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Peer Review Report on the Chromium Mode of Action Studies: Mouse Genomic Studies

Expert Review Organized by Toxicology Excellence for Risk Assessment (TERA)
http://www.tera.org/Peer/index.html

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Note

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Introduction

In July 2009, Toxicology Excellence for Risk Assessment (TERA) convened a Science Advisory Board (SAB) that provided guidance on a protocol for a series of studies investigating the mode of action by which hexavalent chromium (Cr(VI)) is carcinogenic in rats and mice following drinking water exposure. The research project was organized by ToxStrategies The final report of the July peer review was issued in late 2009 (available at: http://www.tera.org/Peer/Chromium/Chromium.htm) and expressed the expert panel's recommendations on studies designed to assess chromium's mode of action following a 90-day drinking water exposure in mice and rats. Following the release of the peer report, ToxStrategies incorporated the panel's recommendations and engaged several research laboratories to conduct the proposed studies. As part of the evaluation of the Cr(VI) MOA, dose-dependent intestinal gene expression was evaluated in female B6C3F1 mice following 7 and 90 days of continuous exposure to chromium in drinking water (the mouse 90-day study reviewed earlier). Intestinal differential gene expression was analyzed for over-represented functions to identify putative key events, and phenotypically anchored to complementary histopathologic, biochemical, and dosimetry data in the small intestine observed in the mouse 90-day study. ToxStrategies has prepared a manuscript reporting the findings of these studies.

Toxicology Excellence for Risk Assessment (TERA) arranged for a written peer review of ToxStrategies' draft manuscript reporting the results of the mouse genomics studies. This review was conducted by four risk assessment experts: two reviewers who were members of the original SAB and two ad hoc reviewers with expertise in genomics studies**.** The scientists who conducted this review are Michael Dourson, TERA; Dave Gaylor, Gaylor and Associates; Lucy Anderson, retired from the National Institutes of Health; Rebecca Fry, University of North Carolina. The objective of the review was to provide ToxStrategies with independent scientific and technical expert opinion and comment on their draft manuscript. The experts provided their own personal opinions, and did not represent the opinions of their employers or other organizations they may be affiliated with. The information in this report does not represent the opinions of Toxicology Excellence for Risk Assessment. This work was done under contract to ToxStrategies and was sponsored by the American Chemistry Council.

The experts received the draft manuscript and charge questions on June 28, 2011. Reviewers were asked to carefully review the document and provide written responses to the charge questions, including a clear rationale and support for their opinions. This report is a compilation of the four reviewers' written comments, organized by the charge questions.

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Appendix B - Citations for the Review

Reviewers' Comments

General Comments

Reviewer 1: *Overall this is a valuable, broad, unique undertaking, involving multi-disciplinary analyses of a target tissue under the same conditions as a tumor-yielding bioassay. There is high potential for meaningful conclusions regarding mode of action (MOA), and hence for improved understanding of human risk.*

The present part of the study under review involved microarray analysis of gene expression in mouse small intestine after exposure to a range of doses of Cr(VI) for 8 or 91 days. The experiments and the analyses appear to be reasonably well done, although there are some questions and issues that need to be addressed (below). A concern is that both the data extractions (selections and groupings of genes for emphasis or as especially informative) and the interpretations may have been too much driven by a hypothesized mode of action (MOA) based in part on their previous experiments (1, 2). This suggested MOA, presented in (1) and (2), is not yet fully compelling (see below). Secondly, microarray analysis is much better suited for hypothesis generation, than for hypothesis testing. With thousands of significant changes in gene expression, one can find support for almost any hypothesis. It is suggested that aspects of the data extractions and of the interpretation be re-worked, as indicated below.

Reviewer 2: *None.*

Reviewer 3: *None.*

Reviewer 4: *This manuscript is a timely and insightful application of genome-wide gene expression technology to evaluate the carcinogenic mechanisms of Cr(IV). The use of genomic approaches in toxicology and risk assessment have the potential to provide tremendous amounts of information about an agent's mode of action, thereby improving the assessment of health risk for exposed human populations. This study uses genome-wide microarray analyses to elucidate the mode of action (MOA) of Cr(IV) in a 90-day drinking water study at Sodium Dichromate Dihydrate (SDD) concentrations ranging from 0.3 to 520 mg/L. Cr(VI) compounds have been classified as a cause of human lung cancer, and alimentary cancers in mice. In this manuscript the authors investigate the effects from oral Cr(VI) on duodenal and jejunal epithelia, two rodent target tissues that develop neoplastic lesions with chronic exposure (NTP 2-year bioassay). Here, gene expression was examined following 7 or 90 days of SDD drinking water exposure.*

