay originate during meiosis. About 25 chemicals have been shown to induce significant increases in MN in exposed mice, and interestingly, four of these chemicals (1,1,-dimethylhydrazine, beta-propiolactone, diethylnitrosoamine and dimethylnitrosoamine) were positive in spermatids but negative in bone marrow [89]. A previous IWGT workshop addressed the utility of the MN spermatid assay and its possible integration with analysis in erythrocytes [90,91]. Although very little work has been done on this aspect, the MN spermatid assay is amenable to integration with other genotoxicity tests such as the recommended experimental design for the transgenic rodent assay (i.e. 28 + 3d) or within an experiment aimed at assessing lacZ mutations in sperm and/or seminiferous tubules.

Limitations: The spermatid MN assay is rarely used because it is labor-intensive, and generally only a few hundred cells per sample are scored providing limited sensitivity to detect small effects. Therefore, an automated procedure for scoring MN as is routinely applied in somatic cells and in vitro should be a focus of future research. A flow-cytometry based method is being developed in which spermatids are first isolated by flow sorting based on DNA content, and then nuclear preparations are analyzed by flow cytometry to detect MN as described for the in vitro MN assay. As in the somatic cell method, a flow-cytometry approach would allow the analysis of several thousands of spermatids per sample providing exquisite sensitivity to detect small effects. Finally, the fate of a sperm cell carrying MN is unclear, and it is unlikely that these would be inherited (the same is true of somatic cells). Nevertheless, the assay provides evidence of genotoxicity in germ cells.

5.4. Sperm comet assay

The comet assay is a simple method for measuring DNA strand breaks in single cells [92]. The OECD has recently adopted a test guideline [93] for conducting the in vivo alkaline comet assay to detect DNA damage. Many chemical and physical genotoxicants have been analyzed using this protocol both in vivo and in vitro, and it is generally used to demonstrate a potential for genotoxic hazard from an exposure [94,95]. Although it has been much more widely used in somatic cells, the assay has been conducted both on mature sperm and on germ cells isolated from the seminiferous tubules [96]. The assay has been applied in numerous studies to demonstrate induced DNA damage in sperm for exposure to genotoxic agents [97]

Limitations: During the development of the OECD in vivo comet assay test guideline, extensive discussions were centered on whether germ cells should be included. However, it was decided that the standard alkaline comet assay as described in the test guideline is not appropriate for measuring DNA strand breaks in mature germ cells. Three factors were considered in reaching this conclusion. First, the proposed exposure regimen for the in vivo comet assay (3 daily doses followed by sample collection 3–6 h later) is not appropriate for sperm because it represents exposure of only the fully mature sperm in which the DNA is highly compacted by protamines. At this stage of development, sperm are extremely resistant to DNA damage [98,99]. Second, analysis of germ cells collected from the seminiferous tubules is not fully validated yet, and only a few studies have applied this approach [96]. In addition, cells collected from the seminiferous tubules contain two different germ cell populations (spermatocytes and elongating spermatids) where DNA double strand breaks are part of the normal process of development (meiotic recombination for the former, chromatin compaction in the latter). Slight variation in the proportion of cells that are analyzed between controls and exposed may produce an apparently significant effect that is coincidental and not related to chemical exposure. Third, comet analysis in mature sperm after a prolonged exposure (i.e. 28 days) could provide some relevant information on whether a chemical induces DNA in germ cells. The method for the comet assay in sperm is more complicated than in somatic cells because it requires an enzymatic digestion to relax the chromatin, and sperm are extremely rich in alkali labile sites. Because of these factors, it is much more difficult to obtain reproducible results with mature sperm. Thus, extensive further validation and development is needed before the comet assay can be routinely employed for determining whether a chemical induces DNA damage in germ cells for regulatory purposes. As with other assays described, the assessment of DNA damage via the comet assay in germ cells does not detect heritable effects but does provide evidence of genotoxicity.

5.5. Sperm chromatin quality assays

Surprisingly, assays that assess chromatin quality are in a more advanced state of validation in humans than in laboratory animals. Assays are available that assess biomarkers of chromatin integrity in human sperm such as DNA damage (i.e. breaks and cross-links), chromatin template function, chromatin structure, and chromatin epigenome [100]. In addition to the comet assay, the sperm chromatin structure assay (SCSA) and the terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL) are among the assays most commonly

Table 2
Summary of the advantages and disadvantages of the newest technologies available to detect germline mutations.

Endpoint	Advantages	Disadvantages
Copy number variant analysis using array CGH and SNP chips	Major phenotypic effects, inherited mutation, relevant to human genetic disease.	New endpoint with no data in germ cell toxicology, currently expensive to measure, requires pedigrees, so far not suitable for measuring somatic mutation in vivo so no direct comparisons can be made, needs extensive validation for work in toxicology.
Whole genome sequencing	Measures broad spectrum of mutations, inherited mutations, clear linkages to health can be made for certain mutations, any species including humans.	Expensive, currently requires pedigrees for interpretation (i.e., sperm analysis not ready yet), bioinformatics challenges, not applied in toxicology yet (no database), extensive validation still required.
HTS for egg aneuploidy (*C. elegans*)	Inexpensive, fast, established model organism in genetics, high degree of conservation in relevant pathways, detects effects in female germ cells.	Relationship to humans is unclear, limited to aneuploidy in eggs measured in embryos at this time, not validated.

used to assess sperm DNA integrity. The SCSA is a flow cytometry based assay first developed over 30 years ago [8] that measures the sensitivity of sperm DNA to acid-induced denaturation. The extent of DNA denaturation is thought to be correlated with the presence of single stranded DNA and is highly associated with infertility [101]; thus, it is potentially indicative of genotoxicity. The TUNEL assay measures DNA breaks in situ as assessed by the incorporation of dUTP at the sites of breaks [102]. Although these assays generally correlate well with each other, they measure different aspects of DNA integrity; therefore, they have different sensitivities. To this end, an international effort is underway to standardize the comet, SCSA/acridine orange and TUNEL assays. This initial validation exercise, which involves about 10 established laboratories around the world, aims to develop fully validated protocols that are sufficiently robust to assure transferability of the assays across laboratories and a high degree of data reproducibility. Once validated in humans, these assays can be readily applied to animal models to provide a fast and sensitive approach to assess the effects of environmental exposure on sperm DNA integrity.

Limitations: Although significant research has been conducted on human sperm DNA integrity over the past decades, our understanding of the mechanisms and consequences of sperm chromatin damage is still incomplete. These assays measure chromatin changes (i.e. premutagenic lesions) in germ cells only, and it is not clear what the implications to offspring are. There are indications that sperm chromatin integrity contributes to healthy pregnancy and offspring health [103–106]. However, there is still a lack of consensus on the cut-off values that identify clinically abnormal parameters. There is also a substantial degree of biological and technical variability that can result in differences across studies/laboratories. The validation exercise for human sperm is expected to provide guidance on both these current limitations that are likely to be applicable (with adaptation) to future rodent based assays.

6. New technologies and approaches

The field of germ cell mutation research is currently undergoing renewed focus predominantly because of the promise of powerful new genomics technologies. There is a great deal of enthusiasm over the opportunities that these new tools bring to the field [107,108] and abundant applications in the clinic demonstrate their power in identifying de novo mutations that cause severe human genetic disorders (e.g. [4,23]). In parallel with increasing sequencing capabilities, a large amount of effort has been put forth to develop high-throughput screening (HTS) tools to identify pathway perturbations [109,110]. HTS is expected to increase chemical testing capacity and greatly reduce animal use. Given that it is not currently possible to carry out a full cycle of gametogenesis in vitro, alternative models must be considered. The potential utility of an HTS *C. elegans* model for egg aneuploidy that shows promise is described below. A summary of the advantages and disadvantages of the new technologies is provided in Table 2.

6.1. Copy number variants

Research in genomics has led to the discovery that approximately 12% of human genetic variation is attributed to CNV [111]. CNVs are a type of structural variation that alters, and in many cases rearranges, the number of copies of specific segments of DNA. CNVs range in size from 50 base pairs to megabases [112,113]. It is widely recognized that CNVs account for a broad range of human genomic disorders [112,114–116]. This can be attributed to the high mutation rates for genomics rearrangements, which affect >1000-fold more nucleotides than point mutations [112]. For example, a genome-wide analysis of CNVs (>100 kbp) in approximately 400 parent- offspring trios found a mutation rate of 1.2×10^{-2} CNVs per generation [117]. Overall, it is apparent that de novo CNVs represent an important source of human genetic diversity that contributes to genetic disorders, and is not captured by existing test methods (for more details see Section 8).

The detection and analysis of CNVs has been greatly facilitated by the development of high- resolution array comparative genomic hybridization (or aCGH) and SNP (single nucleotide polymorphism) microarray technologies [118,119]. These array-based methods are now being used in the clinic to identify the sources of idiopathic diseases [120–124] and are the main technologies used to identify CNVs. However, very little work has been carried out to explore the effect of mutagens on CNV formation. Work in human cells in culture has shown that exposure to chemicals causing replication stress can lead to the formation of CNVs [125–127]. This work includes exposure to hydroxyurea and aphidicolin, in addition to exposure to low doses of ionizing radiation (whereby CNVs are induced through a replication-dependent mechanism, as opposed to replication-independent repair of double strand breaks). In addition, increasing paternal age is associated with increases in de novo CNVs in their offspring through replication-based mechanisms [12]. However, no single comprehensive study has yet undertaken an analysis of induced germline CNVs resulting from mutagen exposure either in an animal model or in humans. Thus, despite promise, the application of this technology to this field is still in its infancy.

The workshop participants agreed that research exploring the effects of mutagens on germ cell CNVs is a critical avenue of research given the high frequency with which CNVs occur, their importance in explaining a large proportion of human genetic disease, and the lack of assessment of CNVs using any of the current technologies.

Limitations: This technology has yet to be applied in the study of induced effects in germ cells or somatic cells in vivo. Thus, extensive development and validation is needed. In addition, the technology is still expensive, and large sample sizes (>100 offspring per group) will be required. The technology must be applied to offspring at

this time, thus parental genomes must be analyzed in addition to the offspring. The participants acknowledged that this should be an area of future research focus.

6.2. Whole genome sequencing

The workshop participants generally agreed that next generation sequencing technologies have matured to a stage where they can be applied to study the effects of mutagens on heritable germ cell mutations. The technologies and bioinformatics tools that have been developed now provide a cost-effective approach to study induced germ cell mutation in a reasonable time-frame. Proof-of-concept is provided in a landmark paper by Kong et al. [10], in which full genome sequencing of 78 Icelandic family trios was used to demonstrate that males pass on an average of two additional mutations to their offspring for each year of their reproductive life, suggesting that the father's age is a dominant factor determining the number of de novo mutations in the child. In addition, the technology is now being used much more routinely in the clinic. Genome-wide mutation spectra and frequencies in rodent models should be comparable to humans, and bioinformatics tools can be used to determine potential phenotypic consequences to the organism. The recommended strategy to develop the appropriate sequencing methodologies for applied genetic toxicology is outlined in a manuscript published by the ENvironmentally Induced Germline Mutation Analysis (ENIGMA) working group in 2013 [4]. There was much enthusiasm among workshop participants in the application of new sequencing tools, and it was recommended that this be a high priority area for applied research.

