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Peer Review Report on the Chromium Mode of Action Studies: Rat Genomics Studies

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

Contact: Jacqueline Patterson
(patterson@tera.org)

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Note

This report was compiled by scientists of Toxicology Excellence for Risk Assessment (TERA). The peer experts served as individuals, representing their own personal scientific opinions. They did not represent their companies, agencies, funding organizations, or other entities with which they are associated. Their opinions should not be construed to represent the opinions of their employers or those with whom they are affiliated.

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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 rat 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 November 17, 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.

Appendix A includes the instructions given to the reviewers. Appendix B includes reviewer annotations of the draft manuscript text.

Reviewers' Comments

General Comments

Reviewer 2: The manuscript under review presents the toxicogenomics microarray results for effects of Cr(VI) in rat intestinal epithelia. It complements parallel results for mice, reviewed recently. These comprehensive surveys provide valuable information to be used toward MOA development, by comparison of a susceptible species, the mouse, with a resistant one, the rat. The current data for the rat, like those for the mouse, are basically sound. Some of the same methodological issues pertain, and will be repeated here for sake of completeness. Comments and suggested small corrections are inserted in the text of the manuscript.

In general, the manuscript is well-written, and is quite complete, presenting and integrating the data in a variety of ways. It may over-reach in its use of the data. There are a few very clearly positive findings, and many others that are more problematic, but all are treated together in presentation and discussion of the results. The use of different statistical cut-offs for different purposes generates some confusion as to what is really real and meaningful. A second suggestion is that there should be more focus on comparison with the mouse, at doses that are carcinogenic in the mouse.

Responses to Charge Questions

Charge Question 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: There are no significant issues. However, I do have some reservation about the thousands of genes identified and whether these are issues of false discovery. More specifically, could the thousands of genes be identified as a result of false positives (was there a stringent false discovery rate correction in the analysis?)

Reviewer 2: a. Materials & Methods, Study Design. The female rats were 4-5 weeks old and acclimated for a minimum of 7 days, so maybe longer, and could have been 5 – 7+ weeks when used. This is a critical time period involving puberty and rapid weight gain. A 5-week female rat is very different from an 8-week one. Was this taken into account?

b. Materials & Methods, RNA Isolation. The test for RNA intactness by gel electrophoresis is reassuring. Was each RNA sample examined in this way? It would be worthwhile to show a set of gels, perhaps in the Supplement, since RNA stability could be a real issue here, in a situation where digestive enzymes abound.

c. Materials & Methods, Microarray Analysis. RNA samples from different individual control and treated rats were used for each set of arrays, a good design. How were these rats selected, considering the possible wide range of weights and ages (see above)? Was each sample pair analyzed only once with each dye configuration?

d. Materials & Methods, QRT-PCR. According to the legend for Fig. 8, QRT-PCR data are from 3 independent replicates. These data are critical, so the method should be given more fully and in the Materials & Methods section. Confirm that these replicates are from individual rats, and state whether they are the same rats as used for the microarray. If not, indicate how they were chosen. How were the housekeeping genes actually used for normalization? QRT-PCR should be shown for these genes for 5 control rats, since the cited reference does not cover intestine.

Reviewer 3: The focus of this manuscript is the effect of SDD on gene expression in rat small intestines. This study is part of a larger study to investigate species-specific differences in gene expression that might explain the different in tumor outcomes between rats and mice following SDD exposure. The authors directly compare the rat gene expression data, described in this manuscript, to mouse data from a manuscript that is not yet published (Kopec, et al). The comparisons and conclusions of this manuscript hinge on the extent to which the methodologies of the two studies are alike. The authors only mention that the mouse study was "similarly designed". In what ways, if any, are the two studies different? Were identical methods used for microarray analysis? Were there differences in methodologies that could contribute to the large differences in gene numbers, and potentially gene expression patters, which distinguish the mouse response from the rat? The authors should explicitly state that the same methodologies were applied to the analysis of both data sets, if it is in fact the case.

Reviewer 4: The statistical analyses employed were appropriate.

- ***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: No, the endpoints and analysis seem sufficient.

Reviewer 2: The range of doses was appropriate. The comprehensive list of endpoints examined in the various arms of the studies is impressive. Longer exposure times clearly would be desirable, since the longest time point, 91 days, is much shorter than the 2-year endpoint for tumors. Gene expression changes were evident between 8 and 91 days, and indeed there is speculation in the manuscript about adaptations over time. Future studies should include 3 month intervals up to 2 years, with coordinated examination of all of the endpoints

Reviewer 3: It appears that the study assessed endpoints appropriate for the scope of the study as it was designed. The mouse methods and data are to be published separately from the rat results, while histologic and pharmacokinetic results are to be published in a separate follow-up study. Although I am aware of this fact as a reviewer, this fact may not be clear to readers once the manuscript is published. Based on the way the abstract and introduction are written, the reader might naturally expect presentation of the methods and data for both species in this manuscript. For clarity, the authors should be explicit about this point in the introduction.

