Models of germ cell development and their application for toxicity studies

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Abstract

Germ cells are unique in their ability to transfer genetic information and traits from generation to generation. As such, the proper development of germ cells and the integrity of their genome are paramount to the health of organisms and the survival of species. Germ cells are also exquisitely sensitive to environmental influences although the testing of germ cell toxicity, especially in females, has proven particularly challenging. In this review, we first describe the remarkable odyssey of germ cells in mammals, with an emphasis on the female germline, from their initial specification during embryogenesis to the generation of mature gametes in adults. We also describe the current methods used in germ cell toxicity testing and their limitations in examining the complex features of mammalian germ cell development. To bypass these challenges, we propose the use of alternative model systems such as *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans* and in vitro germ cell methods that have distinct advantages over traditional toxicity models. We discuss the benefits and limitations of each approach, their application to germ cell toxicity studies, and the need for computational approaches to maximize the usefulness of these models. Together, the inclusion of these alternative germ cell toxicity models will be invaluable for the examination of stages not easily accessible in mammals as well as the large scale, high-throughput investigation of germ cell toxicity.

Keywords

germline; germ cells; meiosis; toxicity; yeast; *Drosophila*; *C. elegans*; stem cell

Introduction

The germ cell lineage is unique as it is the only cell type that bridges generations. Germ cells follow an intricate developmental path that depends on the interaction of several cell types and that spans several months to many years in mammalian species [Lesch and Page 2012]. For these reasons, the recapitulation of germ cell differentiation events in cell culture

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systems has historically proven difficult [Susiarjo et al. 2009]. It is for the same reasons that testing for germ cell toxicity has been mainly limited to either female germ cells of late (non-embryonic) stages of differentiation or to male germ cells where access is facilitated by continuous meiotic differentiation [Susiarjo et al. 2009]. In the present review, we first summarize the key events of mammalian germ cell specification and differentiation and identify current challenges of toxicity testing in germ cells, particularly in the female germline. We then discuss the utility of several model organisms and approaches that may circumvent some of the difficulties inherent to the study of germ cells. We particularly emphasize the benefits of using phenotypic assays in each model system that encompass both genotoxicity and reproductive toxicity, as these aspects of germ cell toxicity share a significant overlap and are mechanistically related.

Mammalian germ cell development, a brief overview

In mammals, several key phases of germ cell development can be recognized. During the specification phase in mice, germ cells emerge at around embryonic day E6 in the epiblast closest to the extraembryonic ectoderm [Chiquoine 1954]. These cells arise through induction by BMP signaling (BMP-4 and BMP-8b) from the extraembryonic ectoderm as well as by canonical WNT signaling in the form of WNT3 from the visceral endoderm adjacent to the epiblast (for review see [Magnusdottir and Surani 2014]). By approximately E7.25, a small number of germ cells (~40) are clearly distinguishable by expression of markers such as *Blimp1* and *Prdm14* [Nakaki et al. 2013].

Following the second phase (the migration to the genital ridge at around E10.5), the female primordial germ cells complete several rounds of mitotic divisions, until E13.5, before arresting. While male germ cells only resume differentiation at puberty, female germ cells directly initiate the third phase: meiotic differentiation [Lesch and Page 2012]. These cells undergo a series of stereotypical, evolutionarily conserved steps inherent to meiotic differentiation. At the chromosomal level, the initiation of meiosis is marked first by the moderate condensation of chromatin into DNA “tracks” which, while not resembling metaphase chromosomes yet, facilitate the interaction of homologues during the first steps of Prophase I [Pawlowski and Cande 2005]. Chromosomal interactions initiate by the alignment and pairing of the homologues, mediated by the tethering, or restriction, of chromosome ends to one side of the germ cell nucleus and coordinated chromosome movement [Scherthan et al. 1996]. When two homologues are in close proximity to each other, they initiate the process of pairing. Pairing varies among species, but commonly involves a sequence-based homology search and the temporary establishment of a homologous chromosome pair [Klutstein and Cooper 2014]. These homologous pairs are then stabilized by the establishment of an intricate protein structure that resembles a ladder, named the synaptonemal complex (SC), which physically locks homologous chromosomes in place [Heyting 1996]. Meiotic recombination is overlaid onto the SC assembly. Recombination is initiated by a programmed double strand break in the DNA performed by the topoisomerase-related protein SPO-11 [Romanienko and Camerini-Otero 1999; Romanienko and Camerini-Otero 2000]. Repair of double strand breaks in somatic cells can be accomplished through several pathways including non-homologous end joining or single strand annealing [Andersen and Sekelsky 2010]. However, meiotic cells principally make

use of the homologous recombination pathway in order to create a minimum of one cross-over event per chromosome [Andersen and Sekelsky 2010]. The establishment of a crossover event is dependent on the repair of the double strand break using the homologous chromosome sequence as a template [Liu and Huang 2014]. Importantly, the repair of at least one break through the cross-over pathway is necessary for the exchange of information between homologous chromosomes as well as for the proper alignment and segregation of chromosomes at Metaphase and Anaphase I [Roeder 1997].

In female mammalian germ cells, meiosis arrests at the end of Prophase I, at the stage of diplotene, and only resumes at puberty with the process of ovulation [Feng et al. 2014]. Female germ cells, i.e. oocytes, first segregate the recombined homologous chromosomes during Meiosis I followed by the segregation of sister chromatids during Meiosis II. Female oocytes arrest again at Metaphase II until fertilization by the sperm which prompts meiotic resumption and activation of the embryonic program.

As noted earlier, many crucial events that guide germ cell specification and early differentiation in mammalian female germ cells take place during embryonic stages, which, due to the small size of the gonad and the small number of cells, renders their isolation and study arduous [Susiarjo et al. 2009]. Early meiotic differentiation in mammals is especially difficult to investigate as the meiotic process unfolds over the course of embryogenesis and a particular meiotic stage is only present at a given stage of embryogenesis. These aspects of mammalian germ cells are difficult to reconcile with the need to assess the toxicity of a large number of chemicals over many stages of germ cell differentiation. For these reasons, the leveraging of alternative models for the study of germ cell toxicity is particularly attractive. Below we review the current tests for reproductive toxicity and germ cell genotoxicity and highlight their advantages as well as some of their current limitations.

Contemporary landscape of toxicity testing in germ cells

Current methodologies for reproductive toxicology testing of pharmaceuticals, as recommended by the International Conference on Harmonisation (ICH; guideline S5:R2), utilize dosing strategies that start four weeks prior to mating in male mice and two weeks in females. This time frame ensures that maturation of spermatocytes and ovulation occurs during the exposure period. Depending upon study design and endpoints, treatment of the mother continues at least through implantation but may continue post-natally through weaning. Such studies aim to identify effects on fertility of the parents as well as early embryonic development through reproductive maturation of the F1 generation. However, these experiments fail to examine the early mitotic and meiotic events of both oocytes and sperm directly, instead relying on endpoints such as offspring lethality and low body weight. Taking into account that these studies generally use only between 16 and 20 mating pairs, abnormalities occurring in low frequency in developing sperm or oocytes may not be detected by measuring fecundity.

The issue of capturing low frequency events also applies to the tried-and-true dominant lethal test (DLT), which is used to detect the ability of chemicals to induce chromosomal abnormalities and mutations in offspring that manifest as embryonic lethality [Epstein

1973]. Males are treated for varying periods up to a full cycle of spermatogenesis, and mated. Mated females are sacrificed during pregnancy and the number of embryos, implants and ovulated eggs is assessed. One approach that can address the low frequency issue is the more sensitive method of fluorescence in situ hybridization (FISH) that can be used to probe for chromosome anomalies and measure their frequency in a high number of sperm cells [Shi and Martin 2000; Tempest et al. 2004; Pereira et al. 2014]. However, the examination of numerous distinct types of rearrangements through FISH is tedious and would also not identify subtle alterations to the genome such as small INDELs or point mutations.