Comments in Response to Charge Questions

1. Study design - Based on your knowledge of toxicological study protocols, please comment on the experimental design of the study:

- **Do you see any significant issues with the test system or test article employed, controls employed, endpoints recorded, terminal procedures, statistical analyses, and quality assurance?**

Reviewer 1: *MATERIALS AND METHODS, page 5. Were the mice weighed at the time of tissue collection, and these results taken into account in the experimental/control pairings or data analysis?*

Preservation of intact RNA requires special precautions for certain tissues, such as pancreas. This could also be true for small intestine. Was the use of TRIzol confirmed to be adequate, by comparison of RNA from fresh tissue with RNA after TRIzol preservation and isolation?

Microarray Analysis, page 6. With microarray, one is at the mercy of the software used to normalize, filter, and process the data obtained from optical signals. The authors have sufficient experience with these applications to inspire confidence. Nevertheless, the potential for type I errors remains, with so many comparisons being made, and the biological significance of 1.2- to 1.5-fold changes can be problematic. For these reasons, for findings to be strongly meaningful for hypothesis-testing, they must be confirmed by protein analysis. Protein analysis is beyond the scope of the present effort but should be considered for next phases, where the data are used for hypothesis testing and/or development. Also, the tissue samples used in the assays described here would include a variety of cell types, such as intestinal epithelial cells at various stages, histiocytes, etc. Not all of these are relevant to the MOA. A further approach would therefore have to be in situ hybridization and/or immunohistochemistry to establish the cell type showing the gene change.

The wide range of starting ages and weights of the mice, as described in (1), among other variables, could have led to considerable variation between mice. More information is required regarding replicates and controls. On page 6, three biological replicates and dye swap are mentioned. On page 8, it stated "an average of the signal data was computed if there were at least three values". We need more explicit detail. Were the three biological replicates individual mice, and was each paired with a different individual control mouse? Was there one RNA sample from each mouse, or several? How many microarray analyses were done for each pair, with each dye configuration? Quantitative real-time PCR (QRT-PCR) was carried out on

"five independent replicates" (legend, Figure 3); were these five individual treated mice and five individual controls?

More information is required regarding the normalization of the QRT-PCR data by use of housekeeping genes (page 9). It would be useful to show actual values for the three genes, Actb, Gapdh, and Hprt, for 5 – 10 samples of individual control mouse small intestine. While Gapdh and Hprt were among the most stable genes studied in the reference cited (3), Actb was not, and no data for small intestine were shown in (3). Were geometric means of the three genes used for standardization, as described in (3)?

Reviewer 2: *The statistical analyses employed were appropriate.*

Reviewer 3: *The exposures, time points, and controls are all suitable. My primary concern is the statistical methods used to identify differentially expressed genes (DEGs). To have thousands of genes differentially expressed makes it very hard to narrow down which pathways may be truly associated with the exposure. It would be helpful to limit these using a more stringent p value/FDR approach. The figures should be modified to more clearly show the players-the NRF2 graphic is very hard to read. The Myc pathway is better but pathway should be zoomed in.*

Reviewer 4: *The authors should mention in their description of microarray methods whether or not a minimum signal intensity cut-off was used for selecting gene probes for further analysis. If so, what cut-off relative to background was used?*

The Agilent microarray is a dual-labeled cDNA microarray gene expression system depending on dye-bias correction. This investigation performed dye-swapping among three biologic replicates, but there were no technical replicates for each sample. Having an odd number of dye-swaps increases risk of labeling bias attributable to defenses in dye incorporation. This raises the concern that type I errors could confound the results, particularly among probes with low signal intensities. Incorporation of split-control hybridization with dye-bias correction might have been one way to cost-effectively overcome this limitation.

Each array is a competition between an untreated control sample and a treatment sample. Please clarify, for each treatment group, the hybridization control used. Were co-hybridizations performed using the same vehicle control samples for all dose levels? Were hybridization controls time-matched? Was there any pooling of control or treatment RNA for hybridization? Please provide more details on the control samples used.

Microarray analysis was performed using mRNA obtained from frozen intestinal epithelial samples obtained from mouse duodenal and jejunal sections. Gene expression comparisons are made among and between exposure levels, exposure times and intestinal locations, particularly in the Quantitative Dose-Response Modeling section. It is understandable that the complexity of the study design and the volume of data obtained makes for a challenging discussion.