The text in the first paragraph of page 9 states that Table 1 links differentially expressed genes to functional categories and histopathology. This is not the case and should be changed. Table 1 only presents differential gene expression and functional categories.

The description given by the authors regarding the total number of microarrays performed is unclear. With regards to the dye swaps. Were there dye swaps for each array (3 x 2 = 6 hybridizations) or did the authors mean that there are 3 independent samples of which one is a dye swap (2 + 1 = 3 arrays total)?

Reviewer 4: This is outside my area of expertise

Charge Question 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: Yes.

Reviewer 2: As noted above, more information on the individual animals would be desirable. The outcomes, however, are reasonable; the summarization is probably sufficient.

Reviewer 3: Yes. The authors have done a reasonable job organizing and summarizing a complicated dataset.

Reviewer 4: The data were summarized appropriately.

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

Reviewer 1: On page 16, biomarkers for intestinal cancer are mentioned-what is the analysis for those biomarkers? Can we call them biomarkers?

Reviewer 2: No**.**

Reviewer 3: No issues. The nomenclature seems adequately defined.

Reviewer 4: Page 6, line 10: Define P1(t).

Page 6, Section 2.4. Define benchmark dose (BMD) and benchmark response (BMR). Explain why BMR=1.349 was selected.

Page 8, Section 3.2, line 4: Define EC_{50}.

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

Reviewer 1: I am concerned about the number of genes detected as differentially expressed. Please take a look at false discovery rate correction to address this.

Reviewer 2: The program used determined the best fit for dose-response among five models. Evidently no differentially-expressed genes fit the linear, exponential or quadratic models. This should be explicitly stated. A substantial number (300-600 probes at day 8, 130-140 probes at day 91) presented a Gaussian pattern. These could be of interest, especially since some of these may be included in functional groupings in the manuscript, e.g., Table 1, and the graph for *Gclc* in Figure 3 looks Gaussian. More information should be provided regarding the Gaussian-model genes.

Reviewer 3: Can the authors provide more detailed information on drinking water consumption on a per animal basis? This would allow actual dose estimation. The authors have only provided drinking water concentration and assumptions have to be made to estimate actual dose. This information will be necessary to phenotypically anchor rat exposure levels to human and mouse endpoints.

Reviewer 4: Adequate statistical information was provided in order to conduct quantitative dose-response modeling.

- ***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: This seems sufficient.

Reviewer 2: From the data in Figure 1 A and D, it is clear that there was dose-response for the number of differentially-expressed genes, in both tissues at both times. The data in Figure 3 show convincingly that *Nrf2*, *Gclc*, and *Gpx2* are among the genes that were dose responsive. These are important and useful conclusions. Beyond this, the relaxation of filtering to allow in genes with 1.2X change and P>0.9 is troublesome. It almost certainly allows in a lot of red herrings, and threatens the reliability of the data processing going forward.

For example, the validity of conclusions implied in Table 1, Table 2, and Supplementary Tables 2 and 3 are mixed and problematic. In Table 1 numerous genes are listed out in functional categories, but only a minority of these on day 8 and very few on day 91 yielded EC50 values. The footnote indicates that the rest did not meet the more stringent cutoff, or had a Gaussian fit.

These must be regarded as uncertain results. In the manuscript text I have highlighted the convincing results in green, others in yellow. Most of the problematic genes show comparable apparent effects in both tissues at day 8, or in duodenum at both times, and these consistencies increase confidence that they are real effects. It could be helpful if SD or SE values were given for each fold-change result, based on the three treated/control comparisons.

The messages in the manuscript, based on the lists, are rather a mixed bag. At present, the genes are listed in alphabetical order within category. It might be more useful to list first the genes for which a treatment-related effect can be strongly concluded, based on modeling, statistics, SE, consistency, etc., then those with weaker reliability, and modify the text accordingly. "Genes A, B and C show clearly Conclusion D; similar possible changes in genes X, Y and Z tend to support this conclusion."

The main reason for the work was to discover any gene changes that might explain why mouse duodenum, but not rat duodenum, is sensitive to tumorigenic effects of Cr(VI). The parts of the manuscript pertaining to this question are poorly developed, disorganized, and incomplete. The data are scattered about among many tables and figures, with differing filtering criteria used in various places. The key figures are Fig. 8 and Suppl. Fig. S4 for day 91. These critical data are missing for day 8 and need to be included. The figures for day 91 show a significant but manageable number of genes with rat vs mouse difference. Four of these were confirmed with nice dose-response QRT-PCR, which is great. The rest are vaguely treated in Table 2, Table 4, Suppl. Tables S3 and S3, and Fig. 9 and in the manuscript in several 3.4 sections (please see my comments in the manuscript). Fig. 9 lacks any indication of statistical significance among the box-and-whisker plots. One has the impression that there has been cherry-picking among the data to try to congeal some function-related meaning. This may not help the ultimate objective, to utilize the results for an integrated MOA assessment.