In transgenerational studies, offspring observations typically end with the F2 generation, and as such only serve to identify effects of direct and *in utero* exposures, thus failing to address the potential for defects in the F3 offspring through epigenetic means. This represents a significant shortcoming of the current approach given the profound epigenetic remodeling of embryonic germ cells and their potential susceptibility to environmental agents at these stages. With whole genome, and epigenome, techniques decreasing in cost and increasing in speed, it will be appropriate to examine one or two generations beyond those exposed *in utero* for evidence of genomic instability (e.g. mutations, CNVs) and changes in patterns of DNA methylation across the genome.

The need to distinguish genotoxicity in germ cells versus somatic cells is also becoming increasingly important as highlighted in the report from the 2013 International Workshop on Genotoxicity Testing [Yauk et al. 2015]. Since the male and female germ lines are uniquely specialized to transfer genetic material from one generation to the next, any specificity of a mutagen on the germline is (assuming the toxicant reaches the gonad in sufficient concentration) of concern regardless of whether it displays mutagenic potential in somatic cells. Current recommendations by the ICH do not address these concerns. The ICH guidelines for genotoxicity recommend a test for gene mutation in bacteria (commonly the Ames test) and an assessment in rodent somatic tissues (guideline S2:R1). The European Medicines Agency (EMA), Pharmaceuticals and Medical Devices Agency (PMDA) and the Federal Drug Administration (FDA) all follow ICH guidelines, indicating that drugs in current development internationally may not be screened directly for germ cell mutagenesis unless the sponsor is specifically asked to do so. Germ cells show differential susceptibility to toxicants in comparison to somatic cells [Ramos-Morales and Rodriguez-Arnaiz 1995; Witt et al. 2003]. Furthermore, there is also clear evidence of sex specificity of germline sensitivity, as the timing of germ cell development, differentiation and epigenetic programming, as well as DNA repair mechanisms, are notably different in oogenesis and spermatogenesis [Ashwood-Smith and Edwards 1996; Olsen et al. 2005; Koubova et al. 2006; Leitch et al. 2013]. Therefore, the accurate identification of chemicals causing germ cell toxicities requires sex-specific assays to identify mutagens that one germ cell lineage (male or female) may be primarily susceptible to. This presents challenges in mammalian species, particularly in females, where oocyte collection is labor-intensive and yields low numbers of germ cells. As a result, there are many more assays available and in development for the analysis of sperm than for the analysis of female germ cells [Yauk et al. 2015].

There are currently several promising advances in alternative model systems that can address some of these gaps in reproductive toxicity testing. These alternative models present distinct advantages over mammalian models, offering endpoints that are not only difficult to observe in traditional models, but also enabling analysis in a way that is high-throughput and cost-effective, factors that make their integration into the current battery of toxicity tests advantageous. Below we present several powerful genetic model systems that have historically illuminated our understanding of germ cell differentiation and function: yeast, *Drosophila*, and *C. elegans*. By contrast to other models such as zebrafish, which has not been used to a great extent in the study of germ cell biology [Tzung et al. 2015], these organisms have provided a vast array of genetic, molecular and cytological tools for the study of germ cells in support of their broad acceptance and application to germ cell toxicity testing. We briefly describe each model and highlight some of their use in toxicology. We also introduce new developments in stem cell and computational toxicology that may add tremendously to our understanding of germline processes and their dysregulation following chemical exposure. We direct the reader to Figure 1 for a description of these models and to Table I for a summary of their strengths and weaknesses.

The budding yeast *Saccharomyces cerevisiae*

While the unicellular organism *Saccharomyces cerevisiae* may not be considered as a germ cell *per se*, it has nonetheless been used extensively as a model to study the cellular machinery that guides the program of cell division both during mitosis and meiosis [Szybalski 2001]. The budding yeast's life cycle includes both a haploid and diploid phase. Under normal conditions, the haploid yeasts proliferate by mitotic division. When in close proximity to each other, haploid yeast cells of the two different mating types (α or a) can respond and attract each other to initiate fusion and amphimixis, or the fusion of their haploid genome, to form a diploid cell [Herskowitz 1988]. Again, under normal conditions, these diploid cells, the favored state of budding yeasts, grow and divide through mitosis. However, upon stressful conditions such as starvation, the cells initiate meiosis to generate a set of four haploid and stress-resistant spores (phenomenon of sporulation) [Herskowitz 1988]. This allows for the synchronization of yeast cells by switching them to nutrient poor media to initiate meiosis. The cells then relatively uniformly complete one round of pre-meiotic S-phase and then proceed through meiosis. The progression through meiosis is fast and completion of Prophase I, which includes synapsis and recombination, occurs within 4 hours after media switching [Brar et al. 2012]. Thus, by collecting samples at various time points, the cells can be observed biochemically, molecularly and cytologically through the different phases of meiotic progression.

At the cytological level, yeast chromosomal dynamics during meiosis can be visualized by a variety of techniques used in other systems such as FISH, immunofluorescence and fluorescent chromosome-tagging [Straight et al. 1996]. The latter is especially attractive as it allows for the *in vivo*, real time visualization of chromosome interactions during meiosis without the need for chromosome preparation or spread. While there are several ways of creating a GFP-tagged chromosome, one popular technique is the creation of a strain that carries multiple copies of a *lacO* sequence. This sequence has the ability to be bound by the Lac repressor (*lacI*), which in this case is expressed as a fusion protein with GFP. Thus, by

inserting the *lacO* sequences at a defined chromosomal site, it is possible to monitor the interaction of the two chromosomal sites (one per homologue). This and similar approaches have been used extensively to examine the kinetics of chromosome pairing during meiosis and its genetic requirement [Brown et al. 2011; Lee et al. 2012].

Importantly, the molecular and cellular events that underlie meiosis in budding yeast are evolutionarily conserved making the budding yeast a relevant model for the study of the meiotic program. For example, while the synaptonemal complex components show little primary sequence homology across phyla, the overall organization of the SC is remarkably similar [Hawley 2011]. Thus, similarly to other species such as *Mus musculus*, *Drosophila* and *C. elegans*, the yeast's SC consists of two lateral elements located against the axes of the homologous chromosomes and of a central element that joins these two lateral elements [Dobson et al. 1994; Lynn et al. 2002]. The role of the SC and its regulation are also well conserved. Indeed, just as in mammals, the proper assembly of the SC along the chromosomal axes is dependent on the creation of double strand breaks as evidenced by a lack of proper SC in a *spo-11* mutant background [Giroux et al. 1989; Bhuiyan and Schmekel 2004]. Beside the structural conservation of the SC, the recombination pathway also shows remarkable evolutionary conservation. As reviewed in Youds and Boulton [Youds and Boulton 2011], a large number of genes implicated in the various aspects meiotic recombination such as double strand break formation, resection, cross-over formation and resolution and anti-cross over activity are highly conserved between yeast, *C. elegans*, *Drosophila*, and vertebrates including mammals. The near universal dependency on the same set of factors to guide homologous recombination therefore allows for the study of these processes when under the influence of deleterious influences such as mutations or chemical exposures.