Consequently, the authors frequently discuss differences and similarities among the jejunal and duodenal results in the context of both time and dose. This discussion is difficult to follow. Since the authors note in the Discussion that "…responses to SDD were similar in both intestinal segments at both time points", is it necessary to discuss both tissues in detail? Would it be possible for the authors to simplify the discussion by focusing on either jejunum or duodenum, perhaps following the high-level discussion of gene expression similarities among doses and exposures between the two locations (Figure 1-2)?

- **In light of the chemical and toxicological profile for chromium (VI), comment on whether there are key physiological/toxicological endpoints that should have been assessed that were not part of the investigation.**

Reviewer 1: *A well-chosen range of doses was used. Gene expression was assessed at days 8 and 91 of treatment. These time points give information about early and intermediate effects. However, the endpoint of interest, tumor development, was assessed at 2 years in the NTP study. For establishment of an MOA it will be essential to follow the various parameters, including gene expression, through the period 3 months to 2 years, at 3-4 month intervals, and correlate with the appearance of any pre-tumorigenic lesions and then tumors. Hopefully this is in progress or at least planned.*

Reviewer 2: *This is outside my area of expertise.*

Reviewer 3: *It seems that given some of the highlighted pathways that include oxidative stress and DNA damage, other measures could be added to strengthen these findings such as DNA damage, or protein levels of some of the DNA damage response players, indicators of oxidative stress, etc.*

Reviewer 4: *Do the dosing regimens used in **this** study produce tumors in mice? Were any tumors observed, and if so, how many tumors were seen and at what dose?*

2. Study Results - Please comment on the strength, credibility, and relevance of the toxicological results of the study under review:

- **Were the individual animal data correctly summarized?**

Reviewer 1: *As noted in the narrative under Study Design, it is not clear in the manuscript exactly how individual animals were used.*

Reviewer 2: *The data were summarized appropriately.*

Reviewer 3: *I am a bit concerned by the number of genes that were identified as differentially expressed. This is a massive change to the genome, and hard to make conclusions about. It is probably possible to reduce these by more strict statistical methods. The dose response analysis is interesting, and appears to be well supported.*

Reviewer 4: .As mentioned above, it is not clear how individual control animals were used for array hybridization. More details are needed to describe the hybridization design since this affects the results and interpretation. *The authors discuss functional analysis of gene expression changes. Does gene enrichment change with dose? Do hierarchical clusters of genes reflect the sigmoidal dose response of individual probes? Could the authors provide insight into the dose-response of gene clusters? This issue has been previously discussed by Mel Andersen (Tox Sci. 118(2); 2010) and Rusty Thomas (Tox Sci. 98(1); 2007). The use of clustered gene expression for dose-response and risk assessment is an area of great interest.*

- **Are there nomenclature issues that need clarification?**

Reviewer 1: *No.*

Reviewer 2:

Page 7, line 4: Define P1(t).
Page 8, line1: Define EC_{50}.
Page 8, line 19: Explain what a BMR=1.349 is accomplishing.

Reviewer 3: *Did not respond.*

Reviewer 4: *The authors should indicate what DAVID and IPA are since many readers in Toxicology are not familiar with these tools. In tables 2 and 3, could the author indicate what "Ratio" and "Molecules" refer to for readers not familiar with DAVID results?*

- **Was adequate statistical information provided for quantitative dose-response analyses?**

Reviewer 1: *Dose-response modeling is extensive and appears to be expertly done. EC_{50} values were obtained for those responses that best fit a sigmoidal model. These were the great majority of the genes showing a >2-fold change at the highest dose. Perhaps a mention should be made of the genes with a Gaussian profile (461 on day 8 and 39 on day 91 for the duodenum, Figure 4 and Supplementary Figure S3), especially if the peak was at 170 mg/L, the lowest dose at which a clear tumorigenic response was first seen in the NTP bioassay.*

Reviewer 2: *Adequate statistical information was provided in order to conduct quantitative dose-response modeling.*

Reviewer 3: *Did not respond.*

Reviewer 4: *Did not respond.*

- **For each lesion or finding presented in the study, please comment on the strength of the evidence supporting the authors' conclusions that the lesion or finding is treatment-related.**

Reviewer 1: *In the context of this study, the findings are treatment-related significant differences in gene expression. The starting point is software-based analysis of paired comparisons between treated and control samples. The analyses included filtering and normalization of the data followed by various tests for significance and presence/type of dose-response. The use of multiple doses greatly increases the certainty of discovering genes with a true treatment-related change in expression. These genes are represented in Figure 4 and Supplementary Figure S3.*

The presentation of the primary data in Supplementary Tables 1-4 may be confusing for some users and could be more extensively described. A "posterior probability" should be defined, and it should be explained why this P1(t) is negative in many cases. According to the text (page 7), P1(t) >0.999 was taken as significant. It could be useful to have these >0.999 values in Supplementary Tables 1-4 highlighted, maybe red or bold font.