Suggestions: use a stringent cut-off (2-fold, P>0.999) and list all the treatment-responsive orthologous genes in duodenum, day 8 and day 91, that were treatment-responsive for rat only, and similarly those that were responsive for mouse only, or in the opposite direction for rat vs mouse. Give the name, the functional category, the EC50 (or the peak dose if Gaussian), and the fold changes at the two highest doses. This will probably not be a very long list; most of the changes listed in the tables are between 1.5- and 2-fold. It is possible that some of the latter are of biological significance, but most likely a change would have to be at least 2-fold to be relevant to MOA. Then use this conservative list for functional groupings, speculations, etc. Place emphasis on the highest doses, where tumorigenesis occurs in mouse but not the rat, and where in fact more Cr(VI) accumulates in mouse duodenum compared with rat (Fig. 10B). This difference in tissue dosing may be the key finding of the whole big project. The gene expression changes can add to this critical observation in important ways, if they can give hypothesis-generating indications of: (1) why more Cr(VI) accumulates in mouse vs rat duodenum at higher doses, and (2) what gene changes occur uniquely in the mouse duodenum at these doses, that are relevant to carcinogenesis. In my opinion the manuscript should be built around these two questions in a very focused way, with all other observations tucked in as peripheral.

Reviewer 3: Overall the authors have done a reasonable job presenting limitations that affect the strength of their findings. One question pertains to the use of the 520 mg/L treatment group

as a basis for identification of genes and pathways of interest because water intake by the high dose group appears to be ~69% of predicted on day 91, based on default consumption values for female Fisher 344 rats. Since the authors do not provide water consumptions data for the dose groups, assumptions must be made if converting water is used to estimate to the actual daily dose. Since the authors appear to have this information they should make it available as a Supplementary table or figure. On page 16 the authors state that "the mg/kg bodyweight SDD doses in the rat 520 mg/L treatment groups at day 8 and 91 were 80 and 60 mg/kg, respectively…". On the basis of this daily dose, the water intake can be calculated using default weight and water consumption for female Fisher rat (~0.124 kg, 0.169 L/kg/day) to be ~68% of the predicted. Similarly, water consumption in the mice is ~64% what would be predicted for female B6CF1 mice (0.0246 kg, 0.264 L/kg/day) in a subchronic study. Thus, there is a concern that the genomic data reflect stress other than that directly related to chromium.

Likewise, the dose of SDD consumed by rats appears to be less than that consumed by mice. The authors found that rats have much lower intestinal chromium concentrations at the same water concentrations. In their final sentences on page 14 the authors state "at 60-520 mg/L SDD the duodenal Cr levels ranged from 18-32 µg Cr/g duodenum in rats, and 34-61 µg/g in mice". Based on default values of rat and mouse water consumption (above) the intestinal concentrations at the 520 mg/L dose appear to be 32 and 61 ug Cr/g tissue for rats and mice respectively. Based on these numbers it appears that rats consume ~64% the amount of SDD relative to mice. While this does not correspond linearly to the intestinal concentration, it does suggest that one factor contributing to higher intestinal concentrations in mice is that mice may be getting ~1.5 times the dose. Presumably, differences in clearance/half-life contribute as well. The authors conclude that it is unknown whether "differences in tissue dosimetry are purely due to pharmacokinetics, or instead reflect adaptive gene expression responses in rats that result in lower tissue dosimetry". The authors should briefly address these issues of daily dose and water consumption and provide summarized BW and water consumption data so that the effect of these parameters can be can be appreciated in the interpretation of the data.

Reviewer 4: Statistical analyses indicated which results were likely treatment related.

Charge Question 3. Study Conclusions:

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

Reviewer 1: I am only a bit concerned by the number of genes that are observed to be changed. Is this an issue of false discovery? There were no contradictory statements.

Reviewer 2: As noted above, the genes most relevant to the purpose of the study, i.e., those showing large, clear differences between rat and mouse in duodenum especially at carcinogenic doses, have not been treated and presented systematically.

Reviewer 3: Was there a reason why different house-keeping genes were used for relative PCR analysis in rats than in mice? This suggests that other differences in genomic analysis may be present.

Reviewer 4: This is outside my area of expertise.