The conservation in the DNA repair machinery in yeast has been exploited to develop new methods to assess chemical toxicity. For example, Schiestl and colleagues developed a del assay that uses the ability of a yeast strain that does not grow on minimal media to revert to a genetic state where it can grow (positive selection) [Schiestl 1989; Brennan and Schiestl 2004]. The del assay is based on a strain containing two copies of a *HIS3* gene that have been partially deleted, one in the 5' end of the gene and one in the 3' end of the gene. Separating these two partially deleted *HIS3* genes is an intervening sequence of DNA. When the strain is exposed to mutagens, carcinogens or clastogens, a high number of breaks are created which, in an attempt to repair the break by recombination, results in the deletion of the intervening sequence and the recreation of a functional *HIS3* gene allowing the yeast to grow on histine-deficient media [Brennan and Schiestl 2004]. This assay has been adapted for high-throughput screening and has also been used to perform chemical screens for differential sensitivity to radiation [Hafer et al. 2010]. Thus, this screen has the ability to identify chemicals that interfere with conserved DNA repair pathways. While the aforementioned research did not examine the modulation of DNA repair in the context of meiosis, it clearly highlights the potential of this model for quickly and reliably examining two crucial processes of germ cell differentiation: SC formation and maintenance, and homologous recombination.

The fly *Drosophila melanogaster*

Drosophila is one of the canonical models of modern genetics and the fundamental discoveries originating from its use have illuminated our understanding of many fields including genomics, signal transduction, embryonic patterning and cancer biology [Rubin and Lewis 2000]. Advantages of the system are a short generation time of about 10 days, low cost of culture and maintenance, and a large collection of genetic mutants, reporter strains and techniques that can be applied to the study of germ cell development and toxicity [Ashburner et al. 2005].

Germline formation in *Drosophila* initiates early during embryogenesis by the precocious cellularization of a portion of the cytoplasm, named the pole plasm, at the posterior end of the otherwise syncytial embryo [Williamson and Lehmann 1996]. The pole plasm contains many factors, mRNA and protein, that are important for the organization of a functional pole plasm that generates germ cells [Williamson and Lehmann 1996]. Such well characterized factors, such as vasa and nanos (homologs of the human genes *VASA* and *NANOS1*, respectively), are evolutionarily conserved and also function in the production of early mammalian germ cells [Fujiwara et al. 1994; Subramaniam and Seydoux 1999; Castrillon et al. 2000; Jaruzelska et al. 2003]. This is surprising as germ cells originate in vastly different ways between mammals and in flies: they are induced in mice and are pre-determined in *Drosophila* (as well as in *C. elegans*) [Lesch and Page 2012]. Nonetheless, mouse mutants for the mouse vasa homologue (Mvh), show an arrest and death of PGCs at the zygotene stage [Tanaka et al. 2000], while Nanos2 mutants fail to enter meiosis completely [Suzuki et al. 2007]. Subsequent to the formation of the pole/germ cells, these cells are internalized during gastrulation and then actively migrate through several tissues layers to reach the posterior mesoderm where surrounded by mesodermal cells, the germ cells form the embryonic gonad and later the ovary (reviewed in [Williamson and Lehmann 1996]).

The adult female fly contains two ovaries that each empty into an oviduct that eventually fuses into a common oviduct. Each ovary is composed of 16 ovarioles that resemble beads on a string. The ovarioles are a chain of egg chambers at various stages of development, preceded by the germarium at the most anterior end where germline stem cells reside. Within each egg chamber a complex of 16 germ cells form a cluster of cells interconnected by cytoplasmic bridges (reviewed in [Williamson and Lehmann 1996]). One of these cells, the one with the greatest number of cytoplasmic connections, differentiates into an oocyte while the others become polyploid and assume nurse cell roles. Nurse cells provide mRNA, proteins and nutrients to the oocyte via cytoplasmic streaming. Eventually, the nurse cells empty all their contents into the oocytes and disappear by apoptosis, leaving one oocyte surrounded by follicle cells that also provide support and factors guiding oocyte patterning [Rongo and Lehmann 1996]. Superimposed onto the differentiation of the oocyte, meiosis also proceeds. Meiosis is initiated within the germarium and progresses as the oocyte moves down the ovariole. Therefore, oocytes at various stages of meiosis can be identified along the length of the ovariole. For example, zygotene-stage germ cells are found in the germarium and in egg chambers of stage 1, while pachytene cells are found in stage 5 egg chambers [Davring and Sunner 1973]. This greatly facilitates the study of meiotic

progression, as ovarioles can be dissected and stained for mRNA or proteins of interest revealing the kinetics of expression of these factors during meiosis.

While in appearance germline formation and germ cell differentiation differ from that of mammals, there is a great level of evolutionary conservation of germ cell processes between species. First, as described above, factors such as nanos and vasa are required for germ cell formation in *Drosophila* and are also required for proper germ cell function in mammals. Second, the epigenetic control of germ cells is also conserved. A shared feature of early germ cell development is the establishment of an overall transcriptionally quiescent state where somatic genes are repressed [Seydoux and Braun 2006]. This is achieved in part by the establishment of a repressive chromatin state, where repressive marks such as H3K9me3 and H3K27me3 are added and active marks such as histone H3K4me2 are removed [Rangan et al. 2011]. The establishment and maintenance of these marks is especially important in *C. elegans* and *Drosophila* as they do not perform or rely on DNA methylation to achieve a repressive germ cell state [Regev et al. 1998]. Finally, as described in the previous section, there is a high degree of conservation of key meiotic processes including sister chromatid cohesion, SC assembly and regulation and homologous recombination. In fact, one of few distinctions between models appears to be the requirement of recombination for proper SC assembly: yeast and mammals require recombination for successful SC assembly while *Drosophila* and *C. elegans* do not (reviewed in [McKim et al. 2002]). Interestingly, the germ cells of male flies do not undergo recombination during meiosis; therefore, the study of conserved meiotic events is restricted to females flies.

Drosophila is a historical model for the detection of induced mutations in germ cells since the characterization of X-ray germ cell mutagenicity by Muller in the late 1920's [Muller 1930]. The comprehensive application of this assay for the screening of chemicals for mutagenicity was later reported in the 1980's [Lee et al. 1983]. The primary assay, named the sex-linked recessive lethal test, takes advantage of several features of genetics in flies: the existence of balancer chromosomes, the use of common non-lethal marker mutations such as white eyes and bar-shaped eyes to mark and follow the balancer chromosome, and the fast generation time that allows the test to run over two generations. Briefly, XY male WT flies are first exposed to a chemical of interest. The exposed males are then mated with XX female flies that are homozygous for the white and bar-shaped eyes recessive mutations. The F1 generation is allowed to intercross to generate an F2 population. In a normal situation, the F2 population is composed of XY males with red round eyes and white bar-shaped eyes and females with red and white bar-shaped eyes. While mutations occur on all chromosomes, mutations in essential genes on the X chromosome are particularly disadvantageous to males as they do not have a second X chromosome (hemizygosity). In the context of the sex-linked lethal test, the mutated X chromosomes are found in the F2 in their hemizygous state only in males with round and red eyes. Therefore, by counting the proportion of males in the F2 with red/round eyes vs white/bar eyes, the germ cell mutagenicity of compounds can be quantified [Adler et al. 1985]. Comparison with other models revealed that the test is highly specific but is unfortunately not as sensitive when compared to other models of mutagenicity [Foureman et al. 1994]. The test has since fallen out of favor, supplanted by the use of the Muta™ Mouse model for germ cell mutation

[Myhr 1991; Tinwell et al. 1997]. The use of the Muta™ Mouse, however, does not allow the testing of chemicals on a large-scale platform and has the same limitations inherent to in vivo mammalian models. Hence, there has been a resurgence of the *Drosophila* model, which allows both complex and large scale examination of alteration of recombination frequencies following exposure or of other effects on germ cells such as chromosome segregation errors or epigenetic alterations [Szabad et al. 2012].