Limitations: Despite rapid declines in cost, the technology is still expensive. However, in contrast to CNVs, smaller sample sizes should generally be required. The technology currently requires pedigrees for analysis, increasing the overall number of samples required and the length of time required. This will be improved once technologies are available to accurately sequence a single gamete genome. Bioinformatics challenges exist that relate to handling/storing the large amounts of data and applying the appropriate filters to remove sequencing artefacts without compromising sensitivity. Full genome sequencing has not been applied in toxicology yet and thus there is no existing database. Extensive validation will be required [4].

6.3. High-throughput analysis of egg aneuploidy in C. elegans

Significant resources are being invested in the development of HTS tools to identify chemicals that perturb molecular pathways that are relevant to human and environmental health (e.g., [4,109]). A major gap in the existing HTS assays is the detection of mutagens and aneugens. The existing assays are limited to assessing the ability of a toxicant to initiate a DNA damage response and demonstrate low sensitivity for identifying mutagens and tumorigens [128]. In addition, mutagenic effects on germ cells are not considered. The working group acknowledged the importance of this gap and suggested that some efforts should be focused on determining the best way to balance the need to understand potential chemical effects on germ cells with higher-throughput, less animal-intensive methodologies.

One assay that partially addresses this gap is a new screening tool in *C. elegans* to measure chromosome segregation errors occurring in eggs [129]. Roundworms offer several advantages for this application because they have a large proportion of germ cells, a short generation time, and are suitable for culturing in 96-well plate format. In addition, there is a good degree of conservation between *C. elegans* and humans in key meiotic pathways and it is an established model system in genetics. In the assay, aneuploidy is examined by observing X-chromosome mis-segregation during meiosis. Embryos that inherit only one X-chromosome are marked by the expression of green fluorescent protein under the control of the X-chromosome counting promoter xol-1. Exposure is performed in 96-well plates, and a 384-well high-content fluorometric approach is used to score the number of aneuploid embryos. The assay takes approximately four days, and given the integration of robotics and culturing in plates, hundreds of chemicals can be analyzed over a very brief timespan. This high-throughput assay is followed by other fast assays, such as DNA staining of the germline, and a germline apoptosis assay, to ensure that aneuploidy originated from disruption of germline processes.

Although the assay is in its infancy and will require further validation, preliminary analysis of a selection of 50 chemicals from ToxCast phase 1 and known chemicals revealed a maximum balanced accuracy (representing the average of sensitivity and specificity) of 69% in predicting the ability of chemicals that cause reproductive toxicity in rodents [9]. The technology was viewed favorably by the workshop participants, and it was recommended that this assay be considered for integration with HTS assays as part of tier 1 screening. It was noted that the model could relatively easily be expanded to apply whole genome sequencing or CNV analysis. Finally, the assay addresses a critical gap in the field: measurement of effects on female germ cells.

Limitations: Although there is some degree of conservation in relevant meiotic and other pathways, the relationship of aneuploidy in *C. elegans* to the same potential outcome in humans is unclear. Issues were also raised relating to pharmacokinetic and dynamic considerations. At this time, the assay is limited to assessing aneuploidy in early embryos.

7. The "blood-testis barrier" and pharmacokinetics in male germ cell toxicity/genotoxicity

Pharmacokinetic and dynamic parameters are important considerations in toxicological testing to determine the extent of exposure of particular cell types/tissues. Within the testes, exposure is affected by the presence of the blood-testis barrier that will result in differential exposures of the various germ cell phases. The testis presents three possible barriers for blood-borne substances to reach the germ cells in the seminiferous epithelium (Fig. 2). These are at the testis vasculature itself, the peritubular myoid cells of the basement membrane, and the tight junctions of the Sertoli cells separating the basal and adluminal compartments of the seminiferous epithelium. Although the nomenclature "blood-testis barrier" is still widely used, unlike the blood-brain barrier, which is actually at the vasculature, the most effective barrier observed in the testes is the one formed by the Sertoli cells.

These barriers divide the testes into three compartments. The interstitial compartment is just outside the vasculature and contains Leydig cells, macrophages, and endothelial cells surrounding the tubules. The boundary between the interstitial and the basal compartment of seminiferous tubules is formed by the basal lamina containing peritubular myoid cells. Within the seminiferous tubules, the basal compartment (containing stem and differentiating spermatogonia and very early spermatocytes) is separated from the adluminal compartment (containing the rest of the spermatocytes, the spermatids, and the spermatozoa that will be released into the epididymis and the ejaculate) by occluding junctions between adjacent Sertoli cells.

Toxicants passing from the testis vasculature into the interstitial compartment cannot be germ cell genotoxicants unless they also pass through the basal lamina of the seminiferous tubules. Genotoxicants reaching the basal compartment of the seminiferous tubules would have a cumulative and permanent effect if they act on stem cells. In addition they would have an acute, but temporary,

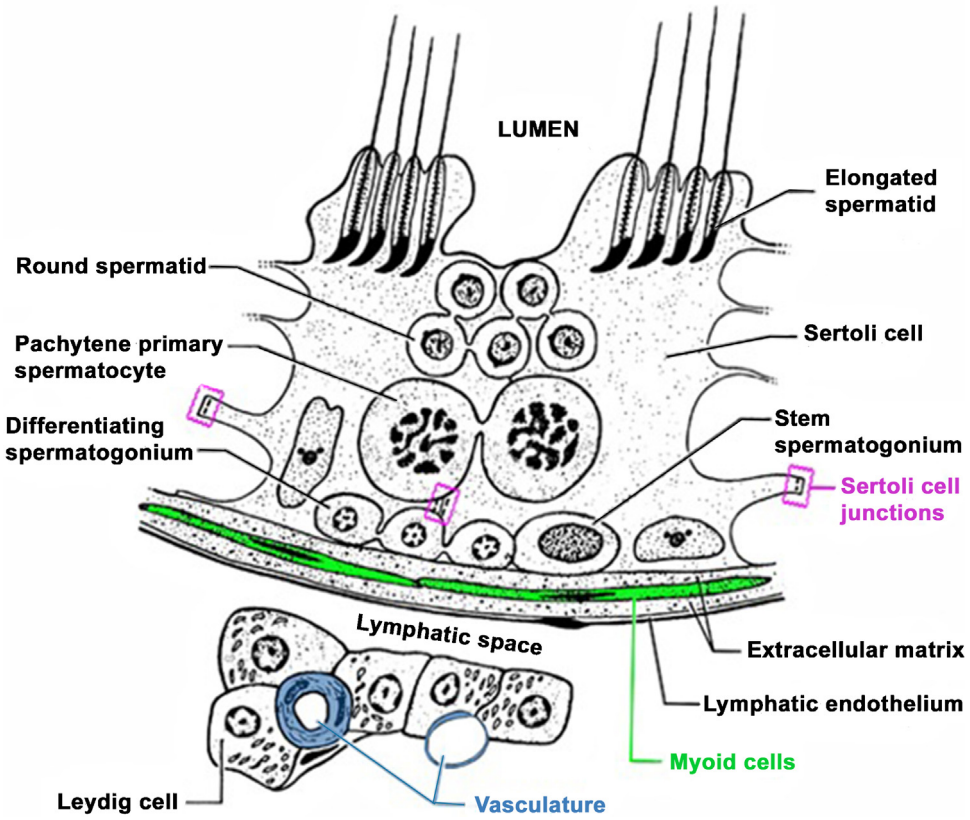

Fig. 2. Barriers to drug diffusion within testis. Vasculature is indicated in blue, peritubular myoid cells in green, and tight junctions between Sertoli cells in red. (Modified from Meistrich, M. Brit. J. Cancer, 55: Supl. VI, 89–101, 33).

effect if they act on differentiating spermatogonia; the effect would remain constant after an exposure time equal to the lifetime of these cells and not increase under further continuous exposure and would disappear soon after exposure to the genotoxicant ceases. The same would hold true for genotoxicants passing through the Sertoli cell barrier and reaching meiotic and post-meiotic germ cells, although the lifetime of these cells would be 8 weeks in human.

Testis capillaries are much more permeable than those in the brain and contribute little to the blood-testis barrier. Most small molecules will pass through the testis capillaries at a similar rate as through capillaries of other tissues, but there is some evidence that acriflavine (MW 259 Da) has limited diffusion and some proteins (MW ~ 30 kDa) have lower concentrations in the interstitial fluid than in plasma [130].

The peritubular myoid cells form a sheet around the seminiferous tubules. In rodents there are occluding junctions between some but not all myoid cells, but in primates there are no such junctions. Many small molecules readily penetrate the myoid cell layer, but proteins (e.g. 44 kDa) only penetrate 15% of tubules in rodents; however, proteins penetrate all tubules in monkeys [131,132].

The major barrier to passage of molecules is the tight junctions between Sertoli cells. It is this barrier that results in the difference in ionic composition in the fluid in the lumen of seminiferous tubules (high K^+, low Na^+) from that of the blood plasma [133]. It also forms a barrier to small molecules and proteins.

The main factors limiting a molecule's passage through the Sertoli cell barrier are its molecular weight, charge, and hydrophilicity; lipophilic molecules apparently pass through the cell membranes and around the junctions more readily. For example, tubular concentrations of lipophilic molecules like thiopental (MW 242 Da) will nearly equal their plasma concentrations within 1 h; whereas, the hydrophilic molecule inulin (MW 5 kDa) will only reach 2% of the plasma concentration [134]. A smaller hydrophilic molecule like urea (MW 60 Da) will reach 70% of plasma concentrations within the tubular lumen in 1 h. Tubular luminal concentrations of genotoxicants like methyl methane sulfonate and busulfan will reach 15%–100% of plasma concentrations in 1 h [135]. Data on DNA adducts from ethylmethanesulfonate show that it forms adducts in germ cells at the same level as in bone marrow, and adducts are detected in late spermatids [136,137].

There are several additional mechanisms affecting the transport of potential genotoxicants into the tubules and the adluminal compartment. For example, active transport or facilitated diffusion with a carrier brings potassium, iron, testosterone, and GM-CSF into the adluminal region [138]. On the other hand, cellular efflux proteins in vascular, peritubular myoid, and Sertoli cells produce partial protection of germ cells from genotoxicants [139]. Furthermore, exposure of the testis to toxicants such as busulfan can increase the porosity of the Sertoli-cell barrier [140], resulting in more exposure of germ cells to this and other toxicants.

Although stem cells are an important target for cumulative and permanent genotoxic damage, it is not possible to measure concentrations of toxicants in the basal tubule compartment or to isolate these cells for measurement of damage. Since the peritubular myoid cells do not form a highly restrictive barrier, measurement of levels of the toxicant in the testis interstitial fluid will give an approximation (possibly an overestimate) of the levels at the stem cells. Killing of the differentiating spermatogonia, which are extremely sensitive to cytotoxicity from anticancer drugs and alkylating agents, or cytogenetic damage to these cells can also be used as qualitative evidence that a genotoxicant has reached the basal compartment of the seminiferous tubules.

Table 3
Summary of the spectrum of de novo genomic changes occurring in humans and associated tests that can be used to measure them.