The next step in confirmation of an effect is application of QRT-PCR. Figure 3 presents data for 4 genes, 3 Cyp isoforms and Myc, with good agreement between microarray and QRT-PCR values. Evidently Gclc and Gpx1 were also tested (Supplementary Table S5) but QRT-PCR results for these important genes are not shown. Many investigators find that a significant percentage of genes with altered expression in microarray cannot be confirmed by QRT-PCR or with other platforms. See for example McHale, et al., Genomics 93:343, where there was only 50% concordance; Severgnini et al., Anal Biochem. 353: 43, 2006, where concordance was ~10%; Wang, et al., BMC Genomics &;59, 2006, where there was perfect concordance with RT-PCR for 30-40% of genes in two different microarray platforms. This sometimes indicates artifacts, or errors in sequence assignment, and in other instances is due to mRNA editing, resulting in different probes for microarray vs QRT-PCR giving different results. In any case, such gene results cannot be used without further work. Bottom line: every gene that is to be taken seriously in the present investigation must be validated by QRT-PCR (or protein analysis).

Reviewer 2: *Statistical analyses indicated which results were likely treatment-related.*

Reviewer 3: *Did not respond.*

Reviewer 4*: Based on the data provided in this manuscript, many of the individual gene expression changes fulfill the Hill criteria of causation. However, it is less clear whether the enriched pathways identified in the lower dose groups (particularly at doses below 14 mg/L; e.g. Table 3 and S8) reliably reflect pathway involvement. Non-significant pathways should be removed since it is difficult to argue that ratios such as 1/35 actually show pathway involvement. Related to this, it is unclear from tables 2 and 3 (and their supplementary counterparts) whether pathways identified in low dose treatments are also present in high dose treatments, but have been moved down in ranking as other responses have kicked in.*

The transcription factor Nrf2 (NFE2L2) is largely regulated post-transcriptionally. Nrf2-regulated genes are activated when proteasomal degradation of the Nrf2 transcription factor, mediated by Keap1, is inhibited. The authors mention that SDD exposure significantly induced several proteasomal genes, potentially indicating endoplasmic reticulum (ER) stress responses and altered protein degradation. Could the authors comment on the contribution of disrupted proteasomal protein degradation as an alternative explanation for antioxidant gene expression?

3. Study Conclusions:

- **Were there critical results or issues that were not addressed? Were there any contradictory statements or observations made?**

Reviewer 1: *Issues that need to be addressed further are mentioned above.*

With regard to conclusions, it is clear from the first paragraph of the Discussion (page 15) that the authors are trying to fit the results to their MOA model presented in a review (2) and in a report of histological and biochemical findings (1). This MOA hypothesis proposes the sequence: oxidative stress → cell proliferation → DNA modification → mutagenesis → tumor. This is essentially the mechanism often considered for non-genotoxic carcinogens, with genetic instability occurring late in the process. The accompanying White Paper makes it clear that they wish to categorize Cr(VI) with non-genotoxic carcinogens.

This MOA differs from an MOA proposed by EPA investigators (4): DNA damage/mutation → cell proliferation → tumor. The important difference between these proposed MOAs is the temporal positioning of DNA changes, i.e., whether mutation is the initiating event, or a late proliferation-generated event. This distinction is critical, as it bears on latency, tissue-, species-, and gender specificity, and role of modifying factors, and also determines whether age-dependent adjustment factors are required (4).

All available data are consistent with either MOA. A substantial part of the argument in (1) was lack of increase in 8-oxo-dG in the Cr(VI)-exposed intestines (data shown in Supplementary Figure S2). These data, obtained with an ELISA kit and showing very high levels of 8-oxo-dG (on the order of 2 lesions/10^3 guanine in controls), are almost certainly unreliable. Measurement of 8-oxo-dG has been a thorny issue for many years, with levels varying over several orders of magnitude for different methods and in different laboratories. Current consensus seems to be that high levels are due mainly to artifactual post-isolation DNA oxidation. With the proper methodology and instrumentation, basal levels of 8-oxo-dG are on the order of 1/10^6 or 1/10^7 guanine. Please see (5) for a recent historical summary and current methodology. It could be worthwhile to repeat the 8-oxo-dG determinations for the Cr(VI)-exposed intestines using this methodology.