The nematode *Caenorhabditis elegans*

C. elegans offers several advantages for the study of germ cells that are anchored in the biology of the organism. First, *C. elegans* has a short life cycle (~4 days at 20°C), which facilitates not only the maintenance of the worms but also the study of fertility and multi-generational effects [Brenner 1974]. Second, the nematode contains a large number, several hundreds, of germ cells totalling about 50% of all cells in an adult worm. Additionally, the worms are transparent, which greatly facilitates the observation of intact germ cell nuclei and chromosomes as they differentiate without the need for complex preparation or dissection. Finally, *C. elegans* displays a temporal-spatial gradient of meiotic differentiation [Zetka and Rose 1995]. This implies that as germline nuclei progress along the length of the elongated gonad, they at the same time progress through the different stages of meiosis. This is in contrast to mammalian germ cells, where germ cells remain in place in the embryonic gonad while progressing through meiosis. In the nematode, this results in the chain-like juxtaposition of germ cells at all stages of meiosis. Combined with the transparency of the organism, this feature allows the observation of the morphology and chromosomal dynamics of germline nuclei in a kinetic fashion [Zetka and Rose 1995; Zetka 2009].

In several studies these features of *C. elegans* have been applied to examine the effect of chemical exposures on the process of germ cell differentiation. In one study, the effect of Bisphenol A (BPA), a known meiotic disruptor in mammals, was tested in *C. elegans* [Allard and Colaiacovo 2010; Allard and Colaiacovo 2011]. In mice, BPA exposure in adults leads to congression failure or the improper alignment of chromosomes on the metaphase plate during meiosis [Hunt et al. 2003]. *In utero* exposure to BPA on the other hand led to the generation of aneuploid oocytes and embryos likely stemming from altered synapsis and recombination during Prophase I [Susiarjo et al. 2007]. *C. elegans* was therefore used to not only verify whether the nematode model recapitulates the mammalian features of germ cell exposure to BPA, but also to provide some mechanistic insight into its effect on the meiotic machinery [Allard and Colaiacovo 2010]. BPA exposure led to the presence of meiotic aberrations in the nematode that are reminiscent of the mouse phenotypes including homologous synapsis and chromosome segregation defects. While entry into meiosis appeared normal, the kinetics of progression through recombination appeared altered as evidence by the delayed removal of the strand invasion marker RAD-51 and activation of the DNA repair checkpoint machinery. These observations suggest a model in which BPA impairs the turnover of double strand breaks and repair through homologous recombination. This study therefore highlights the advantage of using a genetically and cytologically tractable model for rapid analysis of the effects of chemical exposure on germ cell differentiation.

Another study took advantage of both the small size of the worm as well as of the genetic tools that already exist for the study of germline function [Allard et al. 2013]. The goal of the study was to develop an assay that could faithfully detect chromosome anomalies following chemical exposure. By combining a green-fluorescent reporter assay to represent aneuploidy events with high-throughput methodologies, a large number of chemicals can be assayed for their ability to generate embryonic aneuploidy. Groups of 100–200 worms were each exposed to 50 chemicals at 100 μ M for 24 hours and 65 hours in multi-well plates in triplicate. At both time points a number of clear positives were easily identified by GFP expression in embryos visualized by fluorescence microscopy. Follow-up studies verified the presence of aneuploidy and also examined whether these defects originated from disruption of the meiotic program and not from embryonic mitoses. Importantly, the assay bears relevance to mammalian models as the comparison of the nematode data with reproductive toxicity endpoints for these 50 chemicals from US Environmental Protection Agency's ToxRef database revealed a 69% concordance (balanced accuracy). Therefore, this *C. elegans* assay provides a fast strategy for the initial assessment of chemical effects on germline function. Such an assay can be followed by the targeted mechanistic analysis of some of the compounds identified in the screen as highlighted in a recent publication describing the meiotic pathways altered by several of the hits from the aneuploidy screen, including the pesticides Maneb, Diazinon and Fenarimol [Parodi et al. 2015].

In summary, *C. elegans* offers clear advantages for the study of germ cell function including the ability to observe intact chromosomal dynamics as well as the kinetics of meiotic progression. In addition, the genetic tractability of the model facilitates the design of screening tools for the assessment of chemical impact on germ cells. Some caveats of the model include the evolutionary distance between worms and mammals, which inevitably leads to differences in some aspects of meiosis. For example, in *C. elegans* as well as *Drosophila*, synapsis is mostly recombination-independent while it depends on the activity of SPO-11 in other models including mice [Dernburg et al. 1998]. Therefore, it is important to probe the evolutionarily conserved processes and complement a *C. elegans* mechanistic or large scale assay with a mammalian follow up, which is now made easy by recent development in in vitro germ cell culture systems (see below).

In vitro mammalian germ cells: potential for novel toxicity applications

Owing to recent advances in stem cell and germ cell biology, several groups have established methods for the in vitro differentiation of germline cells from embryonic stem cells (ESCs) [Hayashi and Saitou 2014; Magnusdottir and Surani 2014]. These methods rely heavily on our understanding of the embryonic signals that lead to PGC specification and differentiation and aim to recapitulate the embryonic environment in an in vitro context. For example, cultured ESCs have to be sequentially differentiated first into epiblast-like cells (EpiLCs) by a combination of Activin and BMP ligands followed by addition of BMP4/8b, Stem-cell factor (SCF) and EGF for several more days. Remarkably, the resulting PGC-like cells that are generated express the markers of nascent PGCs such as Blimp-1 and Prdm-14. As the overall number of such cells remains low within a mixed aggregated population, this culture method is combined with reporter tools such as the ESC lines called BVSC, which stands for Blimp1-Venus and Stella-Cyan fluorescent protein (CFP) [Hayashi et al. 2012;

Hayashi and Saitou 2013]. Thus, by sorting cells based on their positive status for Venus and CFP, it is possible to quickly and easily identify and isolate cells that have the characteristics of primordial germ cells. These cells are referred to as Primordial Germ Cell Like Cells (PGCLCs).

Beyond marker expression, the PGCLCs also recapitulate events taking place in germ cells at early stages of development [Hayashi et al. 2011]. For example, the dynamic changes in histone methylation and acetylation, and DNA methylation, are observed in the emerging PGCLC population in vitro. Histone H3K9 mono and di-methylation marks and histone acetylation are found at low levels on PGCLC chromatin at all stages of differentiation, while H3K27 methylation becomes enriched in the first four days of culture [Nakaki et al. 2013]. DNA methylation is found in PGCLCs DNA at day 1, and then is rapidly lost from the majority of chromatin between day 2–4 of differentiation with small discrete regions of the genome retaining methylation [Vincent et al. 2011; Hayashi et al. 2012; Hayashi and Saitou 2013; Leitch et al. 2013; Vincent et al. 2013]. Thus, this method allows for the study of the epigenetic changes taking place in germ cells at their early stages of differentiation. Hayashi and colleagues have demonstrated that PGCLCs can be brought further along the differentiation pathway in vitro [Hayashi et al. 2012]. By combining PGCLCs with somatic cells from E12.5 female gonads, the aggregates generated show presence of germ cells that have initiated the meiotic program and display evidence of synapsis, equivalent to E15.5 PGCs. Following transplantation into a host ovary, these aggregates give rise to live offspring indicating that the PGCLCs represent a viable cell population capable of fully recapitulating the meiotic program, fertilization and early development [Hayashi et al. 2012].