Endpoint	Relevant genetic toxicology test
Aneuploidy	Sperm and egg FISH, spermatocyte and oocyte cytogenetics, pedigree DNA microarray or deep sequencing, spermatid MN.
Structural aberrations	Early embryo cytogenetics, sperm FISH, DLT, HTT, some can be identified by pedigree analysis using array CGH, spermatid MN, spermatocyte cytogenetics.
Copy number variants	Pedigree array CGH (microarray) or deep sequencing.
Small molecular rearrangements	Array CGH (as small as 500–5000 bp), pedigree deep sequencing.
Small insertions/deletions	GPT delta transgene mutation (TGR assay), pedigree sequencing.
Tandem repeat gains/losses	ESTR and microsatellite mutation analysis in sperm or pedigrees.
Gene mutations	TGR (OECD TG 488), pedigree DNA deep sequencing.
Non-coding mutations	Pedigree DNA deep sequencing, CNV analysis.

The post-spermatogonial stages are highly sensitive to mutagenic effects [141] since they are undergoing meiosis as well as chromatin remodeling, and they lose DNA repair capabilities. The simplest way to estimate the dose reaching these cells would be to measure concentrations of the genotoxicant in the soluble contents of the seminiferous tubules after removal of the interstitial fluid. However, there may be some overestimation due to retention of some of the agent in the small basal compartment of the tubules. Alternatively, whole testis measurements of the genotoxicant could be performed with correction for the interstitial fluid concentration and volume of interstitial space. More difficult, but more precise, measurements can be done by directly measuring the concentrations in seminiferous tubule fluid; however, measurement of the rete testis fluid may be an alternative.

This information is important for evidence as to whether or not a chemical, or its metabolite(s), reaches target germ cells, which affects the need to conduct germ cell mutagenicity testing.

8. Endpoints most relevant to human genetic risk

Major discussions by the work group were centered on the concept of whether the existing assays (and those in the pipeline) effectively capture the spectrum of mutational events that both occur in humans and are relevant to human health. New genomic tools have allowed for the unprecedented opportunity to assess genome-wide rates of mutation empirically. A comprehensive review of the human germline mutational landscape is given in [112]. Full genome sequencing in human families has been used to directly measure rates of de novo mutations, demonstrating that rates of single nucleotide variants (SNVs) range from $1–1.2 \times 10^{-8}$ SNVs per generation [10,111,142–144], and that 76% of SNVs originate in the paternal lineage. In contrast to SNVs, both the per locus mutation rate and the overall number of nucleotides affected per generation are considerably greater for CNVs [112]. For example, it has been approximated that one large de novo CNV (>100 kbp) occurs per 42 births in humans, compared to an average of 61 new SNVs per birth; however, the average number of base pairs affected by large CNVs is 8–25 kbp per gamete versus 30.5 bp per gamete for SNVs [117]. Moreover, CNVs are often caused by complex chromothripsis events; these mutations involve multiple de novo rearrangements in a single event [111,145–147].

In addition to SNVs and CNVs described above, which may affect coding and non-coding DNA sequences, there are various other types of important functional genomic changes that arise in the human genome; these include small insertions and deletions, mobile element insertions, tandem repeat mutations, translocations and aneuploidies. Microsatellites, in particular, exhibit proportionally higher de novo mutation rates than SNVs, providing an important source of genetic variation [12]. Campbell and Eichler showed that the per generation rates of SNVs, CNVs, mobile element insertions, and aneuploidy, when contrasted with the total number of base pairs affected per gamete, demonstrate an inverse correlation between mutation size and frequency (see Fig. 1 [112]). The figure shows that although more rare, the number of nucleotides affected by large genomic changes including CNVs and aneuploidies is orders of magnitude greater. Overall, an analysis of the rates and spectrum of human mutation reveals a diverse array of important genomic events that should be considered in genetic toxicology which are not currently captured in standard genetic toxicology batteries. Table 3 provides an overview of the endpoints and considers what assays may be used to assess them.

Finally, it is important to note that human epidemiological studies have focused on the ability to measure the phenotypic effects of induced dominant mutations occurring in the descendants of exposed parents. Recent clinical work using advanced genomics technologies has revealed that a large proportion of the mutations occurring in humans are recessive and are not manifest as phenotypes for several generations post-origination until conception occurs with a complementary mutation affecting the same locus [111], or such a mutation occurs in a somatic cell. This should be considered in future study designs.

9. Regulatory requirements for germ cell tests worldwide

Strategies and guidelines for regulatory toxicology testing in various national regulatory jurisdictions, including requirements for germ cell mutation assays, were described extensively by Cimino [148], and have not changed significantly. No jurisdiction requires germ cell testing in an initial test battery (e.g. Tier 1 below).

Genetic toxicology testing strategies across regulatory agencies can generally be separated into three tiers. Tier 1 contains required in vitro and somatic in vivo tests; while, tiers 2 and 3 contain germ cell tests that can be requested for follow-up studies, under certain conditions in many regulatory authorities, e.g. in the U.S.A. (U.S. EPA and U.S. FDA), Canada (Health Canada), the United Kingdom (Committee on Mutagenicity: COM), and Europe (Registration, Evaluation, Authorization and Restriction of Chemicals: REACH). DNA damage assays in the testes or spermatogonia fall into tier 2; whereas, tier 3 assays involve germ cell mutation tests. India and Australia use only tier 1 assays; therefore, they do not require any germ cell assays for regulatory purposes. Other countries generally follow strategies similar to the U.S. EPA guidelines for industrial chemicals. For pharmaceuticals, the ICH Technical Requirements for Registration of Pharmaceuticals for Human Use does not require germ cell tests and assumes that in vivo somatic tests and carcinogenicity data will provide sufficient predictivity/protection for germ cell effects [149].

As described by Eastmond et al. [6], the World Health Organization (WHO)/IPCS Harmonized Scheme notes that if an agent is positive in vivo for somatic cell mutation, then that agent can be considered, or requested, for testing for germ cell mutations; however, such testing is not required. In addition, WHO/IPCS identifies the following as suitable assays in germ cells: transgenic mouse models, the ESTR assay, the spermatogonial chromosome aberration assay, chromosome aberration analysis by FISH, the comet

Table 4
Categorization of mutagens by OECD/GHS/ECHA.
Unique aspects of gametogenesis

Category	Description
1A	Chemicals known to induce heritable mutations in germ cells of humans
1B	Chemicals that should be regarded as if they induce heritable mutations in germ cells of humans
2	Chemicals that cause concern for induction of heritable mutations in germ cells of humans

assay, and assays for DNA adducts. The WHO/IPCS tests in offspring include the ESTR assay, the DLT, the HTT, and the SLT.

The Global Harmonization Scheme (GHS) [150] identifies mutagens according to the categories noted in Table 4. To date 67 countries have implemented this programme and are in the process of integrating it into their relevant regulations. Within the European REACH strategy an agent that is genotoxic in somatic cells is evaluated from the literature to see if it is a potential germ cell mutagen based on bioavailability to the germ cells and appropriate in vivo data. If such an evaluation shows that the literature is insufficient to determine whether the agent is or is not a potential germ cell mutagen, then that agent can be tested in a suitable germ cell genotoxicity assay. Although germ cell testing is not specifically required under the Canadian Environmental Protection Act (CEPA) New Substances Notification Regulations, germ cell mutation tests are requested and evaluated when necessary. For assessments under the New Substance Program from 1994 to 2012, a total of 19 chemicals have been evaluated for germ cell mutagenicity (12 for which the test was submitted, plus 7 for which the test was referenced on the MSDS); this is comparable to the number for which testing in rodent cancer assays was evaluated (i.e. total of 20:17 for which the test was submitted, plus 3 for which test was referenced on the MSDS) (Personal Communication, New Substances Assessment and Control Bureau, Health Canada).

In summary, germ cell mutation is a regulatory endpoint for many organizations, including the IPCS, the regulatory agencies in the U.S., Canada, U.K., and European Union (E.U.). Germ cell mutagens are classified in a manner analogous to that of carcinogens by IARC by Health Canada, GHS, European Chemical Agency (ECHA), and the German Commission for Occupational Health (MAK). Although germ cell mutation is an established regulatory endpoint, the appropriate assays exist, and more than 50 agents have been identified as germ cell mutagens in rodents, no agent has yet to be regulated solely as a germ cell mutagen, or evaluated to be a human germ cell mutagen. As data accumulate showing that cigarette smoke, air pollution, and ionizing radiation are likely human germ cell mutagens, this situation is expected to change soon [2].

10. Discussion

The following questions were discussed at the IWGT workshop. Unless indicated otherwise, a consensus was achieved on the resulting statements below.

10.1. Do genotoxicity and mutagenicity assays in somatic cells predict germ cell effects?

To address this question, the workshop participants revisited a retrospective analysis that was undertaken to assess the performance of selected short-term tests in the discrimination of mammalian germ cell mutagens and non-mutagens [151]. The analysis considered 1080 references with results on germ cell mutagens, and 911 references with results on germ cell non-mutagens. Based on the primary literature available at the time, 23 multi-test, confirmed germ cell mutagens were identified (7 additional chemicals were positive only in a single replicated test). All 23 mutagens were positive in the DLT (15 in mouse, 1 in rat, and 7 in both mouse and rat). Twenty-one multi-test, confirmed germ cell non-mutagens were identified (6 more were negative only in a single replicated test). All 21 chemicals were confirmed negative in the DLT (11 in mouse, 2 in rat and 8 in both). The report suggested the value of the mouse bone marrow MN test as an assay that is predictive of potential germ cell mutagenicity based on mouse DLT results (as confirmation of germ cell damage). Furthermore, regression analysis of the lowest effective doses tested in the two assays demonstrated that the mouse MN test responded at a lower dose than the mouse DLT. Overall, a high degree of sensitivity was found (>90%) for the prediction of germ cell mutagenicity from mouse MN in somatic cells, but it was noted that this may be due to the type of chemicals considered. Specificity was much lower (63–64%), indicating that a proportion of chemicals that was not positive in the DLT was positive in the MN assay in somatic cells. Unfortunately, a more adequate database does not exist at present, and no more recent analyses have been conducted. The current database has some limitations. (a) It is biased towards chemicals that were first found positive in a somatic cell test and is skewed towards alkylating agents. (b) It is limited to a few specific mutational mechanisms or mutation types including SNVs and larger scale chromosomal rearrangements. (c) The database does not include the more recent OECD TG 488 data or other newer germ cell tests. (d) Data are deficient in recently recognized endpoints like CNVs. (e) Lastly, the database contains a number of qualitative and quantitative exceptions as follows: (i) dominant lethal mutations following acrylamide exposure and germ cell tandem repeat mutations following exposure to mainstream tobacco smoke occur in the absence of significant increases in bone marrow or blood MN [5,75]; (ii) four chemicals (1,1-dimethylhydrazine, beta-propiolactone, diethylnitrosoamine and dimethylnitrosoamine) were negative in the bone marrow MN assay, but were found to be positive in the spermatid MN assay [89]; and (iii) three agents, MMS, acrylamide, and ionizing radiation showed quantitatively greater clastogenicity in exposed sperm (detected as chromosomal aberrations in zygotes) than in bone marrow of mice [152–156].