In the first paragraph of the Discussion in the current paper, it is stated that, "The dose-dependent gene expression and their associated functions are consistent with SDD concentrations that elicited histopathological lesions (e.g. cytoplasmic vacuolization) described in Thompson et al. (2011b). Notably, only SDD concentrations >= 60 mg/l were associated with intestinal tumors in the NTP (2008) 2-year bioassay. These findings suggest that gene expression alterations are associated (perhaps causative) with intestinal carcinogenesis." The histological changes do correlate nicely with tumorigenesis. The gene expression changes, however, with median EC_{50} of 56 for the duodenum at day 91, have a more problematic relationship. Tumorigenesis at 60 mg/L was minimal if in fact real. This suggests that the majority of the gene expression changes were NOT associated with tumorigenesis.

The next sentence also needs re-wording. "Importantly, differential gene expression changes were observed as early as day 8 of exposure and were generally preceded by oxidative responses rf2 activation, altered redox status) and thus are likely the result of Cr(VI) exposure as opposed to DNA mutation." The word "preceded" is suggestive of a temporal relationship; there is no time point earlier than day 8. The statement probably means that doses lower than the EC_{50} were causing oxidative responses. The last part of the sentence does not make sense and may be part of the pitch for the non-genotoxic MOA hypothesis. Few would think that DNA mutations would result in gene expression changes under these circumstances. DNA mutations would affect gene expression only in expanded cell populations derived from a mutant, as in a tumor.

For microarray studies, the interpretive management and selective focusing of the results are as important as the data themselves. In the manuscript these applications are given in the sections named Functional Analysis and Phenotypic Anchoring (page 12) and Assessment of DNA Repair Genes and Pathways (page 14) and in the Discussion. I suggest that some additional or alternative approaches be used, as follows.

- *Emphasis is given to canonical pathways and broad functional groupings (DAVID and IPA analysis). These programs are quite useful, but only as a starting point. Their relevance can be specific to cell type and circumstance. So many pathways and functions are presented here (Tables 2 and 3, Supplementary Tables S7 – S10) that no clear conclusions can be drawn. I suggest eliminating these tables, and incorporating the information in other ways. Supplementary Figures S5 and S6 are useful and could be retained.*
- *Genes with a low EC_{50} are listed in Table 1. These are of course of interest in the context of human exposures. I suggest an addition to this, a list of genes showing a significant difference at the lowest dose, with annotation as to function and canonical pathway membership. QRT-PCR should be shown for key genes; these data should be already available for Gclc and Gpx1.*
- *In a further study, white blood cells could be assessed in parallel with intestinal tissue, and the doses extended down to establish a no-effect level. Such data would be quite valuable for human biomarker studies.*
- *A significant tumor yield in the NTP study occurred only at the 172 and 516 mg/L doses in the female mice. [There is a statement on page 18 in the current manuscript that there was an increase in tumors at the 60 mg/L dose relative to historical controls. This could be misleading. 60 mg/L is certainly a minimal effect level at most and clearly distinct from 172 mg/L.] Several histological changes, including vacuolization, atrophy and apoptosis, and crypt cell hyperplasia, were also prominent, mainly at the two highest doses and correlated well with tumorigenesis (1). Therefore, genes which show a change in expression at 170 and 520 but not at 60 mg/L may be of greatest interest for the MOA. We need a table of these genes. This table should include function and membership in a canonical pathway, and be discussed in the text in relation to the histological findings and the tumors. For example, there are two such genes, having an EC_{50} in the range of 170 mg/L at day 90, in the Cell cycle/Growth/Proliferation-related list in Table 4: Cdk1 and E2f2. There should be some apoptosis-related genes, according to Table 3. Supplementary Table S6 shows some genes with large expression changes at 170 and 520 doses but not at the lower doses.*
- *Another way to use the data would be to ask whether there are genes or pathways expected to be cell-protective, that drop out at the two highest doses or after prolonged exposure. For example, in the section headed Assessment of DNA Repair Genes and Pathways (page 14), eight genes involved in oxidative DNA damage repair are noted to be significantly induced in the duodenum on day 8 mainly at the two highest doses. Induction was less evident at day 91, and in the jejunum (which developed fewer tumors). Several of these genes should be verified by QRT-PCR. Apex1, Rad51, and Msh6 would be good choices. These genes could contribute significantly to several aspects of the MOA. At least, they indicate that oxidative*

DNA damage has occurred at the tumorigenic doses in the duodenum at an early time point, and could contribute to tumor initiation. Later in time, the expression of some repair genes for such lesions is reduced or lost. This could mean that oxidative lesions increase at this later time, or that they no longer form because other defenses have been mounted. It is important to continue the study of the nature, time-course and dose-response of DNA damage. Hopefully this is in progress, or could be added to the menu soon.