These approaches for the generation of mammalian germ cells in vitro have yet to be applied to toxicity testing. Furthermore, the yield of cells generated in the production of mammalian germ cells in vitro is still low, especially if meiotic germ cells are needed [Vincent et al. 2011]. Thus, the aforementioned techniques must be developed further if they are to be applied to screen chemicals. However, it is clear that the in vitro generation of mammalian germ cells shows great potential for the study of germ cell toxicity, in line with the increase use of ES cell-based assays in toxicity testing [Hong and Jeung 2013].

Incorporating advances in computational toxicology

Each model described above displays significant strengths but also weaknesses inherent to its biology and model system used (Table I). For example, while all stages of Prophase I can easily be studied in *C. elegans* and incorporated into large scale assays, evolutionary divergence of this model from mammals requires secondary validation in a mammalian species. Conversely, mammalian in vitro models show great relevance but are still mainly limited to the study of pre-meiotic germ cells and are thus most adequate for the examination of epigenetic remodeling. Therefore, there is a need for the coordinated testing of chemicals across several model systems.

Surprisingly, there is currently no published work that makes use of several genetic model systems concomitantly (e.g., yeast, *Drosophila* and *C. elegans* combined) to examine their

predictivity towards mammalian germ cell toxicity. In an ideal scenario, a list of compounds with well-defined mammalian germ cell toxicity, either positive or negative, would be tested across all model systems described above. The data generated in these models would then be compared to the mammalian data and the rates of true and false positives and negatives in each model would be established. A limitation of this scenario is the need to test a large number of chemicals to gain statistical power. However, while there is published evidence of mammalian germ cell toxicants [Hales and Robaire 2001], as a field, we are far from having generated a comprehensive list of chemicals with either obvious germline toxicity or clearly devoid of such activity. One possibility to circumvent this caveat is to make use of the U.S. Environmental Protection Agency's ToxRef database, which compiles a large dataset of in vivo endpoints including multi-generational and reproductive endpoints [Martin et al. 2009; Knudsen et al. 2013]. While ToxRef does not contain germ cell toxicity endpoints, it does include endpoints that can be used as proxies (e.g., ovarian morphology defects or decreased fertility). Comparison against the ToxRef database has been used to calculate the predictivity of the aforementioned *C. elegans* aneuploidy assay towards rat reproductive toxicity. Although the endpoints compared are distinct, they are nonetheless mechanistically linked as germline dysfunction leads to increased aneuploidy and decreased fertility [Hassold and Hunt 2001; Hunt and Hassold 2008].

As the predictive value of mammalian germ cell or reproductive toxicity endpoints is likely to differ between models, the idea of exploring the predictivity of models in combination is particularly attractive. Therefore, a robust statistical model encompassing several alternative organisms and approaches should be established. To build such a model, we propose an approach similar to that described above. A small number of chemicals should be chosen from the Toxref database and tested in each model organism, focusing first on chromosomal errors that are easiest to test in all of the models described above. The predictive value of each model should be calculated independently towards reproductive toxicity endpoints contained in the Toxref database including decreased fertility, litter size and overall reproductive performance. Following this, different combinations of models should be used and their combined predictive value towards mammalian endpoints will be calculated. For such an analysis, an intersectional approach (compounds positive in all models) or an additional approach (all compounds positive in at least one model) should both be considered. A weighted analysis should also be included whereby one model system may carry more weight for the predictivity analysis than another depending on the endpoint. Together, this work will establish the minimum number of (or easiest/least expensive) tests that would need to be performed in order to achieve maximal predictivity towards mammalian reproductive endpoints.

Finally, computational toxicology approaches are crucial to interpreting the wealth of genomics data that can be generated in each model. While this review mainly focused on phenotypic assays, advances in the field of genomics, and epigenomics, have meant that we can correlate powerful phenotypic indicators with whole genome analysis for the identification of the root of phenotypes. This is particularly facilitated by the use of the model organisms discussed in this review as they are isogenic. Thus, with the low cost, coverage and speed of exome or whole-genome sequencing mutations can easily be

identified. In humans, the ongoing 1,000 genomes project has identified 38 million single nucleotide polymorphisms among 1092 culturally diverse individuals [Genomes Project et al. 2012]. Such whole-genome, high resolution approaches have also been used to examine a total of 78 trios of Icelandic families to assess the rate and types of germline *de novo* mutations. The mutation rate was estimated to be 1.20×10^{-8} per nucleotide per generation with a sharp enrichment of transition mutations and within CpG sites and a significant effect from paternal age [Kong et al. 2012]. By employing a similar strategy in our proposed germ cell models, a toxicological dichotomy emerges: not only will whole genome sequencing data reveal mutations caused by a chemical exposure, it can also potentially identify genetic differences between models that result in unique sensitivity or resistance to exposure (a factor to be considered with our proposed weighted predictivity approach). Thus, the genetic homogeneity of these models can be used as a strength when coupled with whole-genome and next generation sequencing techniques. Similarly, large scale analysis of non-genomic effects, such as environmental impact on the methylome, can be rapidly performed in these models. For example, methylated DNA immunoprecipitation-next generation sequencing (MeDIP-seq) has recently been used to determine epigenetic changes in the lung following exposure to irritants [Cheng et al. 2014]. A similar MeDIP-seq analysis would be particularly useful in germ cell toxicity studies in order to predict multigenerational changes.

A corollary to the collection of a vast amount of genomic information is the need to analyze these large data sets and interpret them in a toxicological context. To do this, various computational models are being developed to predict biological responses based on microarray data [Thomas et al. 2013]. For example, in order to analyze toxicogenomic data from the TG-GATEs database in a biologically relevant way, Nagata and colleagues employed the Classification Based on Association (CBA) algorithm. The CBA classifier established by the authors was used to successfully predict whether specific compounds increased liver weight after a 14 day treatment period in rats. Additionally, pathway analysis for sets of genes selected by the qualifier revealed three distinct gene clusters associated with increased liver weight: drug metabolism, histidine degradation and gluconeogenesis pathways [Nagata et al. 2014]. The application of such approaches to the study of germ cell toxicity would open tremendous opportunities. Similar techniques would allow researchers to identify gene signatures of perturbed pathways that lead to endpoints of interest, such as aneuploidy. In conclusion, by combining relevant, cost-effective and high-throughput alternative models with up-and-coming, sensitive techniques, the predictive value of germ cell toxicity assays will see a major leap forward.

Summary and perspectives

While some of the models described above have yet to be applied to the question of germ cell toxicity, their use would be greatly beneficial to the field. Indeed, these models allow (1) examination of stages of germ cell development that are not easily accessible in mammals (specifically early development of the female germline), (2) the investigation of germ cell toxicity on a large-scale, high-throughput platform and (3) the easy mechanistic dissection of altered processes that are evolutionarily conserved. Currently, international standards rely on aging methods to test germ cell toxicity, despite the development of more advanced technologies and powerful alternative models. As the field evolves, more sensitive

assays and endpoints for germ cell toxicity should be implemented as is recommended in the Toxicity Testing in the 21st Century paradigm [National Research Council 2007]. Issues of dose and relevance are inherent to using model systems and do not necessarily diminish the benefits of taking advantage of what these models offer. As the field moves forward, a careful comparative analysis of these models will need to be performed in order to firmly establish these model systems as useful germ cell toxicity tools.