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

Reviewer 1: The authors conclude that the MOA involves oxidative stress, cytotoxicity, cell proliferation and DNA modification. These are supported by the data.

Reviewer 2: I agree with some but not all of the various conclusions and inferences in the Discussion and elsewhere. Please see my comments in the manuscript text.

Reviewer 3: The author's conclusions appear justified in terms of the rat data. On the basis of this manuscript alone, it is difficult to tell whether the comparisons to the mouse data are appropriate. Since the mouse data has not yet been published, and to improve overall clarity, the authors need to clearly state differences in methodology between mouse and rat studies.

One major conclusion is that the large number of genes perturbed by SDD in mice may be due to differences in tissue dosimetry, and may be responsible for incidence of carcinogenesis (page 18). This conclusion appears vague given that the neither the mouse data nor the tissue dosimetry data are available yet. Furthermore, the statement that "The sheer number of significant differential gene expression changes at carcinogenic concentrations is likely responsible for the eventual induction of intestinal tumors" seems to dismiss the hypothesis that particular pathways are critical for carcinogenesis.

Reviewer 4: Somewhat outside my area of expertise, but the conclusions appear to be supported by the results.

Charge Question 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: I do not have the experience to answer this question.

Reviewer 2: The study has major strengths, in terms of the quality and completeness of the assays and basic data processing. Data presentation in its current form is not sufficiently systematic and concise. When the definitely-real facts are sorted out from the possible, with *clear* conclusions on the one hand, and *possible* inferences/speculations on the other, this study could be of great value in the larger MOA context.

An important conclusion thus far is that the rats adapt better than the mice, over the time between day 8 and day 91. This is a really important observation for the MOA and could involve a range

of factors, from organismal to molecular. It seems important to characterize the difference as fully as possible in the present molecular context. For both rat and mouse, there should be lists of genes that are responsive at day 8 but not at day 91, and at day 91 but not day 8. This could be limited to those showing 2-fold change and P>0.999.

Reviewer 3: The authors appear to have been consistent in the comparison of rat and mouse data using the same fold-change cutoffs and level of significance. The fact that both studies were similarly designed and performed by the same research group gives strength to the findings. The companion manuscript by Thompson will provide an important piece of the puzzle. Together, these manuscripts should contribute significantly to our understanding of the MOA and WOE of chromium. However, based on the fact that intestinal concentration appears to be an important factor influencing gene expression patterns, the kinetic differences between the mouse and rat models are going to be essential in estimating human relevance.

Reviewer 4: The study appears appropriate for consideration for an EPA IRIS evaluation.

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

Reviewer 1: The paper is a very thorough comparison of toxicogenomics responses to Cr in rat and mouse intestine.

Reviewer 2: The Abstract gives the impression of a no-clear-result study. The Discussion meanders and gives the same impression. This approach under-sells the results! There are a few really clear interesting differences that should be emphasized; the discussion of the rest can be minimized. In other words, firmly stress a few strong hypothesis-generating findings and don't let them get lost in a morass of this-and-that. Others may pick up and pursue these hypotheses. Also, the relationships to toxicokinetics and toxicodynamics are critical and worth emphasizing; more interesting results might emerge if these aspects are pursued further (see above).

Reviewer 3: The manuscript is generally well written (see marked up file for a few editorial suggestions). The data is clearly organized and clearly discussed. The figures provide sufficient detail to give the reader confidence that the experiments were well executed and the findings are reliable.

Reviewer 4: None.

Appendix A

Instructions to Reviewers

Dear Reviewers,

Thank you again for your willingness to provide a peer review of the rat 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 rat genomics 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).

If possible, please send your written review by email to me by Friday, December 16, 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

Reviewers' Annotated Draft Manuscripts

Reviewer 2 Comments

ABSTRACT (250)

Continuous exposure to high concentrations of hexavalent chromium [Cr(VI)] in drinking water results in intestinal tumors in mice but not rats. Concentration-dependent gene expression effects were evaluated in female F344 rat duodenal and jejunal epithelia following 7 and 90 days of exposure to 0.3-520 mg/L $Na_2Cr_2O_7 \bullet H_2O$ (SDD) in drinking water. Whole-genome microarrays identified 3269 and 1815 duodenal, and 4557 and 1534 jejunal differentially expressed genes at 8 and 91 days, respectively, with significant overlaps between the intestinal segments. Functional annotation identified gene expression changes associated with oxidative stress, cell cycle, cell death, and immune response that were consistent with reported changes in redox status and histopathology. Comparative analysis with B6C3F1 mouse data from a similarly designed study identified 2790 rat orthologs differentially expressed in the duodenum compared to 5013 mouse orthologs at day 8. Comparable numbers of orthologs were altered in the jejunum of both species (~3500). Approximately 40% fewer orthologs were altered at day 91. Automated dose-response modeling resulted in similar median EC_{50s} in the rodent duodenal and jejunal mucosae at day 91 (39-55 mg/L SDD). Comparative evaluation of the small number of divergently regulated orthologs, and several oncogenes and tumor suppressors revealed few differences that would likely explain the disparate intestinal tumor outcomes. Comparable numbers of differentially expressed genes were observed at equivalent Cr concentrations (μg Cr/g duodenum); however, at ≥170 mg/L SDD mice accumulated higher Cr levels than rats, resulting in a dramatic ~2-fold increase in the number of differentially expressed genes (~10% of the genome). This abstract could be a lot more interesting a forceful. Focus on the genes that show a very clear change in mouse duodenum, but not rat, at carcinogenic doses, and vice versa, especially at day 91, and on how these differences could relate to Cr(VI) clearance and to tumorigenesis.