- *Supplementary Table S6 could also be used for this part of the exercise: what potentially protective genes are highly expressed up to 14 or 60 mg/L, then not at higher doses? Cyp1A and Gclc could be candidates, for example. Known tumor suppressor genes, especially those with established roles in intestinal tumorigenesis, should be examined one by one.*

In summary, the focused data extraction and presentation and the Discussion need to be less concerned with supporting a particular MOA, and less wide-ranging, and more concentrated on (1) changes at the lowest dose, as relevant to human exposure and (2) changes that correlate with tumorigenesis and its associated histological parameters.

Reviewer 2: *Did not respond.*

Reviewer 3: *I would tone down any mention that the gene expression identified is "causative" of the phenotype. We simply can't say this, but could say associated.*

Reviewer 4: *In the introduction the authors state "Microarray analysis enables the simultaneous assessment of all gene expression changes in a cell or tissue, which can be used to support the elucidation of the MOA as a function of dose." In the methods the authors mention that BMDExpress can group genes by functional categories. This implies that the functional categories displayed are dose-responsive. What are the BMDs and BMDLs for the major functional categories identified by the authors? Although many individual genes are dose-responsive, I do not believe the functional categories are. If the authors focus on functional categories of genes that are dose responsive, many of the pathways and functions that as noted by reviewers 1 and 2, that may not be MOA-related, would be removed.*

- **Do you agree with the authors' conclusions of the study?**

Reviewer 1: *See above.*

Reviewer 2: *Although this question is somewhat outside my area of expertise, the conclusions appear to be supported by the results*

Reviewer 3: *Did not respond.*

Reviewer 4: *Did not respond.*

4. **Study Reliability – Describe the reliability of the study for consideration in the derivation of EPA IRIS quantitative health benchmarks and the qualitative characterization of cancer risk. Describe any major strengths or uncertainties with this study that might preclude it from being used as consideration for determination of the mode-of-action and weight-of evidence for chromium (VI)'s cancer risk and derivation of a cancer slope factor.**

Reviewer 1: *Overall, the study provides a reliable set of gene expression data and dose-response information. A major strength is the use of a suitable range of doses and sophisticated dose-response modeling. This insures that gene probes showing a clear dose-response are in fact influenced by treatment. More QRT-PCR validation (or other assay) of particular genes are needed before they can be used for definitive support (or refutation) of a particular MOA. For full and complete validation, protein analysis and in situ hybridization/immunohistochemistry will be required.*

Reviewer 2: *The study appears appropriate for consideration with an EPA IRIS evaluation.*

Reviewer 3: *This is a complicated question. It relates primarily to the use of gene expression data to be used as predictors of cancer risk. Given the number of genes changed in these experiments, and the need for some more assays (e.g. DNA damage assessment, oxidative stress measures) I would hesitate to say that the gene expression changes are indicators of cancer risk.*

Reviewer 4: *This investigation reinforces the results of the 2-year NTP bioassay which showed that only SDD concentrations >60 mg/L were associated with intestinal tumors in mice. Furthermore, it supports the current EPA maximum contaminant level of 0.1 mg/L of TOTAL chromium (according to the authors) which was shown here to have little effect on gene transcription. It is particularly important to note that Cr(IV) is normally a fraction of the total Cr present in drinking water.*

5. Please identify and discuss any other relevant scientific issues or comments not addressed by the above questions.

Reviewer 1: *I have a suggestion regarding a model system that could be used to shed light on the MOA, in particular on the key issue of early vs late genotoxicity/mutagenicity. The Apc (min/+) mouse develops small intestinal tumors, associated with loss of the normal + allele. On appropriate genetic backgrounds these tumors can be promoted by a variety of chemicals and X-*

rays. They can also be induced by a single dose of a genotoxic carcinogen or X-rays. Thus this model might be used to determine, for Cr(VI), both early (initiating) and late (promotional, progressing) DNA damage effects for a specific gene target.

A protocol could be as follows. To test for initiation, mice would be given a single i.g. dose of Cr(VI) over a range of doses. A known direct-acting intestinal carcinogen would be given to a parallel group as a positive control. To test for promotion/progression, Cr(VI) would be given in the drinking water as a series of doses. As a positive control, a parallel group would receive a known promoter. In a third set, mice would be given both the single i.g. doses and then chronic treatment in drinking water. Groups of mice would be euthanized at frequent intervals (3 months) until a high yield of tumors is obtained. Tumors would be assessed for Apc LOH.