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Reference

- Adler ID, Cole J, Danford N, Ehling U, Parry M, Roderick R, Venitt S, Vogel W, Waters R. Guide to short-term tests for detecting mutagenic and carcinogenic chemicals. 1985
- Allard P, Colaiacovo MP. Bisphenol A impairs the double-strand break repair machinery in the germline and causes chromosome abnormalities. *Proc Natl Acad Sci U S A*. 2010; 107(47):20405–20410. [PubMed: 21059909]
- Allard P, Colaiacovo MP. Mechanistic insights into the action of Bisphenol A on the germline using *C. elegans*. *Cell Cycle*. 2011; 10(2):183–184. [PubMed: 21228622]
- Allard P, Kleinstreuer NC, Knudsen TB, Colaiacovo MP. A Screening Platform for the Rapid Assessment of Chemical Disruption of Germline Function. *Environ Health Perspect*. 2013
- Andersen SL, Sekelsky J. Meiotic versus mitotic recombination: two different routes for double-strand break repair: the different functions of meiotic versus mitotic DSB repair are reflected in different pathway usage and different outcomes. *Bioessays*. 2010; 32(12):1058–1066. [PubMed: 20967781]
- Ashburner, M.; Golic, KG.; Hawley, RS. *Drosophila : a laboratory handbook*. 2nd edition. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 2005. p. 1409xxviii
- Ashwood-Smith MJ, Edwards RG. DNA repair by oocytes. *Mol Hum Reprod*. 1996; 2(1):46–51. [PubMed: 9238657]
- Bhuiyan H, Schmekel K. Meiotic chromosome synapsis in yeast can occur without Spo11-induced DNA double-strand breaks. *Genetics*. 2004; 168(2):775–783. [PubMed: 15514052]
- Brar GA, Yassour M, Friedman N, Regev A, Ingolia NT, Weissman JS. High-resolution view of the yeast meiotic program revealed by ribosome profiling. *Science*. 2012; 335(6068):552–557. [PubMed: 22194413]
- Brennan RJ, Schiestl RH. Detecting carcinogens with the yeast DEL assay. *Methods Mol Biol*. 2004; 262:111–124. [PubMed: 14769958]
- Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics*. 1974; 77(1):71–94. [PubMed: 4366476]
- Brown MS, Zanders S, Alani E. Sustained and Rapid Chromosome Movements Are Critical for Chromosome Pairing and Meiotic Progression in Budding Yeast. *Genetics*. 2011; 188(1):U21–U47.
- Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. *Proc Natl Acad Sci U S A*. 2000; 97(17):9585–9590. [PubMed: 10920202]
- Cheng RY, Shang Y, Limjunyawong N, Dao T, Das S, Rabold R, Sham JS, Mitzner W, Tang WY. Alterations of the lung methylome in allergic airway hyper-responsiveness. *Environ Mol Mutagen*. 2014; 55(3):244–255. [PubMed: 24446183]
- Chiquoine AD. The identification, origin, and migration of the primordial germ cells in the mouse embryo. *Anat Rec*. 1954; 118(2):135–146. [PubMed: 13138919]
- Davring L, Sunner M. Female meiosis and embryonic mitosis in *Drosophila melanogaster*. I. Meiosis and fertilization. *Hereditas*. 1973; 73(1):51–64. [PubMed: 4218221]

- Dernburg AF, McDonald K, Moulder G, Barstead R, Dresser M, Villeneuve AM. Meiotic recombination in *C. elegans* initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis. *Cell*. 1998; 94(3):387–398. [PubMed: 9708740]
- Dobson MJ, Pearlman RE, Karaïskakis A, Spyropoulos B, Moens PB. Synaptonemal Complex Proteins - Occurrence, Epitope Mapping and Chromosome Disjunction. *J Cell Sci*. 1994; 107:2749–2760. [PubMed: 7876343]
- Epstein SS. Use of the dominant-lethal test to detect genetic activity of environmental chemicals. *Environ Health Perspect*. 1973; 6:23–26. [PubMed: 4592376]
- Feng CW, Bowles J, Koopman P. Control of mammalian germ cell entry into meiosis. *Mol Cell Endocrinol*. 2014; 382(1):488–497. [PubMed: 24076097]
- Foureman P, Mason JM, Valencia R, Zimmering S. Chemical mutagenesis testing in *Drosophila*. X. Results of 70 coded chemicals tested for the National Toxicology Program. *Environ Mol Mutagen*. 1994; 23(3):208–227. [PubMed: 8162896]
- Fujiwara Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, Noce T. Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila* vasa and its specific expression in germ cell lineage. *Proc Natl Acad Sci U S A*. 1994; 91(25):12258–12262. [PubMed: 7991615]
- Genomes Project C, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, Handsaker RE, Kang HM, Marth GT, McVean GA. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012; 491(7422):56–65. [PubMed: 23128226]
- Giroux CN, Dresser ME, Tiano HF. The Spo11 Gene Is Required Specifically for Chromosome Synapsis during Yeast Meiosis. *Genetics*. 1989; 122(2):S35–S35.
- Hafer K, Rivina Y, Schiestl RH. Yeast DEL Assay Detects Protection against Radiation-Induced Cytotoxicity and Genotoxicity: Adaptation of a Microtiter Plate Version. *Radiation Research*. 2010; 174(6):719–726. [PubMed: 21128795]
- Hales BF, Robaire B. Review - Paternal exposure to drugs and environmental chemicals: Effects on progeny outcome. *J Androl*. 2001; 22(6):927–936. [PubMed: 11700855]
- Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet*. 2001; 2(4):280–291. [PubMed: 11283700]
- Hawley RS. Solving a Meiotic LEGO (R) Puzzle: Transverse Filaments and the Assembly of the Synaptonemal Complex in *Caenorhabditis elegans*. *Genetics*. 2011; 189(2):405–409. [PubMed: 21994217]
- Hayashi K, Ogushi S, Kurimoto K, Shimamoto S, Ohta H, Saitou M. Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science*. 2012; 338(6109):971–975. [PubMed: 23042295]
- Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M. Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell*. 2011; 146(4):519–532. [PubMed: 21820164]
- Hayashi K, Saitou M. Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. *Nat Protoc*. 2013; 8(8):1513–1524. [PubMed: 23845963]
- Hayashi K, Saitou M. Perspectives of germ cell development in vitro in mammals. *Anim Sci J*. 2014; 85(6):617–626. [PubMed: 24725251]
- Herskowitz I. Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiol Rev*. 1988; 52(4):536–553. [PubMed: 3070323]
- Heyting C. Synaptonemal complexes: structure and function. *Curr Opin Cell Biol*. 1996; 8(3):389–396. [PubMed: 8743892]
- Hong EJ, Jeung EB. Assessment of Developmental Toxicants using Human Embryonic Stem Cells. *Toxicol Res*. 2013; 29(4):221–227. [PubMed: 24578791]
- Hunt PA, Hassold TJ. Human female meiosis: what makes a good egg go bad? *Trends Genet*. 2008; 24(2):86–93. [PubMed: 18192063]
- Hunt PA, Koehler KE, Susiarjo M, Hodges CA, Ilagan A, Voigt RC, Thomas S, Thomas BF, Hassold TJ. Bisphenol A exposure causes meiotic aneuploidy in the female mouse. *Curr Biol*. 2003; 13(7):546–553. [PubMed: 12676084]