Overall, the workshop participants acknowledged that based on the available (limited) data, somatic cell tests appear to predict germ cell effects quite well but with relatively low specificity. However, there are notable exceptions and of particular concern is the broad spectrum of new mutational endpoints that are emerging as critical to human health that are not captured by existing tests. Accordingly, caution should be exercised at present in drawing broad conclusions around the question of the predictivity of somatic in vivo tests for mutagenic effects in germ cells. More work needs to be done to expand the available database to include the most relevant tests and endpoints, to understand the prevalence of exceptions, as well as to expand beyond the presently biased chemical space covered in the database, prior to drawing a firm conclusion on this question. Finally, it was suggested that retrospective analyses be conducted that consider more quantitative metrics (e.g. mathematical models to derive points of departure – see more details in Section 11). Such refined quantitative analyses can provide more information on the low end of the dose-response curve to yield an improved understanding of the toxicological sensitivity of germ cells versus somatic cells, and to determine if there are true hazards for heritable effects based on animal data.

10.2. Should germ cell tests be done, and when?

As with other target tissues, if there is no significant risk that humans or their germ cells will be exposed, then there is no need for

germ cell testing. Furthermore, in accord with the approach in the OECD test guidelines for somatic in vivo tests, if there is evidence that a chemical, or its metabolite(s), will not reach target germ cells or gonadal tissue, it is not appropriate to conduct germ cell tests with either in vivo positive or negative somatic mutation outcomes. However, because of uncertainties discussed regarding the predictivity of somatic cell assays, it was recommended by a very large majority that negative somatic cell mutagens with clear evidence for gonadal exposure and evidence of toxicity in germ cells could be considered for germ cell mutagenicity testing, which would also serve to fill critical data gaps. Signals of concern from repeat dose toxicity tests and reproductive toxicity tests include germ cell loss in testicular histopathology or decreased sperm counts, any evidence of reduced fertility and/or implant loss in mating studies, and especially increased post implantation losses. For somatic mutagens that are known to reach the gonadal compartments and result in the exposure of germ cells, a large majority agreed that it could be assumed that the test substance is likely a germ cell mutagen without further testing. Nevertheless, germ cell mutagenicity testing would still be needed if quantitative germ cell risk assessment is required, since there are data indicating that germ cell effects occur at lower doses than somatic cell mutagenicity, or within endpoints not currently studied in somatic cells.

It was noted that the male germ cell compartment is rarely included in PB/PK studies. The group recommends that more consideration be given to germ cell exposure, and that male germ cell compartment should become part of the PB/PK measurements or modelling.

10.3. What new assays should be implemented and how?

This discussion expanded to include various points relating to what assays should be recommended as we move forward. Clearly, there are existing assays that need further development/validation as well as new assays that can be recommended as previously discussed. The workshop also considered what existing reproductive toxicology assays should be used to identify potential germ cell hazards, which is a shift in thinking in this field. In addition, within these discussion points, the participants recommended what assays should be integrated with existing tests.

10.3.1. Germ cell genotoxicity/mutagenicity tests

Based on discussions pertaining to the limitations of existing assays and the human mutational spectrum assessed by these assays, the participants noted that there is an immediate need for research on the application of whole genome sequencing in heritable mutation analysis and for genome wide assays of de novo mutational events (both SNV and CNV). The group agreed that integration of whole genome sequencing with existing assays is best facilitated through reproductive toxicology testing (see Section 10.3.2 for this discussion). The egg aneuploidy assay in *C. elegans* was also endorsed as a promising avenue for tier 1 high throughput screening.

However, in parallel with the application of these new assays, there is a perceived, immediate need for the currently available germ cell tests to be integrated with somatic cell genotoxicity tests to address gaps in this field. Specifically, it was recommended that further research is needed to develop an integrated approach for the TGR assay (to assess both somatic and male germ cell mutation concurrently), and the spermatid MN assay (with the somatic cell MN test). The integration of germ cell gene mutation analysis using the TGR assay (TG 488 [41]) was noted as a particular opportunity to expand our existing database of knowledge relating to germ cell versus somatic cell effects. A large majority of participants felt that it should be recommended that the relevant germ cell tests be run routinely in parallel with somatic cell tests when conducting transgenic mutation studies. Objections to this were based on the need to have a stronger database before this recommendation can be made (thus, requiring more research). Some discussion also centered on the possibility of capturing more endpoints of relevance by analyzing clastogenicity within the same TGR animals. A large majority of participants recommended the use of the spermatid MN assay for this purpose; they also felt that a germ cell chromosomal aberration assay should be carried out in parallel with bone marrow chromosome aberration assays. Finally, given the rapid pace of implementation of sperm chromatin damage assays in the clinic, a general need exists to standardize the protocols for the sperm comet assay, TUNEL and the SCSA to provide readily comparable rodent and human data. There was consensus that integration of these tests with sub-chronic assays in both genetic toxicology and reproductive toxicology is an obvious opportunity and starting point.

The purpose of the above recommendations is to enhance understanding of what constitutes a germ cell mutagen. The approaches proposed above provide a toolbox of options that the participants recommend can be used to address the existing gaps. In particular, there is an urgent need for a more mechanistically diverse database. This will require continued integration of germ and somatic cell genetic toxicology tests. However, the group expressed concern over where the resources might come from to address these various gaps and build (enhance) the database.

10.3.2. Reproductive toxicology tests

There was unanimous consent that current one-generation and multi-generational reproductive toxicology tests provide endpoints of relevance to germ cell genotoxicity in addition to other possible reproductive effects (e.g. endocrine effects). The relevant endpoints noted were: (a) detrimental effects on fecundity/fertility (number of successful litters and number of pups per litter); (b) severe effects on sperm count; (c) altered testicular histopathology; and (d) decreased testicular weight. It was also agreed that alterations to sperm morphology do not provide information on germ cell genotoxicity (but may indicate that a chemical can reach the testes). There was consensus that if there was a significant reduction in male reproductive performance, the chemical should be assessed using a DLT approach (where assessment of male mediated post-implantation loss is quantified after the males have been dosed for an appropriate period of time, see Fig. 1). At its simplest, this could be by mating males after they have been dosed for all of spermatogenesis, which could be integrated with current repeat dose toxicity tests [58] or reproductive toxicity tests. Overall, the group recommended that an important retrospective analysis would be to study the existing reproductive toxicology databases in detail to mine the data on well-known compounds and identify associations between reproductive toxicology endpoints and germ cell mutagenesis. It was recommended that a consortium consider this as a potential project that would be of great value to this field.

Although there was general enthusiasm for the application of whole genome sequencing to pedigrees collected as part of standard reproductive toxicology testing, the participants noted that it is too early to make such a recommendation and acknowledged the need for basic research on this application first. The group unanimously supported the promotion of such experiments as part of future research programs and recommended that appropriately frozen specimens begin to be collected from standard reproductive toxicology assays for such purposes. However, modifications to existing protocols are likely required for effective analysis of germ cell mutations in pedigrees from reproductive toxicity assays, which relates to the requirement to haplotype new mutations to assign parental origin. This is difficult in inbred rodents (which would lack the SNVs required for haplotyping) and would require the use of two different strains in a reproductive toxicology assay.

Finally, workshop participants noted that studies of effects in females are a major gap. It was unanimously recommended that maternal exposures (in utero in addition to prior to pregnancy) should be performed more regularly and considered for future research using whole genome sequencing. One final opportunity that was noted by the group was the possibility of using TGR mouse models for reproductive toxicity testing. This was acknowledged as an opportunity but it was felt that high costs might currently be prohibitive. However, the participants recommended further exploring this opportunity.

11. Considerations in risk assessment

Historically, germ cell risk has been estimated quantitatively via several rodent-based approaches: the doubling dose (indirect method; [157]), the parallelogram method [158], and the direct method [157], which provide estimates of relative genetic risk rather than the frequency of affected offspring. Subsequently, a method was developed to estimate the frequency of genetically abnormal offspring, which provides a more realistic, quantitative estimate of the actual risk to future generations of affected parents [159]. These approaches relied mostly on assays that are generally no longer used to provide data for the estimation of risk to offspring, and required broad assumptions to extrapolate from rodents to humans. Accordingly, new approaches for characterizing germ cell risk must be explored. Germ cell risk assessment must rely on the many types of tests described above that range from direct effects on parental germ cells (e.g. aberrations or MN in spermatogonia or spermatids; mutation of transgenes in germ cells), developmental and reproduction tests (e.g. DLT; 1- or multi-generation reproduction studies), and effects on support cells of the germinal tissues. Absorption, distribution, metabolism, and excretion (ADME) must be considered to provide evidence of germ cell exposure. From most of these studies, dose-response data can be generated for endpoints of concern related to germ cells. These data can then be evaluated similarly to other toxicity data using commonly accepted metrics and mathematical models.

Probably the most useful and commonly used approach today begins with modeling the dose-response data to generate a point of departure (POD) metric. The POD is the point on the dose-response curve that marks the starting point for low-dose extrapolation to exposure levels of concern for human risk [160]. Recent evaluations of appropriate models to generate PODs recommend that the benchmark approach (see EPA, 2012 for guidance [159]) is the most appropriate for modeling genetic toxicity data [161–164].

Once a POD is generated from the dose-response modeling on any test data related to potential adverse germ cell effects, a characterization of the mode of action (MOA) for biological understanding of what the effects are, and how they may be adverse, is indicated. The MOA is the sequence of key events and processes that lead to an adverse outcome. ADME information is very useful for providing target cell exposure data when evaluating possible MOAs. Based on the MOA, and other relevant information, the appropriate form of extrapolation below the POD can be employed.

If the MOA supports a linear low-dose extrapolation, then the slope provides an estimate of risk per unit of dose. However, if a linear extrapolation is not indicated by the MOA, either a margin of exposure (MOE) approach, or an uncertainty factor approach, is commonly used to generate a reference dose (RfD), depending on the risk management needs being addressed. The MOE compares the POD to the current or predicted human exposure (i.e. ratio of the POD to the human exposure) to determine how close the human exposures are to the dose (POD) associated with adverse effects. The RfD is derived by dividing the dose at the POD by uncertainty factors to determine a dose below which the risk becomes of low concern. These two approaches for genetic toxicity using in vitro and in vivo data from somatic cells are discussed more fully in Johnson et al. [163], and can be considered valid for characterizing the risk from germ cell-related data. Although these concepts were introduced at the IWGT meeting, there was insufficient time for a full discussion. However, the presently available data and current understanding of germ cell responses to mutagens, including pharmacokinetics, and interspecies extrapolation, do not currently offer grounds to reject the use of these approaches for characterizing the risk to germ cells from potential mutagens.

12. Concluding remarks

In summary, the importance of protecting humans from heritable mutation hazards and risks, and the determination of the causes of de novo mutations in offspring was emphasized in this workshop. An updated review of the advantages and disadvantages of the existing assay for germ cell and heritable effects highlighted a number of gaps. Various existing and new assays show great promise to help meet these needs. Newly recognized types of genomic changes, such as CNVs, need to be explored to understand their relevance in germ cell genetic toxicology. Such tests will require further development and validation, as well as research efforts to establish the best integrated testing approach. We must work to enhance the current database to identify the most effective approaches. This effort should include harnessing available data from reproductive toxicology assays that have not been used historically for these purposes. Induced mutations that do not cause a phenotype in the first generation must also be considered for causing disease in future generations. Moreover, intergenerational mutational events occurring by exposure of germ cells during embryologic development, which can result in genetic disease, should also be investigated [124,165]. Accordingly, applying new genomics technologies to evaluate animals, including humans, exposed to mutagens should be a priority.

