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Identifying germ cell mutagens using OECD test guideline 488 (transgenic rodent somatic and germ cell gene mutation assays) and integration with somatic cell testing

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ABSTRACT

The Organisation for Economic Co-operation and Development Test Guideline 488 (TG 488) provides recommendations for assessing germ cell and somatic cell mutagenicity using transgenic rodent (TGR) models. However, important data gaps exist for selecting an optimal approach for simultaneously evaluating mutagenicity in both cell types. It is uncertain whether analysis of germ cells from seminiferous tubules (hereafter, tubule germ cells) or caudal sperm within the recommended design for somatic tissues (i.e., 28 days of exposure plus three days of fixation time, 28 + 3d) has enough sensitivity to detect an effect as compared with the analysis of sperm within the recommended design for germ cells (i.e., 28 + 49d and 28 + 70d for mouse and rat, respectively). To address these data gaps, the Germ Cell workgroup of the Genetic Toxicology Technical Committee of the Health and Environmental Sciences Institute reviewed the available TGR mutagenicity data in male germ cells, and, characterized the exposure history of tubule germ cells for different sampling times to evaluate its impact on germ cell mutagenicity testing using TG 488. Our analyses suggest that evaluating mutant frequencies in: i) sperm from the cauda epididymis at 28 + 3d does not provide meaningful mutagenicity data; ii), tubule germ cells at 28 + 3d provides reliable mutagenicity data only if the results are positive; and iii) tubule germ cells at 28 + 28d produces reliable positive and negative results in both mice and rats. Thus, the 28 + 28d regimen may provide an approach for simultaneously assessing mutagenicity in somatic tissues and germ cells from the same animals. Further work is required to support the 28 + 28d protocol for tissues other than slowly proliferating tissues as per current TG 488. Finally, recommendations are provided to guide the experimental design for germ cell mutagenicity data for regulatory submission, as well as other possible approaches to increase the reliability of the TGR assay.

1. Foreward

It is our pleasure to contribute this paper, and its companion paper [1], to the Special Issue in honor of Dr. David DeMarini. David's passion for the field of genetic toxicology and translating that to global public policy is evidenced in the commentary he published after receiving the prestigious 2011 Alexander Hollaender Award from the Environmental Mutagenesis and Genomics Society. In his commentary [2], David calls for preparedness within the international scientific community to apply data from emerging genomic technologies to address the long-standing issue of heritable mutations in humans. David's breadth of knowledge is demonstrated by building on the evidence provided by over 80 years of genetic toxicology and germ cell biology research. The Transgenic Rodent Somatic and Germ Cell Gene Mutation Assay (TGR) (OECD Test Guideline 488) has recently found renewed interest within the genetic toxicology testing and regulatory community, in large part for its unique ability to assess chemically-induced mutations in germ cells. As such, our work to address data gaps for the optimal experimental design

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to detect germ cell mutagens is timely and in line with one of David's passions, namely promoting human health by protecting against genetic damage.

2. Introduction

Promising technical breakthroughs enabling direct measurement of germ cell and inherited *de novo* mutations have rekindled interest in identifying and characterizing human germ cell mutagens [2,3]. The Organisation for Economic Co-operation and Development (OECD) adopted Test Guideline (TG) 488 [4] that includes recommendations for assessing the mutagenic effects of chemicals in germ cells using transgenic rodent (TGR) models. These TGR mutation models harbor transgenic reporter genes that are carried on vectors designed to be easily recovered from any tissue in the body [5]. Recovered transgenes can be rapidly processed for assessment of mutations that impact the function of the reporter gene using *in vitro* bacterial assays. The TGR assay allows the determination of the mutagenic effects of the test chemicals at both site-of-contact and distal tissues including male germ cells. This provides a means to directly compare the response in germ cells to somatic tissues. Furthermore, germ cell mutagenicity is now a health hazard criterion in the Globally Harmonized System (GHS) of classification and labelling [6]. This has resulted in an increased demand for germ cell mutagenicity studies for regulatory submission purposes. Thus, it is critical to assure that germ cell mutagenicity data submitted for regulatory purposes are robust and allow regulators to make appropriate risk assessment/risk management decisions. However, important data gaps still exist on the optimal experimental design (including appropriate germ cell samples and time points) to detect germ cell mutagens with TGR models. These data gaps hamper the integration of germ cell testing with ongoing somatic cell testing.