- Jaruzelska J, Kotecki M, Kusz K, Spik A, Firpo M, Reijo Pera RA. Conservation of a Pumilio-Nanos complex from *Drosophila* germ plasm to human germ cells. *Dev Genes Evol.* 2003; 213(3):120–126. [PubMed: 12690449]
- Klutstein M, Cooper JP. The Chromosomal Courtship Dance-homolog pairing in early meiosis. *Curr Opin Cell Biol.* 2014; 26:123–131. [PubMed: 24529254]
- Knudsen T, Martin M, Chandler K, Kleinstreuer N, Judson R, Sipes N. Predictive models and computational toxicology. *Methods Mol Biol.* 2013; 947:343–374. [PubMed: 23138916]
- Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, Magnusson G, Gudjonsson SA, Sigurdsson A, Jonasdottir A, Jonasdottir A, Wong WS, Sigurdsson G, Walters GB, Steinberg S, Helgason H, Thorleifsson G, Gudbjartsson DF, Helgason A, Magnusson OT, Thorsteinsdottir U, Stefansson K. Rate of de novo mutations and the importance of father's age to disease risk. *Nature.* 2012; 488(7412):471–475. [PubMed: 22914163]
- Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC. Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proc Natl Acad Sci U S A.* 2006; 103(8):2474–2479. [PubMed: 16461896]
- Lee CY, Conrad MN, Dresser ME. Meiotic Chromosome Pairing Is Promoted by Telomere-Led Chromosome Movements Independent of Bouquet Formation. *PLoS Genet.* 2012; 8(5)
- Lee WR, Abrahamson S, Valencia R, von Halle ES, Wurgler FE, Zimmering S. The sex-linked recessive lethal test for mutagenesis in *Drosophila melanogaster*. A report of the U.S. Environmental Protection Agency Gene-Tox Program. *Mutat Res.* 1983; 123(2):183–279. [PubMed: 6413857]
- Leitch HG, Tang WW, Surani MA. Primordial germ-cell development and epigenetic reprogramming in mammals. *Curr Top Dev Biol.* 2013; 104:149–187. [PubMed: 23587241]
- Lesch BJ, Page DC. Genetics of germ cell development. *Nat Rev Genet.* 2012; 13(11):781–794. [PubMed: 23044825]
- Liu T, Huang J. Quality control of homologous recombination. *Cellular and Molecular Life Sciences.* 2014; 71(19):3779–3797. [PubMed: 24858417]
- Lynn A, Koehler KE, Judis L, Chan ER, Cherry JP, Schwartz S, Seftel A, Hunt PA, Hassold TJ. Covariation of synaptonemal complex length and mammalian meiotic exchange rates. *Science.* 2002; 296(5576):2222–2225. [PubMed: 12052900]
- Magnusdottir E, Surani MA. How to make a primordial germ cell. *Development.* 2014; 141(2):245–252. [PubMed: 24381195]
- Martin MT, Mendez E, Corum DG, Judson RS, Kavlock RJ, Rotroff DM, Dix DJ. Profiling the reproductive toxicity of chemicals from multigeneration studies in the toxicity reference database. *Toxicol Sci.* 2009; 110(1):181–190. [PubMed: 19363143]
- McKim KS, Jang JK, Manheim EA. Meiotic recombination and chromosome segregation in *Drosophila* females. *Annual Review of Genetics.* 2002; 36:205–232.
- Muller HJ. Types of visible variations induced by x-rays in *Drosophila*. *Journal of Genetics.* 1930; 22(3):U299–U297.
- Myhr BC. Validation studies with Muta Mouse: a transgenic mouse model for detecting mutations in vivo. *Environ Mol Mutagen.* 1991; 18(4):308–315. [PubMed: 1836178]
- Nagata K, Washio T, Kawahara Y, Unamia A. Toxicity prediction from toxicogenomic data based on class association rule mining. *Toxicology Reports.* 2014; 1:1133–1142.
- Nakaki F, Hayashi K, Ohta H, Kurimoto K, Yabuta Y, Saitou M. Induction of mouse germ-cell fate by transcription factors in vitro. *Nature.* 2013; 501(7466):222–226. [PubMed: 23913270]
- National Research Council. *Toxicity Testing in the 21st Century: A Vision and a Strategy.* The National Academies Press; 2007.
- Olsen AK, Lindeman B, Wiger R, Duale N, Brunborg G. How do male germ cells handle DNA damage? *Toxicol Appl Pharmacol.* 2005; 207(2 Suppl):521–531. [PubMed: 16051290]
- Parodi DA, Sjarif J, Chen Y, Allard P. Reproductive toxicity and meiotic dysfunction following exposure to the pesticides Maneb, Diazinon and Fenarimol. *Toxicology Research.* 2015
- Pawlowski WP, Cande WZ. Coordinating the events of the meiotic prophase. *Trends Cell Biol.* 2005; 15(12):674–681. [PubMed: 16257210]

- Pereira CS, Juchniuk de Vozzi MS, Dos Santos SA, Vasconcelos MA, de Paz CC, Squire JA, Martelli L. Smoking-induced chromosomal segregation anomalies identified by FISH analysis of sperm. *Mol Cytogenet.* 2014; 7(1):58. [PubMed: 25264457]
- Ramos-Morales P, Rodriguez-Arnaiz R. Genotoxicity of two arsenic compounds in germ cells and somatic cells of *Drosophila melanogaster*. *Environ Mol Mutagen.* 1995; 25(4):288–299. [PubMed: 7607183]
- Rangan P, Malone CD, Navarro C, Newbold SP, Hayes PS, Sachidanandam R, Hannon GJ, Lehmann R. piRNA production requires heterochromatin formation in *Drosophila*. *Curr Biol.* 2011; 21(16):1373–1379. [PubMed: 21820311]
- Regev A, Lamb MJ, Jablonka E. The role of DNA methylation in invertebrates: Developmental regulation or genome defense? *Molecular Biology and Evolution.* 1998; 15(7):880–891.
- Roeder GS. Meiotic chromosomes: it takes two to tango. *Genes Dev.* 1997; 11(20):2600–2621. [PubMed: 9334324]
- Romanienko PJ, Camerini-Otero RD. Cloning, characterization, and localization of mouse and human SPO11. *Genomics.* 1999; 61(2):156–169. [PubMed: 10534401]
- Romanienko PJ, Camerini-Otero RD. The mouse Spo11 gene is required for meiotic chromosome synapsis. *Mol Cell.* 2000; 6(5):975–987. [PubMed: 11106738]
- Rongo C, Lehmann R. Regulated synthesis, transport and assembly of the *Drosophila* germ plasm. *Trends Genet.* 1996; 12(3):102–109. [PubMed: 8868348]
- Rubin GM, Lewis EB. A brief history of *Drosophila*'s contributions to genome research. *Science.* 2000; 287(5461):2216–2218. [PubMed: 10731135]
- Scherthan H, Weich S, Schwegler H, Heyting C, Harle M, Cremer T. Centromere and telomere movements during early meiotic prophase of mouse and man are associated with the onset of chromosome pairing. *J Cell Biol.* 1996; 134(5):1109–1125. [PubMed: 8794855]
- Schiestl RH. Nonmutagenic Carcinogens Induce Intrachromosomal Recombination in Yeast. *Nature.* 1989; 337(6204):285–288. [PubMed: 2643057]
- Seydoux G, Braun RE. Pathway to totipotency: lessons from germ cells. *Cell.* 2006; 127(5):891–904. [PubMed: 17129777]
- Shi Q, Martin RH. Aneuploidy in human sperm: a review of the frequency and distribution of aneuploidy, effects of donor age and lifestyle factors. *Cytogenet Cell Genet.* 2000; 90(3–4):219–226. [PubMed: 11124518]
- Straight AF, Belmont AS, Robinett CC, Murray AW. GFP tagging of budding yeast chromosomes reveals that protein-protein interactions can mediate sister chromatid cohesion. *Current Biology.* 1996; 6(12):1599–1608. [PubMed: 8994824]
- Subramaniam K, Seydoux G. nos-1 and nos-2, two genes related to *Drosophila* nanos, regulate primordial germ cell development and survival in *Caenorhabditis elegans*. *Development.* 1999; 126(21):4861–4871. [PubMed: 10518502]
- Susiarjo M, Hassold TJ, Freeman E, Hunt PA. Bisphenol A exposure in utero disrupts early oogenesis in the mouse. *PLoS Genet.* 2007; 3(1):e5. [PubMed: 17222059]
- Susiarjo M, Rubio C, Hunt P. Analyzing mammalian female meiosis. *Methods Mol Biol.* 2009; 558:339–354. [PubMed: 19685334]
- Suzuki A, Tsuda M, Saga Y. Functional redundancy among Nanos proteins and a distinct role of Nanos2 during male germ cell development. *Development.* 2007; 134(1):77–83. [PubMed: 17138666]
- Szabad J, Bellen HJ, Venken KJ. An assay to detect in vivo Y chromosome loss in *Drosophila* wing disc cells. *G3 (Bethesda).* 2012; 2(9):1095–1102. [PubMed: 22973547]
- Szybalski W. My road to Ojvind Winge, the father of yeast genetics. *Genetics.* 2001; 158(1):1–6. [PubMed: 11333213]
- Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T. The mouse homolog of *Drosophila* Vasa is required for the development of male germ cells. *Genes Dev.* 2000; 14(7):841–853. [PubMed: 10766740]
- Tempest HG, Homa ST, Dalakiouridou M, Christopikou D, Wright D, Zhai XP, Griffin DK. The association between male infertility and sperm disomy: evidence for variation in disomy levels