Conflict of interest

None.

Funding

Funding for this workshop was provided by the IWGT.

Acknowledgements

We thank Bernard Robaire for assisting with the development of the workshop programme and for helpful discussions, and numerous internal reviewers for their thoughtful comments on the manuscript.

References

[1] B.N. Ames, W.E. Durston, E. Yamasaki, F.D. Lee, Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection, Proc. Natl. Acad. Sci. U. S. A. 70 (1973) 2281–2285.
[2] D.M. DeMarini, Declaring the existence of human germ-cell mutagens, Environ. Mol. Mutagen. 53 (2012) 166–172.
[3] T.M. Singer, C.L. Yauk, Germ cell mutagens: risk assessment challenges in the 21st century, Environ. Mol. Mutagen. 51 (2010) 919–928.
[4] C.L. Yauk, J. Lucas, S.S. Argueso, P. Auerbach, S.R. Awadalla, D.M. Davis, G.R. DeMarini, Y.E. Douglas, R.K. Dubrova, T.W. Elespuru, B.F. Glover, M.E. Hales, C.B. Hurles, J.R. Klein, D.K. Lupski, F. Manchester, A. Marchetti, J.J. Montpetit, B. Mulvihill, W.A. Robaire, G.A. Robbins, D.T. Rouleau, C.M. Shaughnessy, J.G.t. Somers, J. Taylor, M.D. Trasler, T.E. Waters, K.L. Wilson, J.B. Witt Bishop, Harnessing genomics to identify environmental determinants of heritable disease, Mutat. Res. 752 (2013) 6–9.
[5] K.L. Witt, L.A. Hughes, L.T. Burka, A.F. McFee, J.M. Mathews, S.L. Black, J.B. Bishop, Mouse bone marrow micronucleus test results do not predict the

germ cell mutagenicity of N-hydroxymethylacrylamide in the mouse dominant lethal assay, Environ. Mol. Mutagen. 41 (2003) 111–120.
[6] D.A. Eastmond, A. Hartwig, D. Anderson, W.A. Anwar, M.C. Cimino, I. Dobrev, G.R. Douglas, T. Nohmi, D.H. Phillips, C. Vickers, Mutagenicity testing for chemical risk assessment: update of the WHO/IPCS harmonized scheme, Mutagenesis 24 (2009) 341–349.
[7] N. Verhofstad, J.O. Linschooten, J. van Benthem, Y.E. Dubrova, H. van Steeg, F.J. van Schooten, R.W. Godschalk, New methods for assessing male germ line mutations in humans and genetic risks in their offspring, Mutagenesis 23 (2008) 241–247.
[8] R.T. Schulte, D.A. Ohl, M. Sigman, G.D. Smith, Sperm DNA damage in male infertility: etiologies, assays, and outcomes, J. Assisted Reprod. Genet. 27 (2010) 3–12.
[9] P. Allard, N.C. Kleinstreuer, T.B. Knudsen, M.P. Colaiacovo, A C. elegans screening platform for the rapid assessment of chemical disruption of germline function, Environ. Health Perspect. 121 (2013) 717–724.
[10] A. Kong, M.L. Frigge, G. Masson, S. Besenbacher, P. Sulem, G. Magnusson, S.A. Gudjonsson, A. Sigurdsson, A. Jonasdottir, A. Jonasdottir, W.S. Wong, G. Sigurdsson, G.B. Walters, S. Steinberg, H. Helgason, G. Thorleifsson, D.F. Gudbjartsson, A. Helgason, O.T. Magnusson, U. Thorsteinsdottir, K. Stefansson, Rate of de novo mutations and the importance of father's age to disease risk, Nature 488 (2012) 471–475.
[11] B.M. D'Onofrio, M.E. Rickert, E. Frans, R. Kuja-Halkola, C. Almqvist, A. Sjolander, H. Larsson, Lichtenstein, paternal age at childbearing and offspring psychiatric and academic morbidity, JAMA Psychiatry 71 (2014) 432–438.
[12] J.X. Sun, A. Helgason, G. Masson, S.S. Ebenesersdottir, H. Li, S. Mallick, S. Gnerre, N. Patterson, A. Kong, D. Reich, K. Stefansson, A direct characterization of human mutation based on microsatellites, Nat. Genet. 44 (2012) 1161–1165.
[13] J.Y. Hehir-Kwa, B. Rodriguez-Santiago, L.E. Vissers, N. de Leeuw, R. Pfundt, J.K. Buitelaar, L.A. Perez- Jurado, J.A. Veltman, De novo copy number variants associated with intellectual disability have a paternal origin and age bias, J. Med. Genet. 48 (2011) 776–778.
[14] Y.E. Dubrova, R.I. Bersimbaev, L.B. Djansugurova, M.K. Tankimanova, Z. Mamyrbaeva, R. Mustonen, C. Lindholm, M. Hulten, S. Salomaa, Nuclear weapons tests and human germline mutation rate, Science 295 (2002) 1037.
[15] Y.E. Dubrova, G. Grant, A.A. Chumak, V.A. Stezhka, A.N. Karakasian, Elevated minisatellite mutation rate in the post-chernobyl families from ukraine, Am. J. Hum. Genet. 71 (2002) 801–809.
[16] J.O. Linschooten, N. Verhofstad, K. Gutzkow, A.K. Olsen, C. Yauk, Y. Oligschlager, G. Brunborg, F.J. van Schooten, R.W. Godschalk, Paternal lifestyle as a potential source of germline mutations transmitted to offspring, FASEB J. 27 (2013) 2873–2879.
[17] S. Frias, P. Van Hummelen, M.L. Meistrich, X.R. Lowe, F.B. Hagemeister, M.D. Shelby, J.B. Bishop, A.J. Wyrobek, NOVP chemotherapy for Hodgkin's disease transiently induces sperm aneuploidies associated with the major clinical aneuploidy syndromes involving chromosomes X, Y, 18, and 21, Cancer Res. 63 (2003) 44–51.
[18] W.A. Robbins, M.L. Meistrich, D. Moore, F.B. Hagemeister, H.U. Weier, M.J. Cassel, G. Wilson, B. Eskenazi, A.J. Wyrobek, Chemotherapy induces transient sex chromosomal and autosomal aneuploidy in human sperm, Nat. Genet. 16 (1997) 74–78.
[19] W.A. Robbins, M.F. Vine, K.Y. Truong, R.B. Everson, Use of fluorescence in situ hybridization (FISH) to assess effects of smoking, caffeine, and alcohol on aneuploidy load in sperm of healthy men, Environ. Mol. Mutagen. 30 (1997) 175–183.
[20] C.S. Ku, E.K. Tan, D.N. Cooper, From the periphery to centre stage: de novo single nucleotide variants play a key role in human genetic disease, J. Med. Genet. 50 (2013) 203–211.
[21] R.P. Kuiper, M.J. Ligtenberg, N. Hoogerbrugge, A. Geurts van Kessel, Germline copy number variation and cancer risk, Curr. Opin. Genet. Dev. 20 (2010) 282–289.
[22] C. Gonzaga-Jauregui, J.R. Lupski, R.A. Gibbs, Human genome sequencing in health and disease, Annu. Rev. Med. 63 (2012) 35–61.
[23] C. Gilissen, J.Y. Hehir-Kwa, D.T. Thung, M. van de Vorst, B.W. van Bon, M.H. Willemsen, M. Kwint, I.M. Janssen, A. Hoischen, A. Schenck, R. Leach, R. Klein, R. Tearle, T. Bo, R. Pfundt, H.G. Yntema, B.B. de Vries, T. Kleefstra, H.G. Brunner, L.E. Vissers, J.A. Veltman, Genome sequencing identifies major causes of severe intellectual disability, Nature 511 (2014) 344–347.
[24] J.R. Lupski, New mutations and intellectual function, Nat. Genet. 42 (2010) 1036–1038.
[25] D.G. MacArthur, S. Balasubramanian, A. Frankish, N. Huang, J. Morris, K. Walter, L. Jostins, L. Habegger, J.K. Pickrell, S.B. Montgomery, C.A. Albers, Z.D. Zhang, D.F. Conrad, G. Lunter, H. Zheng, Q. Ayub, M.A. DePristo, E. Banks, M. Hu, R.E. Handsaker, J.A. Rosenfeld, M. Fromer, M. Jin, X.J. Mu, E. Khurana, K. Ye, M. Kay, G.I. Saunders, M.M. Suner, T. Hunt, I.H. Barnes, C. Amid, D.R. Carvalho-Silva, A.H. Bignell, C. Snow, B. Yngvadottir, S. Bumpstead, D.N. Cooper, Y. Xue, I.G. Romero, C. Genomes Project, J. Wang, Y. Li, R.A. Gibbs, S.A. McCarroll, E.T. Dermitzakis, J.K. Pritchard, J.C. Barrett, J. Harrow, M.E. Hurles, M.B. Gerstein, C. Tyler-Smith, A systematic survey of loss-of-function variants in human protein-coding genes, Science 335 (2012) 823–828.
[26] M. Hurles, Older males beget more mutations, Nat. Genet. 44 (2012) 1174–1176.
[27] E.P. Consortium, An integrated encyclopedia of DNA elements in the human genome, Nature 489 (2012) 57–74.
[28] J.N. Groen, D. Capraro, K.V. Morris, The emerging role of pseudogene expressed non-coding RNAs in cellular functions, Int. J. Biochem. Cell Biol. 54C (2014) 350–355.
[29] OECD, Test No. 485: Mouse Heritable Translocation Assay, OECD Guidelines for Testing of Chemical Section 4, OECD Publishing, Paris, France, 1986.
[30] L.B. Russell, B.E. Matter, Whole-mammal mutagenicity tests: evaluation of five methods, Mutat. Res. 75 (1980) 279–302.
[31] A.P. Davis, M.J. Justice, An oak ridge legacy: the specific locus test and its role in mouse mutagenesis, Genetics 148 (1998) 7–12.
[32] S. Green, K.S. Lavappa, M. Manandhar, C.J. Sheu, E. Whorton, J.A. Springer, A guide for mutagenicity testing using the dominant lethal assay, Mutat. Res. 189 (1987) 167–174.
[33] OECD, Test No. 478: Rodent Dominant Lethal Test, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 1984.
[34] I.D. Adler, D. Anderson, Dominant lethal effects after inhalation exposure to 1,3-butadiene, Mutat. Res. 309 (1994) 295–297.
[35] J.G. Brewen, H.S. Payne, K.P. Jones, R.J. Preston, Studies on chemically induced dominant lethality I. The cytogenetic basis of MMS-induced dominant lethality in post-meiotic male germ cells, Mutat. Res. 33 (1975) 239–250.
[36] F. Marchetti, J.B. Bishop, L. Cosentino, D. Moore, A.J. Wyrobek 2nd, Paternally transmitted chromosomal aberrations in mouse zygotes determine their embryonic fate, Biol. Reprod. 70 (2004) 616–624.
[37] C.H. Guo, Y.F. Lu, G.S. Hsu, The influence of aluminum exposure on male reproduction and offspring in mice, Environ. Toxicol. Pharmacol. 20 (2005) 135–141.
[38] OECD, Test No. 483: Mammalian Spermatogonial Chromosome Aberration Test, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 1997.
[39] F. Marchetti, A.J. Wyrobek, Mechanisms and consequences of paternally-transmitted chromosomal abnormalities, birth defects research, Part C Embryo Today Rev. 75 (2005) 112–129.
[40] F. Marchetti, A.J. Wyrobek, PAINT/DAPI analysis of mouse zygotes to detect paternally transmitted chromosomal aberrations, Adv. Exp. Med. Biol. 518 (2003) 131–145.
[41] OECD, Test No. 488: Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 2013.
[42] I.B. Lambert, T.M. Singer, S.E. Boucher, G.R. Douglas, Detailed review of transgenic rodent mutation assays, Mutat. Res. 590 (2005) 1–280.
[43] T.M. Singer, I.B. Lambert, A. Williams, G.R. Douglas, C.L. Yauk, Detection of induced male germline mutation: correlations and comparisons between traditional germline mutation assays, transgenic rodent assays and expanded simple tandem repeat instability assays, Mutat. Res. 598 (2006) 164–193.
[44] OECD, Detailed Review of Transgenic Rodent Gene Mutation Assays, Series on Testing and AssessmentNo. 103, ENV/JM/MONO(2009)7, OECD Publishing, Paris, France, 2009.
[45] L. Simon, D.T. Carrell, Sperm DNA damage measured by comet assay, Methods Mol. Biol. 927 (2013) 137–146.
[46] G.A. Sega, Unscheduled DNA synthesis in the germ cells of male mice exposed in vivo to the chemical mutagen ethyl methanesulfonate, Proc. Natl. Acad. Sci. U. S. A. 71 (1974) 4955–4959.
[47] D.P. Evenson, Sperm chromatin structure assay (SCSA(R)), Methods Mol. Biol. 927 (2013) 147–164.
[48] A.J. Wyrobek, T.E. Schmid, F. Marchetti, Cross-species sperm-FISH assays for chemical testing and assessing paternal risk for chromosomally abnormal pregnancies, Environ. Mol. Mutagen. 45 (2005) 271–283.
[49] ICH, Detection Of Toxicity to Reproduction for Medicinal Products & Toxicity to Male Fertility S5(R2). ICH harmonised tripartite guideline, International Conference on Harmonization, Geneva, Switzerland, 2005.
[50] OECD, Test No. 421: Reproduction/Developmental Toxicity Screening Test, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 1995.
[51] OECD, Test No. 414: Prenatal Developmental Toxicity Study, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 2001.
[52] D.K. Gulati, E. Hope, J. Teague, R.E. Chapin, Reproductive toxicity assessment by continuous breeding in Sprague-Dawley rats: a comparison of two study designs, Fundam. Appl. Toxicol. 17 (1991) 270–279.
[53] OECD, Test No. 416: Two-Generation Reproduction Toxicity, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 2001.
[54] OECD, Test No. 415: One-Generation Reproduction Toxicity Study, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 2001.
[55] OECD, Test No. 443: Extended One-Generation Reproductive Toxicity Study, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 2011.
[56] OECD, Guidance Document on The Current Implementation of Internal Triggers in Test Guideline 443 for an Extended One Generation Reproductive Toxicity Study in the United States and Canada, Series on Testing and Assessment No. 117, OECD Publishing, Paris, France, 2011.
[57] OECD, Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 2001.