If the Cr(VI) has an initiating effect, the single i.g. treatment should result in shorter latency and/or increased tumor yield, with the expected LOH in the tumors. If only the chronic treatment is effective and over a long time course, the promotion/progression non-genotoxic model is supported. If the i.g. single treatment and the drinking water treatment synergize, then contribution of Cr(VI) to both initiation and promotion/progression would be indicated.

Reviewer 2: *Did not respond.*

Reviewer 3: *Did not respond.*

Reviewer 4: *Figure 3 should be moved to supplementary data.*

Page 4, last paragraph: In general, these differential gene expression studies showed what?

Page 8, line 4: Please clarify why only the choice of the sigmoidal model here, as opposed to the plethora of models stated immediately above.

In the first paragraph of the discussion, how do the authors know these changes to be nonlinear? Have these results been tested in a statistical model? Or is the authors' surmise observational?

In the first paragraph of the discussion, and more generally, how can it be that EC50 values are in the same range as BMDL values at 91 days?

We suggest rewriting the ending to the first paragraph of the discussion as:

> *Importantly, differential gene expression changes were observed as early as day 8 of exposure and were generally preceded by oxidative responses (e.g. Nrf2 activation, altered redox status). Furthermore, focal proliferation or neoplastic lesions do not occur in two 90-day drinking water studies (NTP, 2007; Thompson et al., 2011b). These facts suggest strongly that the tumorigenic findings from Cr(VI) lifetime exposures are not due to direct DNA mutation*

Page 4, Line 11. Use "propose" instead of "draft."

Page 4, Line 13. Add "in the NTP study" after "assessed."

In general, these studies showed what?

Page 38, Table 4. The precision in the values found in this table should be consistent with that from derived values, in this case, the administered doses.

6. Comments on white paper: Principal Components and Cluster Analyses of Cr(VI) Day 8 Duodenal Gene Expression Data

Reviewer 1: *This was a reasonable exercise to undertake, and the authors have been careful to be explicit with some caveats in the Discussion, pp. 7-8. The choices of dose and time were reasonable.*

More validation is needed for the 116 genes used. Did all of them show unequivocal dose-response? At least some of them should be confirmed by QRT-PCR.

I do not have direct personal experience with these analyses. The paper must be examined by a statistical expert who can attest that all steps were done correctly.

The outcome, as shown in Figures 1 and 2, is impressive. However, these results must be interpreted with additional cautions. The four genotoxic carcinogens are all organic compounds, and all form DNA base adducts and cause mutation via mispairing. They are thus distinct from Cr(VI), the genotoxicity of which is ascribed to oxidative DNA damage and/or non-specific DNA adducts and binary/tertiary cross-linking, strand breaks, error-prone double-strand break repair, etc. One would expect the gene expression profiles to be different even if they were due mainly to DNA damage effects. It would be of interest to repeat the exercise with a known inorganic carcinogen such as cisplatin, with DNA interactions more similar to those of Cr(VI).

Secondly, the non-genotoxic carcinogens all cause reactive oxygen species (ROS) to some degree, to which their effects are often imputed. Stimulation of ROS-responsive genes by Cr(VI), early and at low doses, is established in the current manuscript. Commonality in gene expression profiles is thus not surprising. It would be useful to have more detailed and specific information about which genes are in which cluster (Figure 1) and in the various areas of the heat map (Figure 2). In fact each gene should be labeled in Figure 2.

In my opinion this White Paper material should not be included in the manuscript, which is already full enough. It could certainly go in the final Mode of Action paper. It could even be published separately as a Note or Letter.

Reviewer 2: *Statistical principal components analyses and cluster analyses are appropriate and useful for identifying significant factors in large and complex data sets. Comparing the Cr(VI) gene expression data with the gene expression data from four genotoxic carcinogens and four non-genotoxic carcinogens provides useful data for evaluating the mode of action for Cr(VI). I agree with the conclusion expressed by the authors that the analyses suggest that Cr(VI) may act through a non-mutagenic MOA in the mouse small intestine.*

Since the approach in the principal components analysis (*PCA) White Paper is different from that in the mouse genomics manuscript, I suggest that the PCA White Paper should be separate from the mouse genomics manuscript. Clearly, the results from the PCA White Paper should be used in the final MOA manuscript.*

Reviewer 3: *The white paper is interesting and helpful. The approach to compare the genes expression changes induced by chromium to those induced by genotoxic and non-genotoxic agents is a good one. The PCA approach however is not one of the strongest analytical approaches to use. The hierarchical clustering approach is more straightforward and I would include that in the genomics manuscript. An alternative method would be to perform two-class prediction analysis using the gene expression from the chromium experiments.*

Reviewer 4: *The white paper makes a significant contribution to the literature and should be published as a separate technical commentary, but in the same journal issue as the mouse genomics paper. The main reason for the separate publication is that the methods sections of these two texts are completely different.*

The methods text in the white paper will need to be slightly expanded to more fully explain the analysis. For example, further explication of PCA, Horn's parallel analysis and eigenvalues are all needed. The authors need to remember that their methods section should give sufficient information so that the rest of us can replicate the analysis.