Key words: chromium, Cr(VI), microarray, intestine, toxicogenomics, phenotypic anchoring

1. INTRODUCTION (353)

Hexavalent chromium [Cr(VI)] is a recognized lung carcinogen (IARC, 1990). In contrast, oral exposure to Cr(VI) at environmentally relevant exposure levels is thought not to pose a cancer risk due to reduction of Cr(VI) to Cr(III) by bodily fluids and cellular constituents (U.S. EPA, 1991; Proctor et al., 2002). However, chronic exposure to high concentrations of Cr(VI), in the form of sodium dichromate dihydrate (SDD), results in alimentary canal tumors in rodents (NTP, 2008). Oral mucosa tumors were reported in Fisher 344 rats at SDD concentrations ≥172 mg/L, and duodenal tumors in B6C3F1 mice at ≥57 mg/L (NTP, 2008). Notably, these concentrations were associated with significant reductions in water intake in both species that was attributed, in part, to unpalatability (NTP, 2008).

To further elucidate the key events involved in the mode of action (MOA) of intestinal tumor development, a complementary series of dose-dependent comparative drinking water studies were conducted in female F344 rats and B6C3F1 mice (Thompson et al., 2011a; Thompson et al., 2011b; Thompson et al., In press). In both species, intestinal lesions included evidence of oxidative stress, villous cytotoxicity, and crypt hyperplasia. Given the similar phenotypic (or apical) responses in our rat and mice studies, it is critical to compare genomic responses to Cr(VI) in order to assess whether there are species-specific responses that may explain the different tumor outcomes.

Messenger RNA (mRNA) [actually total RNA was extracted] was extracted from rat duodenal and jejunal epithelial scrapings and analyzed using whole-genome Agilent oligonucleotide microarrays following continuous exposure to SDD in drinking water for 7 and 90 days. Differential gene expression was analyzed for over-represented functions and phenotypically anchored to published gross physiology, histopathology, and biochemistry data from complementary studies (Thompson et al., In press). In addition, the rat toxicogenomic data were compared to B6C3F1 mouse gene expression data collected using the same study design, exposure regimen, tissue collection, and gene expression analysis methods (Kopec et al., submitted). Significant qualitative and quantitative differences in the number and types of differentially expressed gene were identified in rat and mouse toxicogenomic dataset comparisons. These results support a proposed MOA involving oxidative stress, cytotoxicity, cell proliferation, and DNA modification.

2. MATERIALS & METHODS

2.1 Animal Husbandry and Study Design

Detailed description of the test substance, animal husbandry, and study design has been previously described (Thompson et al., 2011b; Thompson et al., In press). Briefly, Southern Research Institute (Birmingham, AL) obtained 4-5 week old female Fischer rats (Charles Rivers Laboratories International, Stone Ridge, NY), which were acclimated for a minimum of 7 days and fed *ad libitum* with irradiated NTP-200 wafers (Zeigler Bros, Gardners, PA). So the rats could have been 5 – 7+ weeks when used. This is a critical time period involving puberty and rapid weight gain. A 5-week female rat is very different from an 8-week one. Was this taken into account? Animals were continuously exposed to sodium dichromate dihydrate (SDD) dissolved in tap water at 0, 0.3, 4, 60, 170 and 520 mg/L, corresponding to 0, 0.1, 1.4, 20.9, 59.3, and 181 mg/L Cr(VI) for 7 and 90 days (referred to as day 8 and day 91). Rodents were then euthanized using CO_2 and intestinal sections were collected and flushed with ice-cold phosphate

buffered saline. Duodenal and jejunal sections were cut longitudinally and the epithelium was scraped using disposable sterile plastic spatulas (VWR International) into vials containing ~1 ml of TRIzol (Invitrogen, Carlsbad, CA) and snap-frozen in liquid nitrogen. The samples were stored at –80°C and shipped on dry ice to Michigan State University for gene expression analysis. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee at Southern Research Institute.