The recommended protocol for mutation analysis in somatic tissues in TG 488 involves treating TGR rodents for 28 days followed by tissue collection 3 days after the last exposure (i.e., 28 + 3d protocol) [4]. This protocol is considered an optimal compromise for detecting mutations in both fast- (e.g., bone marrow) and slow-dividing (e.g., liver) tissues [5,7,8]. TG 488 also includes protocols for the assessment of mutations in male germ cells. The focus is on male germ cells because insufficient numbers of oocytes can be recovered from female rodents to conduct the TGR assay. In addition, because oocytes in mature females are not undergoing DNA synthesis, assessment of mutagenic effects in female germ cells would require exposure during *in utero* development when oogonia stem cells proliferate [9]. TG 488 provides two major recommendations for male germ cell mutation analysis: 1) analyses of sperm from the cauda epididymis and/or vas deferens at a minimum of 49 days (i.e., 28 + 49d) for the mouse or 70 days (i.e., 28 + 70d) for the rat, to measure effects in spermatogonial stem cells; or 2) analysis of germ cells collected from seminiferous tubules (hereafter, tubule germ cells) and sperm from the cauda epididymis at 28 + 3d. Specifically, the guideline states that "*sampling cells from seminiferous tubules in addition to spermatozoa from the vas deferens/cauda epididymis following only a 28 + 3 day sampling regimen would provide some coverage of cells exposed across the majority of phases of germ cell development, and may be useful for detecting some germ cell mutagens*". Because the database for chemicals that have been evaluated in germ cells with TGR systems is limited, these recommendations have some limitations, especially for rat germ cells, as discussed in more detail below. Therefore, an OECD Standard Protocol Submission Form (SPSF) project is currently active to address them and modify TG 488 with an emphasis on germ cell analysis. From a practical standpoint, any recommendation for revision of TG 488 must take into account that the assessment of effects in germ cells at a time point different from that used for somatic tissues requires doubling the number of animals needed for testing, which invokes financial, time, and ethical implications. At the same time, decisions based on regulatory tests to evaluate whether chemicals are germ cell mutagens using the approach described in TG 488 should carefully consider the biology of spermatogenesis and the experimental variables that can affect the detection of mutations in germ cells.

To address issues in the SPSF, the Germ Cells workgroup of the Health and Environmental Sciences Institute (HESI) Genetic Toxicology Technical Committee (GTTC) has been working toward: 1) identifying appropriate time points (i.e., sampling times) for assessing mutagenicity in rodent germ cells; 2) determining if a single sampling time would allow the simultaneous assessment of mutagenicity in somatic tissues and germ cells; and, 3) making recommendations about potential modifications to the current recommended protocol for germ cell testing in TG 488. To achieve these objectives, we: 1) conducted a literature search to gather and review all available TGR mutagenicity data in male germ cells; 2) applied a mathematical model to quantify the exposure history of the germ cell population collected from seminiferous tubules at different sampling times [1]; and, 3) developed a list of issues that are specific to germ cell mutagenicity testing using TG 488 and how these may affect the detection of mutations in germ cells. It is concluded that the 28 + 3d design has serious limitations for germ cell testing and that the available data are still too limited to define the most effective approach for simultaneously assessing somatic and germ cell mutagenicity using TG 488. Recommendations are provided for specific factors that should be taken into account when developing experimental designs for determining whether a chemical is a germ cell mutagen, and for additional studies that should be undertaken to develop a definitive testing approach for the simultaneous assessment of somatic and germ cells mutagenicity using TG 488.

3. Mutagenicity data in male germ cells using TGR models

TGR models have primarily been used to assess mutagenicity in somatic tissues and for the majority of the agents that have been tested there are no germ cell mutagenicity data. The detailed review paper on the TGR mutation assay [5] and the subsequent 2009 OECD report [8] prepared in support of the development of the original TG 488 summarized experiments from 228 agents. Of these, only 41 agents were tested in germ cells, 14 of which yielded significant increases in mutant frequencies. In addition, one study tested the combined effects of two positive agents (hydroxyurea and X-rays). Here, we conducted a literature search to update the germ cell data from the OECD report and identified 11 papers that were published after 2009. As of March 2018, there are 61 scientific papers that have germ cell mutagenicity data on 45 agents (Table 1). Since the OECD report [8], four additional chemicals (benzo(a)pyrene (BaP), di-(2-ethylhexyl)-phtalate (DEHP), glycidamide and N-hydroxymethylacrylamide (NHMA)) have been tested for germ cell mutagenicity, with the first three producing positive results. In addition, two agents (γ rays and acrylamide), included in the OECD report as negative for mutagenicity in germ cells, have new data using different experimental designs showing them to be positive. In total, there are currently 19 agents with positive TGR mutagenicity results in germ cells (Table 2). Table 1 lists the 45 agents tested in germ cells with information about the TGR model used, reporter gene, tissue sampled, exposure and sampling times, and the qualitative results that were obtained. Of these 45 agents, 43 were tested in mouse TGR models and only 3 (acrylamide, glycidamide and 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline) were tested in rat TGR models, with the last two being the only agents that have no data in mice. Complete details about tissue samples analyzed, doses used, sampling times and mutant frequencies generated in the 61 studies are provided in Supplementary Table 1, which includes a total of 410 experimental records (293 of these were present in the OECD report). An experimental record is defined as an individual data point within a study (e.g., a study that investigated three doses at two sampling times in two types of samples has 12 experimental records).

Analyses of mutations in germ cells have been conducted using three types of samples each of which results in collection of different germ cell populations (Table 1): whole testis (with or without removing