[58] OECD, Test No. 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, OECD Guidelines for Testing of Chemicals, Section 4, OECD Publishing, Paris, France, 2001.
[59] T. Mitchard, P. Jarvis, J. Stewart, Assessment of male rodent fertility in general toxicology 6-month studies, Birth Defects Res. (Part B) 65 (2012) 410–420.
[60] R.E. Chapin, R.A. Sloane, J.K. Haseman, The relationships among reproductive endpoints in Swiss mice, using the reproductive assessment by continuous breeding database, Fundam. Appl. Toxicol. 38 (1997) 129–142.
[61] R.E. Chapin, R.A. Sloane, J.K. Haseman, Reproductive endpoints in general toxicity studies: are they predictive? Reprod. Toxicol. 12 (1998) 489–494.
[62] L. Cosentino, J.A. Heddle, Differential mutation of transgenic and endogenous loci in vivo, Mutat. Res. 454 (2000) 1–10.
[63] J.H. van Delft, A. Bergmans, F.J. van Dam, A.D. Tates, L. Howard, D.J. Winton, R.A. Baan, Gene-mutation assays in lambda lacZ transgenic mice: comparison of lacZ with endogenous genes in splenocytes and small intestinal epithelium, Mutat. Res. 415 (1998) 85–96.
[64] L.B. Barnett, R.W. Tyl, B.S. Shane, M.D. Shelby, S.E. Lewis, Transmission of mutations in the lacI transgene to the offspring of ENU-treated big blue male mice, Environ. Mol. Mutagen. 40 (2002) 251–257.
[65] P. Bois, J. Williamson, J. Brown, Y.E. Dubrova, A.J. Jeffreys, A novel unstable mouse VNTR family expanded from SINE B1 elements, Genomics 49 (1998) 122–128.
[66] R.J. Hardwick, M.V. Tretyakov, Y.E. Dubrova, Age-related accumulation of mutations supports a replication-dependent mechanism of spontaneous mutation at tandem repeat DNA loci in mice, Mol. Biol. Evol. 26 (2009) 2647–2654.
[67] M. Shanks, L. Riou, P. Fouchet, Y.E. Dubrova, Stage-specificity of spontaneous mutation at a tandem repeat DNA locus in the mouse germline, Mutat. Res. 641 (2008) 58–60.
[68] R.C. Barber, R.J. Hardwick, M.E. Shanks, C.D. Glen, S.K. Mughal, M. Voutounou, Y.E. Dubrova, The effects of in utero irradiation on mutation induction and transgenerational instability in mice, Mutat. Res. 664 (2009) 6–12.
[69] Y.E. Dubrova, A.J. Jeffreys, A.M. Malashenko, Mouse minisatellite mutations induced by ionizing radiation, Nat. Genet. 5 (1993) 92–94.
[70] Y.E. Dubrova, M. Plumb, J. Brown, E. Boulton, D. Goodhead, A.J. Jeffreys, Induction of minisatellite mutations in the mouse germline by low-dose chronic exposure to gamma-radiation and fission neutrons, Mutat. Res. 453 (2000) 17–24.
[71] Y.E. Dubrova, M. Plumb, J. Brown, J. Fennelly, P. Bois, D. Goodhead, A.J. Jeffreys, Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 6251–6255.
[72] Y.J. Fan, Z. Wang, S. Sadamoto, Y. Ninomiya, N. Kotomura, K. Kamiya, K. Dohi, R. Kominami, O. Niwa, Dose-response of a radiation induction of a germline mutation at a hypervariable mouse minisatellite locus, Int. J. Radiat. Biol. 68 (1995) 177–183.
[73] C.D. Glen, A.G. Smith, Y.E. Dubrova, Single-molecule PCR analysis of germ line mutation induction by anticancer drugs in mice, Cancer Res. 68 (2008) 3630–3636.
[74] C. Vilarino-Guell, A.G. Smith, Y.E. Dubrova, Germline mutation induction at mouse repeat DNA loci by chemical mutagens, Mutat. Res. 526 (2003) 63–73.
[75] F. Marchetti, A. Rowan-Carroll, A. Williams, A. Polyzos, M.L. Berndt-Weis, C.L. Yauk, Sidestream tobacco smoke is a male germ cell mutagen, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 12811–12814.
[76] C.M. Somers, B.E. McCarry, F. Malek, J.S. Quinn, Reduction of particulate air pollution lowers the risk of heritable mutations in mice, Science 304 (2004) 1008–1010.
[77] C.M. Somers, C.L. Yauk, P.A. White, C.L. Parfett, J.S. Quinn, Air pollution induces heritable DNA mutations, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 15904–15907.
[78] C. Yauk, A. Polyzos, A. Rowan-Carroll, C.M. Somers, R.W. Godschalk, F.J. Van Schooten, M.L. Berndt, I.P. Pogribny, I. Koturbash, A. Williams, G.R. Douglas, O. Kovalchuk, Germ-line mutations, DNA damage, and global hypermethylation in mice exposed to particulate air pollution in an urban/industrial location, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 605–610.
[79] C.L. Yauk, M.L. Berndt, A. Williams, A. Rowan-Carroll, G.R. Douglas, M.R. Stampfli, Mainstream tobacco smoke causes paternal germ-line DNA mutation, Cancer Res. 67 (2007) 5103–5106.
[80] C.L. Yauk, Y.E. Dubrova, G.R. Grant, A.J. Jeffreys, A novel single molecule analysis of spontaneous and radiation-induced mutation at a mouse tandem repeat locus, Mutat. Res. 500 (2002) 147–156.
[81] I.L. Berg, R. Neumann, K.W. Lam, S. Sarbajna, L. Odenthal-Hesse, C.A. May, A.J. Jeffreys, PRDM9 variation strongly influences recombination hot-spot activity and meiotic instability in humans, Nat. Genet. 42 (2010) 859–863.
[82] C.A. May, K. Tamaki, R. Neumann, G. Wilson, G. Zagars, A. Pollack, Y.E. Dubrova, A.J. Jeffreys, M.L. Meistrich, Minisatellite mutation frequency in human sperm following radiotherapy, Mutat. Res. 453 (2000) 67–75.
[83] J.A. Armour, M.H. Brinkworth, A. Kamischke, Direct analysis by small-pool PCR of MS205 minisatellite mutation rates in sperm after mutagenic therapies, Mutat. Res. 445 (1999) 73–80.
[84] S.M. Mirkin, Expandable DNA repeats and human disease, Nature 447 (2007) 932–940.
[85] M.A. Beal, T.C. Glenn, S.L. Lance, C.M. Somers, Characterization of unstable microsatellites in mice: no evidence for germline mutation induction following gamma-radiation exposure, Environ. Mol. Mutagen. 53 (2012) 599–607.
[86] S.D. Dertinger, D.K. Torous, M. Hayashi, J.T. MacGregor, Flow cytometric scoring of micronucleated erythrocytes: an efficient platform for assessing in vivo cytogenetic damage, Mutagenesis 26 (2011) 139–145.
[87] S.L. Avlasevich, S.M. Bryce, S.E. Cairns, S.D. Dertinger, In vitro micronucleus scoring by flow cytometry: differential staining of micronuclei versus apoptotic and necrotic chromatin enhances assay reliability, Environ. Mol. Mutagen. 47 (2006) 56–66.
[88] A.D. Tates, Validation studies with the micronucleus test for early spermatids of rats. A tool for detecting clastogenicity of chemicals in differentiating spermatogonia and spermatocytes, Mutagenesis 7 (1992) 411–419.
[89] I. Cliet, C. Melcion, A. Cordier, Lack of predictivity of bone marrow micronucleus test versus testis micronucleus test: comparison with four carcinogens, Mutat. Res. 292 (1993) 105–111.
[90] M. Hayashi, J.T. MacGregor, D.G. Gatehouse, I.D. Adler, D.H. Blakey, S.D. Dertinger, G. Krishna, T. Morita, A. Russo, S. Sutou, In vivo rodent erythrocyte micronucleus assay II. Some aspects of protocol design including repeated treatments, integration with toxicity testing, and automated scoring, Environ. Mol. Mutagen. 35 (2000) 234–252.
[91] T. Morita, J.T. MacGregor, M. Hayashi, Micronucleus assays in rodent tissues other than bone marrow, Mutagenesis 26 (2011) 223–230.
[92] A. Azqueta, A.R. Collins, The essential comet assay: a comprehensive guide to measuring DNA damage and repair, Arch. Toxicol. 87 (2013) 949–968.
[93] OECD, Test No. 489: In Vivo Mammalian Alkaline Comet Assay, OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, Paris, France, 2014.
[94] S.M. Duty, N.P. Singh, M.J. Silva, D.B. Barr, J.W. Brock, L. Ryan, R.F. Herrick, D.C. Christiani, R. Hauser, The relationship between environmental exposures to phthalates and DNA damage in human sperm using the neutral comet assay, Environ. Health Perspect. 111 (2003) 1164–1169.
[95] P. Moller, L.E. Knudsen, S. Loft, H. Wallin, The comet assay as a rapid test in biomonitoring occupational exposure to DNA-damaging agents and effect of confounding factors, Cancer Epidemiol. Biomarkers Prev. 9 (2000) 1005–1015.
[96] E. Cordelli, P. Eleuteri, M.G. Grollino, B. Benassi, G. Blandino, C. Bartoleschi, M.C. Pardini, E.V. Di Caprio, M. Spano, F. Pacchierotti, P. Villani, Direct and delayed X-ray-induced DNA damage in male mouse germ cells, Environ. Mol. Mutagen. 53 (2012) 429–439.
[97] G. Speit, M. Vasquez, A. Hartmann, The comet assay as an indicator test for germ cell genotoxicity, Mutat. Res. 681 (2009) 3–12.
[98] G.A. Haines, J.H. Hendry, C.P. Daniel, I.D. Morris, Increased levels of comet-detected spermatozoa DNA damage following in vivo isotopic- or X-irradiation of spermatogonia, Mutat. Res. 495 (2001) 21–32.
[99] G.A. Haines, J.H. Hendry, C.P. Daniel, I.D. Morris, Germ cell and dose-dependent DNA damage measured by the comet assay in murine spermatozoaa after testicular X-irradiation, Biol. Reprod. 67 (2002) 854–861.
[100] D.P. Evenson, Z. Darzynkiewicz, M.R. Melamed, Relation of mammalian sperm chromatin heterogeneity to fertility, Science 210 (1980) 1131–1133.
[101] E.S. Sills, J.T. Fryman, M. Perloe, K.B. Michels, M.J. Tucker, Chromatin fluorescence characteristics and standard semen analysis parameters: correlations observed in andrology testing among 136 males referred for infertility evaluation, J. Obstetrics Gynaecol. 24 (2004) 74–77.
[102] W. Gorczyca, J. Gong, Z. Darzynkiewicz, Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays, Cancer Res. 53 (1993) 1945–1951.
[103] R.J. Aitken, R. Bronson, T.B. Smith, G.N. De Iuliis, The source and significance of DNA damage in human spermatozoa; a commentary on diagnostic strategies and straw man fallacies, Mol. Hum. Reprod. 19 (2013) 475–485.
[104] R.J. Aitken, G.N. De Iuliis, R.I. McLachlan, Biological and clinical significance of DNA damage in the male germ line, Int. J. Androl. 32 (2009) 46–56.
[105] S.E. Lewis, L. Simon, Clinical implications of sperm DNA damage, Hum. Fertil. 13 (2010) 201–207.
[106] L. Robinson, I.D. Gallos, S.J. Conner, M. Rajkhowa, D. Miller, S. Lewis, J. Kirkman-Brown, A. Coomarasamy, The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis, Hum. Reprod. 27 (2012) 2908–2917.
[107] M.A. Beal, T.C. Glenn, C.M. Somers, Whole genome sequencing for quantifying germline mutation frequency in humans and model species: cautious optimism, Mutat. Res. 750 (2012) 96–106.
[108] P.M. Boone, C.A. Bacino, C.A. Shaw, P.A. Eng, P.M. Hixson, A.N. Pursley, S.H. Kang, Y. Yang, J. Wiszniewska, B.A. Nowakowska, D. del Gaudio, Z. Xia, G. Simpson-Patel, L.L. Immken, J.B. Gibson, A.C. Tsai, J.A. Bowers, T.E. Reimschisel, C.P. Schaaf, L. Potocki, F. Scaglia, T. Gambin, M. Sykulski, M. Bartnik, K. Derwinska, B. Wisniowiecka-Kowalnik, S.R. Lalani, F.J. Probst, W. Bi, A.L. Beaudet, A. Patel, J.R. Lupski, S.W. Cheung, P. Stankiewicz, Detection of clinically relevant exonic copy-number changes by array CGH, Hum. Mutat. 31 (2010) 1326–1342.
[109] R. Kavlock, K. Chandler, K. Houck, S. Hunter, R. Judson, N. Kleinstreuer, T. Knudsen, M. Martin, S. Padilla, D. Reif, A. Richard, D. Rotroff, N. Sipes, D. Dix, Update on EPA's ToxCast program: providing high throughput decision support tools for chemical risk management, Chem. Res. Toxicol. 25 (2012) 1287–1302.