2.2 RNA Isolation

Frozen rat intestinal samples were homogenized using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated according to the manufacturer's protocol with an additional acid phenol:chloroform extraction. Isolated RNA was resuspended in RNA storage solution (Ambion Inc., Austin, TX), quantified (A_{260}), and quality was assessed by evaluation of the A_{260}/A_{280} ratio and by visual inspection of 1 µg total RNA on a denaturing gel. Was each RNA sample examined in this way? It would be worthwhile to show a set of gels, perhaps in the Supplement, since RNA stability could be a real issue here, in a situation where digestive enzymes abound.

2.3 Microarray Analysis

Dose-dependent changes in gene expression were examined using rat 4x44 K Agilent whole-genome oligonucleotide microarrays (version 1, Agilent Technologies, Inc., Santa Clara, CA). Treated samples were co-hybridized with vehicle controls to individual arrays according to manufacturer's protocol (Agilent Manual: G4140-90050 v. 5.0.1). All hybridizations were performed with three independent biological replicates for treated and control tissues (i.e., RNA samples were not pooled) and independent labeling of each sample (Cy3 and Cy5, including dye swap) for each treatment group at each time point (8 and 91 days). How were these rats selected, considering the possible wide range of weights and ages (see above)? Was each sample pair analyzed only once with each dye configuration?

Microarray slides were scanned at 532 nm (Cy3) and 635 nm (Cy5) on a GenePix 4000B scanner (Molecular Devices, Union City, CA). Images were analyzed for feature and background intensities using GenePix Pro 6.0 software (Molecular Devices). All data passed our laboratory quality assurance protocol (Burgoon et al., 2005) and were deposited in TIMS dbZach data management system (Burgoon and Zacharewski, 2007). Microarray data were normalized using a semi-parametric approach (Eckel et al., 2005) and the posterior probabilities were calculated using an empirical Bayes method based on a per gene and dose basis using model-based *t* values (Eckel et al., 2004). Gene expression data were ranked and prioritized using |fold change|>1.5 and statistical P1(*t*) value >0.999 criteria to identify differentially expressed genes.

2.4 Dose-Response Modeling

Dose-response modeling was performed using the ToxResponse Modeler, which identifies the best-fit between five different mathematical models (linear, exponential, Gaussian, sigmoidal, quadratic) (Burgoon and Zacharewski, 2008). The algorithm then identifies the best-fit from the five best in-class models for subsequent EC_{50} calculations. Microarray data sets were first sorted using more stringent criteria (|fold change|>2 and P1(*t*)>0.999 cut-off in the 520 mg/L SDD group), and then modeled to identify genes exhibiting sigmoidal dose-response profiles. EC_{50} values were only determined for genes

exhibiting a sigmoidal dose-response curve. Evidently no differentially-expressed genes fit the linear, exponential or quadratic models. This should be explicitly stated. A substantial number (300-600 probes at day 8, 130-140 probes at day 91) presented a Gaussian pattern. These could be of interest, especially since some of these may be included in functional groupings in the manuscript, e.g., Table 1. More information should be provided regarding the Gaussian-model genes.

BMDExpress was also used to model individual gene responses at day 91 via benchmark dose (BMD) modeling using a modified version of a previously published procedure (Thomas et al., 2007; Kopec et al., submitted). Hill, power, linear and 2° polynomial models were fit assuming constant variance and the benchmark response (BMR) factor was set to 1.349; the best fitting models for each probe were collated and probes with poor model fits or BMD values outside of experimental dose range were excluded.

2.5 Quantitative Real-Time PCR (QRT-PCR)

QRT-PCR was used to confirm the differential expression of selected genes identified in the microarray analysis (Kopec et al., submitted). What was the basis for gene selection? Briefly, total RNA was reverse transcribed to cDNA and PCR amplified on an Applied Biosystems PRISM 7500 Sequence Detection. **Supplementary Table S1** provides the names, gene symbols, accession numbers, forward and reverse primer sequences, and amplicon sizes. cDNAs were quantified using a standard curve approach and the copy number of each sample was standardized to 3 housekeeping genes (mouse: *Actb*, *Gapdh*, *Hprt;* rat: *ActB, Hprt, Rpl13a*) to control for differences in RNA loading, quality, and cDNA synthesis (Vandesompele et al., 2002). For graphing purposes (GraphPad Prism 5.0), the relative expression levels were scaled such that the expression level of the time-matched control group was equal to one. According to the legend for Fig. 8, QRT-PCR data are from 3 independent replicates. These data are critical, so the method should be given more fully and in this section. Confirm that these replicates are from individual rats, and state whether they are the same rats as used for the microarray. If not, indicate how they were chosen. How were the housekeeping genes actually used for normalization? QRT-PCR should be shown for these genes for 5 control rats, since the cited reference does not cover intestine.