Both figures will need to be enhanced. For example, label the axes in Figure 1 and show the various chemicals associated with their respective points. Also, give a heading and description to both figures. Finally, the Cr results of Figure 2 heat-map do not obviously "cluster" with the non-mutagenic carcinogens. Perhaps the authors could provide a table that gives the number of times that the Cr results match either one group of carcinogens or another and how many times that the match does not help.

The authors will need to do a quick review on the likely MOA for the 8 chemicals from the Ellinger-Ziegelbauer et al. (2005) study. This review does not have to be extensive.

The attached mark-up gives some suggested changes. In addition, the authors will need to refine the transition between the methods and results sections. Also, I was not impressed with the ending of this text, but a technical commentary probably does not need refinement here.

Appendix A: Instructions to Reviewers

Dear Reviewers,

Thank you again for your willingness to provide a peer review of the mouse genomics study, which has been conducted as part of the series of studies investigating the mode of action by which hexavalent chromium is carcinogenic in rats and mice following drinking water exposure. This research project was organized by ToxStrategies. This email provides you with the review materials and instructions. The study and charge questions are attached.

The subject of this review is a draft manuscript that has been prepared by ToxStrategies describing the mouse 90-day study. Following your peer review, ToxStrategies will be submitting the draft manuscript for publication.

For this peer review, the reviewers are asked to carefully review the manuscript and provide written responses to the charge questions for each assessment. Please address each charge question (as appropriate given your expertise). Also, please comment on any aspect of the genomics study that is not specifically addressed by the charge. For all comments, provide **clear rationales and support** for your opinions. Please identify the page number and line number of the text that you are commenting on to allow ToxStrategies easy reference to the specific text. We will need an electronic copy of your comments preferably in MS Word. We prefer that you use the attached charge file as a template and add your answers to it. If you would like a copy of any cited references, please send your request to me (Strawson@tera.org or 910-528-9768).

Your written review should be sent by email to me by no later than **Monday, July 25, 2011**. After the reviews are submitted, we may schedule a follow-up with ToxStrategies to resolve any issues or to answer any clarifying questions that ToxStrategies may have for the reviewers. The need for follow up will be determined upon receipt of the reviews. A draft compiled report with the other experts' comments on this study will be forwarded to you and you will be provided the opportunity (albeit brief) to revise your comments if you feel that is needed. At the completion of this review, ***please destroy any copies of the review materials***, as they are draft and are not for distribution outside of the review panel.

If you have questions regarding the review, please contact me. Thank you again for being willing to do this review in such a short time frame. ToxStrategies very much appreciates your assistance.

Joan Strawson
Review Coordinator
910-528-9768

Appendix B: Citations for the Review

Reviewer 1:

1. Thompson, C.M., Proctor, D.M., Haws, L.C., Herbert, C.D., Grimes, S.D., Shertzer, H.G., Kopec, A.K., Hixon, J.G., Zacharewski, T.R., and Harris, M.A. (2011) Investigation of the mode of action underlying the tumorigenic response induced in B6C3F1 mice exposed orally to hexavalent chromium. *Toxicol. Sci*., in press.

2. Thompson, C.M., Haws, L.C., Harris, M.A., Gatto, N.M., and Proctor, D.M. (2011) Application of the U.S. EPA mode of action framework for purposes of guiding future research: A case study involving the oral carcinogenicity of hexavalent chromium. *Toxicol. Sci*. **119**: 20-40.

3. Vandesompele, J., De Peter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol*. **3**: RESEARCH0034.

4. McCarroll, N., Keshava, N., Chen, J., Akerman, G., Kligerman, A., and Rinde, E. (2010) An evaluation of the mode of action framework for mutagenic carcinogens case study II: Chromium (VI). *Environ. Mol. Mutagen*. **51**: 89-111.

5. Mangal, D., Vudathala, D., Park, J.-H., Lee, S.H., Penning, T.M., and Blair, I.A. (2009) Analysis of 7,8-dihydro-8-oxo-2'-deoxyguanosine in cellular DNA during oxidative stress. *Chem. Res. Toxicol*. **22**: 788-797.