[110] D.J. Dix, K.A. Houck, M.T. Martin, A.M. Richard, R.W. Setzer, R.J. Kavlock, The ToxCast program for prioritizing toxicity testing of environmental chemicals, Toxicol. Sci. 95 (2007) 5–12.
[111] P. Stankiewicz, J.R. Lupski, Structural variation in the human genome and its role in disease, Annu. Rev. Med. 61 (2010) 437–455.
[112] C.D. Campbell, E.E. Eichler, Properties and rates of germline mutations in humans, Trends Genet. TIG 29 (2013) 575–584.
[113] R.E. Mills, K. Walter, C. Stewart, R.E. Handsaker, K. Chen, C. Alkan, A. Abyzov, S.C. Yoon, K. Ye, R.K. Cheetham, A. Chinwalla, D.F. Conrad, Y. Fu, F. Grubert, I. Hajirasouliha, F. Hormozdiari, L.M. Iakoucheva, Z. Iqbal, S. Kang, J.M. Kidd, M.K. Konkel, J. Korn, E. Khurana, D. Kural, H.Y. Lam, J. Leng, R. Li, Y. Li, C.Y. Lin, R. Luo, X.J. Mu, J. Nemesh, H.E. Peckham, T. Rausch, A. Scally, X. Shi, M.P. Stromberg, A.M. Stutz, A.E. Urban, J.A. Walker, J. Wu, Y. Zhang, Z.D. Zhang, M.A. Batzer, L. Ding, G.T. Marth, G. McVean, J. Sebat, M. Snyder, J. Wang, K. Ye, E.E. Eichler, M.B. Gerstein, M.E. Hurles, C. Lee, S.A. McCarroll, J.O. Korbel, P. Genomes, Mapping copy number variation by population-scale genome sequencing, Nature 470 (2011) 59–65.
[114] S. Girirajan, E.E. Eichler, Phenotypic variability and genetic susceptibility to genomic disorders, Hum. Mol. Genet. 19 (2010) R176–R187.
[115] J. Sebat, B. Lakshmi, J. Troge, J. Alexander, J. Young, P. Lundin, S. Maner, H. Massa, M. Walker, M. Chi, N. Navin, R. Lucito, J. Healy, J. Hicks, K. Ye, A. Reiner, T.C. Gilliam, B. Trask, N. Patterson, A. Zetterberg, M. Wigler, Large-scale copy number polymorphism in the human genome, Science 305 (2004) 525–528.
[116] J.R. Lupski, Genomic rearrangements and sporadic disease, Nat. Genet. 39 (2007) S43–S47.
[117] A. Itsara, H. Wu, J.D. Smith, D.A. Nickerson, I. Romieu, S.J. London, E.E. Eichler, De novo rates and selection of large copy number variation, Genome Res. 20 (2010) 1469–1481.
[118] Y. Shen, B.L. Wu, Microarray-based genomic DNA profiling technologies in clinical molecular diagnostics, Clin. Chem. 55 (2009) 659–669.
[119] A.E. Oostlander, G.A. Meijer, B. Ylstra, Microarray-based comparative genomic hybridization and its applications in human genetics, Clin. Genet. 66 (2004) 488–495.
[120] P. Dittwald, T. Gambin, P. Szafranski, J. Li, S. Amato, M.Y. Divon, L.X. Rodriguez Rojas, L.E. Elton, D.A. Scott, C.P. Schaaf, W. Torres-Martinez, A.K. Stevens, J.A. Rosenfeld, S. Agadi, D. Francis, S.H. Kang, A. Breman, S.R. Lalani, C.A. Bacino, W. Bi, A. Milosavljevic, A.L. Beaudet, A. Patel, C.A. Shaw, J.R. Lupski, A. Gambin, S.W. Cheung, P. Stankiewicz, NAHR-mediated copy-number variants in a clinical population: mechanistic insights into both genomic disorders and mendelizing traits, Genome Res. 23 (2013) 1395–1409.
[121] J. Wiszniewska, W. Bi, C. Shaw, P. Stankiewicz, S.H. Kang, A.N. Pursley, S. Lalani, P. Hixson, T. Gambin, C.H. Tsai, H.G. Bock, M. Descartes, F.J. Probst, F. Scaglia, A.L. Beaudet, J.R. Lupski, C. Eng, S. Wai Cheung, C. Bacino, A. Patel, Combined array CGH plus SNP genome analyses in a single assay for optimized clinical testing, Eur. J. Hum. Genet. EJHG 22 (2014) 79–87.
[122] S.W. Cheung, C.A. Shaw, W. Yu, J. Li, Z. Ou, A. Patel, S.A. Yatsenko, M.L. Cooper, P. Furman, P. Stankiewicz, J.R. Lupski, A.C. Chinault, A.L. Beaudet, Development and validation of a CGH microarray for clinical cytogenetic diagnosis, Genet. Med. 7 (2005) 422–432.
[123] P.M. Boone, Z.T. Soens, I.M. Campbell, P. Stankiewicz, S.W. Cheung, A. Patel, A.L. Beaudet, S.E. Plon, C.A. Shaw, A.L. McGuire, J.R. Lupski, Incidental copy-number variants identified by routine genome testing in a clinical population, Genet. Med. 15 (2013) 45–54.
[124] J. Pham, C. Shaw, A. Pursley, P. Hixson, S. Sampath, E. Roney, T. Gambin, S.H. Kang, W. Bi, S. Lalani, C. Bacino, J.R. Lupski, P. Stankiewicz, A. Patel, S.W. Cheung, Somatic mosaicism detected by exon-targeted, high-resolution aCGH in 10 362 consecutive cases, Eur. J. Hum. Genet. EJHG 22 (2014) 969–978.
[125] M.F. Arlt, J.G. Mulle, V.M. Schaibley, R.L. Ragland, S.G. Durkin, S.T. Warren, T.W. Glover, Replication stress induces genome-wide copy number changes in human cells that resemble polymorphic and pathogenic variants, Am. J. Hum. Genet. 84 (2009) 339–350.
[126] M.F. Arlt, A.C. Ozdemir, S.R. Birkeland, T.E. Wilson, T.W. Glover, Hydroxyurea induces de novo copy number variants in human cells, Proc. Natl. Acad. Sci. U. S. A. 108 (2011) 17360–17365.
[127] M.F. Arlt, S. Rajendran, S.R. Birkeland, T.E. Wilson, T.W. Glover, Copy number variants are produced in response to low-dose ionizing radiation in cultured cells, Environ. Mol. Mutagen. 55 (2014) 103–113.
[128] A.W. Knight, S. Little, K. Houck, D. Dix, R. Judson, A. Richard, N. McCarroll, G. Akerman, C. Yang, L. Birrell, R.M. Walmsley, Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast chemicals, Regul. Toxicol. Pharmacol. RTP 55 (2009) 188–199.
[129] P. Allard, M.P. Colaiacovo, Bisphenol A impairs the double-strand break repair machinery in the germline and causes chromosome abnormalities, Proc. Natl. Acad. Sci. ICH U. S. A. 107 (2010) 20405–20410.
[130] T.T. Turner, C.P. Rhoades, Testicular capillary permeability: the movement of luteinizing hormone from the vascular to the interstitial compartment, J. Androl. 16 (1995) 417–423.
[131] M. Dym, The fine structure of the monkey (Macaca) sertoli cell and its role in maintaining the blood-testis barrier, Anatomical Rec. 175 (1973) 639–656.
[132] M. Dym, D.W. Fawcett, The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium, Biol. Reprod. 3 (1970) 308–326.
[133] B.P. Setchell, The functional significance of the blood-testis barrier, J. Androl. 1 (1980) 3–10.
[134] K. Okumura, I.P. Lee, R.L. Dixon, Permeability of selected drugs and chemicals across the blood-testis barrier of the rat, J. Pharmacol. Exp. Ther. 194 (1975) 89–95.
[135] G.M. Waites, A.R. Jones, S.J. Main, T.G. Cooper, The entry of antifertility and other compounds into the testis, Adv. Biosci. 10 (1973) 101–116.
[136] A.A. van Zeeland, A. de Groot, A. Neuhauser-Klaus, DNA adduct formation in mouse testis by ethylating agents: a comparison with germ-cell mutagenesis, Mutat. Res. 231 (1990) 55–62.
[137] G.A. Sega, J.G. Owens, Ethylation of DNA and protamine by ethyl methanesulfonate in the germ cells of male mice and the relevancy of these molecular targets to the induction of dominant lethals, Mutat. Res. 52 (1978) 87–106.
[138] R.N. McLay, W.A. Banks, A.J. Kastin, Granulocyte macrophage-colony stimulating factor crosses the blood-testis barrier in mice, Biol. Reprod. 57 (1997) 822–826.
[139] J. Wijnholds, G.L. Scheffer, M. van der Valk, P. van der Valk, J.H. Beijnen, R.J. Scheper, P. Borst, Multidrug resistance protein 1 protects the oropharyngeal mucosal layer and the testicular tubules against drug-induced damage, J. Exp. Med. 188 (1998) 797–808.
[140] N. Levine, D.J. Marsh, Micropuncture study of the fluid composition of 'sertoli cell-only' seminiferous tubules in rats, J. Reprod. Fertil. 43 (1975) 547–549.
[141] M.L. Meistrich, Potential genetic risks of using semen collected during chemotherapy, Hum. Reprod. 8 (1993) 8–10.
[142] C.D. Campbell, J.X. Chong, M. Malig, A. Ko, B.L. Dumont, L. Han, L. Vives, B.J. O'Roak, P.H. Sudmant, J. Shendure, M. Abney, C. Ober, E.E. Eichler, Estimating the human mutation rate using autozygosity in a founder population, Nat. Genet. 44 (2012) 1277–1281.
[143] D.F. Conrad, J.E. Keebler, M.A. DePristo, S.J. Lindsay, Y. Zhang, F. Casals, Y. Idaghdour, C.L. Hartl, C. Torroja, K.V. Garimella, M. Zilversmit, R. Cartwright, G.A. Rouleau, M. Daly, E.A. Stone, M.E. Hurles, P. Awadalla, P. Genomes, Variation in genome-wide mutation rates within and between human families, Nat. Genet. 43 (2011) 712–714.
[144] J.C. Roach, G. Glusman, A.F. Smit, C.D. Huff, R. Hubley, P.T. Shannon, L. Rowen, K.P. Pant, N. Goodman, M. Bamshad, J. Shendure, R. Drmanac, L.B. Jorde, L. Hood, D.J. Galas, Analysis of genetic inheritance in a family quartet by whole-genome sequencing, Science 328 (2010) 636–639.
[145] P. Liu, C.M. Carvalho, P.J. Hastings, J.R. Lupski, Mechanisms for recurrent and complex human genomic rearrangements, Curr. Opin. Genet. Dev. 22 (2012) 211–220.
[146] P. Liu, A. Erez, S.C. Nagamani, S.U. Dhar, K.E. Kolodziejska, A.V. Dharmadhikari, M.L. Cooper, J. Wiszniewska, F. Zhang, M.A. Withers, C.A. Bacino, L.D. Campos-Acevedo, M.R. Delgado, D. Freedenberg, A. Garnica, T.A. Grebe, D. Hernandez-Almaguer, L. Immken, S.R. Lalani, S.D. McLean, H. Northrup, F. Scaglia, L. Strathearn, P. Trapane, S.H. Kang, A. Patel, S.W. Cheung, P.J. Hastings, P. Stankiewicz, J.R. Lupski, W. Bi, Chromosome catastrophes involve replication mechanisms generating complex genomic rearrangements, Cell 146 (2011) 889–903.
[147] J.R. Lupski, J.W. Belmont, E. Boerwinkle, R.A. Gibbs, Clan genomics and the complex architecture of human disease, Cell 147 (2011) 32–43.
[148] M.C. Cimino, Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes, Environ. Mol. Mutagen. 47 (2006) 362–390.
[149] ICH, Guidance On Genotoxicity Testing And Data Interpretation For Pharmaceuticals Intended For Human Use S2(R1). ICH Harmonised Tripartite Guideline, International Conference on Harmonization, Geneva, Switzerland, 2011.
[150] UN, Globally Harmonized System of Classification and Labelling of Chemicals (GHS), United Nations, New York, USA, 2013.
[151] M.D. Waters, H.F. Stack, M.A. Jackson, B.A. Bridges, I.D. Adler, The performance of short-term tests in identifying potential germ cell mutagens: a qualitative and quantitative analysis, Mutat. Res. 341 (1994) 109–131.
[152] I.D. Adler, I. Ingwersen, U. Kliesch, A. el Tarras, Clastogenic effects of acrylamide in mouse bone marrow cells, Mutat. Res. 206 (1988) 379–385.
[153] G.C. Jagetia, Radiation-induced chromosomal aberrations in the bone marrow of mice exposed to various doses of gamma-radiation, Radiat. Environ. Biophys. 32 (1993) 109–117.
[154] Y. Matsuda, N. Seki, T. Utsugi-Takeuchi, I. Tobari, X-ray and mitomycin c (MMC)-induced chromosome aberrations in spermiogenic germ cells and the repair capacity of mouse eggs for the X-ray and MMC damage, Mutat. Res. 211 (1989) 65–75.
[155] F. Pacchierotti, C. Tiveron, M. D'Archivio, B. Bassani, E. Cordelli, G. Leter, M. Spano, Acrylamide- induced chromosomal damage in male mouse germ cells detected by cytogenetic analysis of one-cell zygotes, Mutat. Res. 309 (1994) 273–284.
[156] N. Tanaka, M. Katoh, S. Iwahar, Formation of chromosome-type aberrations at first cleavage MMS treatment in late spermatids of mice, Cytogenet. Cell Genet. 31 (1981) 145–152.
[157] U.H. Ehling, Quantification of the genetic risk of environmental mutagens, Risk Anal. 8 (1988) 45–57.
[158] F. Sobels, The Parallelogram: An Indirect Approach for the Assessment of Genetic Risk from Chemical Mutagens, in: K.C. Bora, G.R. Douglas, E.R. Nestmann (Eds.), Elsevier, Amsterdam, 1982, pp. 323–327.
[159] J. Favor, D. Layton, G. Sega, J. Wassom, J. Burkhart, G. Douglas, K. Dearfield, D. Brusick, International commission for protection against environmental