2.6 Functional Gene Annotation and Statistical Analysis

Annotation and functional categorization of differentially regulated genes was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al., 2003) and Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA). For cross-species comparisons, HomologeneID was used to identify differentially expressed orthologous genes. Hierarchical clustering (average linkage method; Pearson correlation) was performed using MultiExperiment Viewer (MeV v. 4.6.0) implemented in the TM4 microarray software suite (Saeed et al., 2003). QRT-PCR statistical analyses were performed with SAS 9.2 (SAS Institute, Cary, NC). Unless stated otherwise, all data were analyzed by analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Differences between treatment groups were considered significant when $p<0.05$.

3. RESULTS

3.1 Effects of SDD on Gene Expression in Rat Small Intestine

3.1.1 Rat Intestinal Differential Gene Expression at Day 8

Rat intestinal gene expression was evaluated using 4x44K Agilent oligonucleotide microarrays containing 17,142 unique annotated genes. Microarray analysis (|fold change|>1.5, P1(*t*)>0.999) identified 3269 duodenal and 4557 jejunal differentially expressed genes, identified at one or more doses, at day 8. The number of differentially expressed duodenal and jejunal genes increased with dose (**Fig. 1A**). Comparative analysis identified 2312 genes that were differentially expressed (|fold change|>1.5, P1(*t*)>0.999) in both intestinal sections following SDD exposure (**Fig. 1B**). Attention should be focused on the duodenum, the target site for tumors in the mice. Relaxing the filtering criteria to |fold change|>1.2, and P1(*t*)>0.9 to avoid exclusion of genes bordering the stringent cut-offs, increased the number of overlapping genes to 4240 (**Fig. 1C**). This degree of relaxation of the filtering criteria almost certainly brings in many red herrings. This suggests that SDD elicited the differential expression of the same genes in the rat duodenum and jejunum. However, duodenal differential gene expression exhibited greater fold changes (-31.2- to 54.5-fold) compared to the jejunum (-41.7- to 16.6-fold). Is not the -41.7 a greater fold change than -31.2? The top 10 most induced and repressed genes for duodenum and jejunum at each concentration are shown in **Supplementary Tables S2A-B**. These are potentially the genes of greatest interest, since they are the most likely to show major changes in protein expression. We need to know more about them, especially those in duodenum at the two highest doses. These should be listed out, with their full names and descriptions and dose-response information. Their behavior should be compared with that of the mouse ortholog. This could be a good place to list the most changed mouse genes, and show what happens to these genes in rat.

3.1.2 Rat Intestinal Differential Gene Expression at Day 91

Microarray analysis (|fold change|>1.5, P1(*t*)>0.999) identified 1815 duodenal and 1534 jejunal differentially expressed genes at day 91 (**Fig. 1D**). This represents a 56% and 66% decrease in unique differentially expressed genes, respectively, compared to day 8. Approximately 765 genes overlapped between the duodenal and jejunal epithelia, which increased to 2151 genes when the criteria were relaxed to |fold change|>1.2, and P1(*t*)>0.9 (**Fig. 1E-F**). The same suggestions apply as above, that is, concentrate on the duodenum and on the strictly filtered genes. Relative fold induction at the highest dose was comparable for both tissues (up to 19.4-fold), but duodenal epithelium showed greater suppression (-26.5-fold) of gene expression relative to jejunum (-12.4-fold). The top 10 most induced and repressed genes for duodenum and jejunum at each concentration are shown in **Supplementary Tables S3A-B**. Again, those at the two highest doses should be described in detail and compared with their behavior in the mouse.

3.2 Dose-Response Modeling of Rat Intestinal Differential Gene Expression

Differentially expressed probes meeting the criteria of |fold change|>2 and P1(*t*)>0.999 at 520 mg/L SDD were selected for dose-response analysis using ToxResponse modeler (Burgoon and Zacharewski, 2008). Of the 1572 probes (943 unique genes) meeting the criteria at day 8, 1269 (744 unique genes) exhibited a sigmoidal-dose response profile with ~67% of all EC_{50s} between 0.3 and 10 mg/L SDD (**Fig. 2A**). Jejunum analysis identified 1934 sigmoidal probes (1021 unique genes) with 65% having EC_{50s} between 10-100 mg/L SDD (**Fig. 2B**). DAVID analysis of