among individuals and a correlation between particular semen parameters and disomy of specific chromosome pairs. *Reprod Biol Endocrinol*. 2004; 2:82. [PubMed: 15598347]

- Thomas R, Thomas RS, Auerbach SS, Portier CJ. Biological networks for predicting chemical hepatocarcinogenicity using gene expression data from treated mice and relevance across human and rat species. *PLoS One*. 2013; 8(5):e63308. [PubMed: 23737943]
- Tinwell H, Lefevre P, Williams CV, Ashby J. The activity of ENU, iPMS and MMS in male mouse germ cells using the Muta Mouse positive selection transgenic mutation assay. *Mutat Res*. 1997; 388(2–3):179–185. [PubMed: 9057879]
- Tzung KW, Goto R, Saju JM, Sreenivasan R, Saito T, Arai K, Yamaha E, Hossain MS, Calvert ME, Orban L. Early depletion of primordial germ cells in zebrafish promotes testis formation. *Stem Cell Reports*. 2015; 4(1):61–73. [PubMed: 25434820]
- Vincent JJ, Huang Y, Chen PY, Feng S, Calvopina JH, Nee K, Lee SA, Le T, Yoon AJ, Faull K, Fan G, Rao A, Jacobsen SE, Pellegrini M, Clark AT. Stage-specific roles for tet1 and tet2 in DNA demethylation in primordial germ cells. *Cell Stem Cell*. 2013; 12(4):470–478. [PubMed: 23415914]
- Vincent JJ, Li Z, Lee SA, Liu X, Etter MO, Diaz-Perez SV, Taylor SK, Gkoutela S, Lindgren AG, Clark AT. Single cell analysis facilitates staging of Blimp1-dependent primordial germ cells derived from mouse embryonic stem cells. *PLoS One*. 2011; 6(12):e28960. [PubMed: 22194959]
- Williamson A, Lehmann R. Germ cell development in *Drosophila*. *Annu Rev Cell Dev Biol*. 1996; 12:365–391. [PubMed: 8970731]
- Witt KL, Hughes LA, Burka LT, McFee AF, Mathews JM, Black SL, Bishop JB. 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*. 2003; 41(2):111–120. [PubMed: 12605380]
- Yauk CL, Aardemab MJ, van Benthem J, Bishop JB, Dearfield KL, DeMarini DM, Dubrova YE, Honma M, Lupski JR, Marchetti F, Meistrich ML, Pacchierotti F, Stewart J, Waters MD, Douglas GR. Approaches for identifying germ cell mutagens: Report of the 2013 IWGT workshop on germ cell assays. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 2015
- Youds JL, Boulton SJ. The choice in meiosis - defining the factors that influence crossover or non-crossover formation. *J Cell Sci*. 2011; 124(4):501–513. [PubMed: 21282472]
- Zetka M. Homologue pairing, recombination and segregation in *Caenorhabditis elegans*. *Genome Dyn*. 2009; 5:43–55. [PubMed: 18948706]
- Zetka M, Rose A. The genetics of meiosis in *Caenorhabditis elegans*. *Trends Genet*. 1995; 11(1):27–31. [PubMed: 7900192]

a. Yeast meiotic differentiation

b. The *C. elegans* germline

c. *Drosophila* oogenesis

d. *In vitro* generation of mouse primordial germ cells

Figure 1. Germ cell development and meiotic differentiation in several model systems

a. Yeast (*S. cerevisiae*) meiotic differentiation. Within the span of 6 hours, induced diploid cells undergo a last round of pre-meiotic S before initiating meiosis. During Prophase I, synapsis and recombination events occur before homologous chromosome segregation at Anaphase I. **b. The *C. elegans* germline.** In the nematode, germline nuclear differentiation is continuous. As the nuclei move along the gonad they concomitantly progress through the various stages of Prophase I starting with Leptotene (Lept) and Zygotene in the most distal region to diakinesis in the most proximal region. Oocytes (orange shading) are fertilized by

going through the spermathecal (Sp) to generate embryos. **c. *Drosophila* oogenesis.** Germline stem-cells residing in the germarium undergo a division to give rise to another stem cell or a cell that undergoes meiosis. The egg chambers, each containing one oocyte (orange shading) and nurse cells then progress through meiosis as they move forward through the ovariole. By stage 9 of oogenesis, the oocytes have completed Prophase I. **d. In vitro generation of mouse PGCs.** ES cells in culture are first induced into an epiblast like state by application in the culture media of several factors including FGF, Activin and Knock-out Serum Replacement (KSR). From that stage, PGC-like cells (PGCLC) are induced by application of a combination of BMP4/8b, FGF and KSR.

Table I

Strengths and weaknesses of models of germ cell development

Model system considered	Advantages	Limitations
<i>Saccharomyces cerevisiae</i>	<ul style="list-style-type: none"> • Conservation of meiotic processes with mammals • Spo11-dependent SC formation (as in mammals) • Fast differentiation • Inexpensive, easy to culture • Vast number of genetic mutants and deletions available • High-throughput • Amenable to biochemical analyses • Direct observation of chromosome dynamics possible 	<ul style="list-style-type: none"> • Temporal differentiation requires time-series analyses • Evolutionarily distant from mammals • Toxicokinetics parameters are likely distinct from mammals • Unicellular organism
<i>Drosophila melanogaster</i>	<ul style="list-style-type: none"> • Conservation of meiotic processes with mammals • Vast number of genetic mutants available • High-throughput 	<ul style="list-style-type: none"> • Difficult to observe chromosome morphology in intact germline • Evolutionarily distant from mammals • Toxicokinetics parameters are likely distinct from mammals • <i>Spo-11</i>-independent SC formation
<i>Caenorhabditis elegans</i>	<ul style="list-style-type: none"> • Conservation of meiotic processes with mammals • Vast number of genetic mutants available • Access to full kinetics of meiotic differentiation in each gonad • Direct observation of chromosome morphology and dynamics possible • High-throughput 	<ul style="list-style-type: none"> • Evolutionarily distant from mammals • Toxicokinetic parameters are likely distinct from mammals • <i>Spo-11</i>-independent SC formation
In vitro mammalian germ cell differentiation	<ul style="list-style-type: none"> • Higher relevance to humans compared to other models • Amenable to observation of epigenetic changes in PGCs • Potential for high-throughput screening 	<ul style="list-style-type: none"> • Not high-throughput (yet) • Difficult to examine meiotic differentiation • More expensive to culture than other model organisms