mutagens and carcinogens. Genetic risk extrapolation from animal data to human disease. A taskgroup report. No. 105, Mutat. Res. 330 (1995) 23–34.
[160] U.S. EPA, Benchmark Dose Technical Guidance, EPA/100/R-12/001.2012.
[161] J.T. MacGregor, R. Frötschl, P.A. White, K.S. Crump, D.A. Eastmond, S. Fukushima, M. Guérard, M. Hayashi, L. Soeteman-Hernandez, T. Kasamatsu, D.D. Levy, T. Morita, L. Müller, R. Schoeny, M.J. Schuler, V. Thybaud, G.E. Johnson, IWGT report on quantitative approaches to genotoxicity risk assessment I. Methods and metrics for defining exposure-response relationships and points of departure (PoDs), Mutat. Res. 783 (2015) 55–65.
[162] J.T. MacGregor, R. Frötschl, P.A. White, K.S. Crump, D.A. Eastmond, S. Fukushima, M. Guérard, M. Hayashi, L. Soeteman-Hernandez, G.E. Johnson, T. Kasamatsu, D.D. Levy, T. Morita, L. Müller, R. Schoeny, M.J. Schuler, V. Thybaud, IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk, Mutat. Res. 783 (2015) 66–78.
[163] G.E. Johnson, L.G. Soeteman-Hernandez, B.B. Gollapudi, G. Bodger, K.L. Dearfield, R.H. Heflich, J.G. Hixon, D.P. Lovell, J.T. MacGregor, L.H. Pottenger, C.M. Thompson, L. Abraham, V. Thybaud, J.Y. Tanir, E. Zeiger, J. van Benthem, P.A. White, Derivation of point of departure (PoD) estimates in genetic toxicology studies and their potential applications in risk assessment, Environ. Mol. Mutagen. 8 (2014) 609–623.
[164] B.B. Gollapudi, G.E. Johnson, L.G. Hernandez, L.H. Pottenger, K.L. Dearfield, A.M. Jeffrey, E. Julien, J.H. Kim, D.P. Lovell, J.T. Macgregor, M.M. Moore, J. van Benthem, P.A. White, E. Zeiger, V. Thybaud, Quantitative approaches for assessing dose–response relationships in genetic toxicology studies, Environ. Mol. Mutagen. 54 (2013) 8–18.
[165] I.M. Campbell, J.R. Stewart, R.A. James, J.R. Lupski, P. Stankiewicz, P. Olofsson, C.A. Shaw, Am. J. Hum. Genet. 95 (2014) 345–359.