the 858 duodenal genes with EC_{50s} <10 mg/L SDD identified over-represented functions associated with protein synthesis, translation and ribosome-related genes. Sustained induction (~2-fold) across 4-520 mg/L SDD was observed for eukaryotic translation elongation and initiation factors (*Eef1b2, Eef1e1*, *Eif2b3*, *Eif2s1*, and *Eif2s2)* and the ribosomal proteins (*Rpl13*, *Rps3*, *Rps5*, *Rps10*, and *Rps27*) that exhibited EC_{50s} <10 mg/L. These do not match the listings in Table 1 under Protein Synthesis. At day 91, only 310 duodenal and 167 jejunal genes exhibited sigmoidal dose-response profiles with >72% having EC_{50s} between 10 and 100 mg/L SDD (**Supplementary Fig. 1**). Overall, the day 8 median duodenal EC_{50} value was ~10 times lower compared to the median jejunal EC_{50} value (5 vs. 52 mg/L SDD), while at day 91, the median EC_{50s} were comparable for rat duodenum and jejunum (49 vs. 52 mg/L SDD).

3.3 Phenotypic Anchoring of Rat Differential Gene Expression Responses

DAVID and IPA were used to associate functions with differential gene expression and phenotypically anchor significantly expressed genes to complementary histopathology and biochemical data (**Table 1**). For example, reductions in the GSH/GSSG ratio suggested that the rat intestinal epithelium experienced oxidative stress (Thompson et al., In press). Induction of *Nrf2* (*Nfe2l2*) ~2.6-fold and subsequent induction (up to 2.7-fold) of downstream targets are also suggestive of oxidative stress. For example, ubiquitination and proteasomal degradation of proteins (*Vcp*, *Usp14* and *Ube2k*), chaperone and stress proteins (*Stip1*, *Cct7*, *Erp29*), and antioxidant proteins (*Atf4*, *Gpx2*, and *Prdx1*) are all suggestive of oxidative stress. Induction of *Nrf2* and its target genes between the duodenum and jejunum was comparable at day 8. Interestingly, the calculated EC_{50} values for *Nrf2* were 4.2 and 14.2 mg/L SDD in the duodenum and jejunum at day 8, respectively. The ToxResponse modeler also calculated EC_{50} values below 5.0 mg/L SDD for *Nrf2*-regulated *Usp14*, *Cct7*, and *Erp29* at day 8. The *Nrf2*-mediated oxidative stress response was also observed at day 91 and select genes were verified by QRT-PCR, including induction of *Nrf2*, *Gclc* and *Gpx2* (**Fig. 3**). Compared to day 8, the induction was more modest, but overall the efficacy (maximum fold change) in the jejunum was slightly higher, in agreement with more oxidative stress and lower GSH/GSSG levels (Thompson et al., In press). Above, results for genes highlighted in green are reliable, based on modeling/statistics or RT-PCR, those in yellow are not. Four additional genes in Table 1 are in the low-reliability category and may be there because they are interesting rather than convincing. Overall this collection is not particularly strong as support for oxidative stress. It is fine to do phenotypic anchoring and to relate to outcomes from other arms of the study, but misleading to do so with weak data.

Genes associated with immune response (e.g., *Acp5*, *Anxa2* *Anxa5 in the table*, *Blnk* not in Table 1, *Ccl24* *Cxcl12*, *Kitlg*, *Il1rl1*, *Il33* and *C1qa*) were also differentially expressed (**Table 1**), consistent with the mild to marked histiocytic infiltration at days 8 and 91. Interestingly, *Il1rl1* (5- to 10.9- fold) and *Il33* (4.5- to 5.9-fold) exhibited the greatest fold expression with EC_{50} values of 6.8 and 5.4 mg/L SDD, respectively in the duodenum at day 8. The same genes were also among the highest induced immune response in the mouse, although their maximum fold induction (efficacy) was lower with higher EC_{50s} (Kopec et al., submitted). Four additional genes in this list are problematic. There are enough strong clear responses for this category; could leave out the weak ones.

Differentially expressed genes involved in cell cycle, growth and proliferation exhibited dose-dependent induction in the rat including *Myc*, *Tp53* and their downstream regulated genes. SDD also induced cyclin-dependent kinases and cell division associated proteins including *Cdc20*, *Cdc26*, *Cdc37*, *Ccnb1*, *Cdk2*, *Cdk4*, and *Cdk105* up to 3.2-fold with EC_{50} values between

3.9 and 115 mg/L SDD. Moreover, insulin-like growth factor 1 (*Igf1*), trefoil factor 1 (*Tff1*; $EC_{50\ Duodenum\ and\ Jejunum\ Day\ 8}$ = 4.2 and 35.3 mg/L SDD) and proliferating cell nuclear antigen (*Pcna*; $EC_{50\ Duodenum\ and\ Jejunum\ Day\ 8}$ = 4.1 and 5.0 mg/L SDD) were also significantly induced in the rat intestine. The mouse intestinal epithelium showed comparable *Pcna* induction (~2-fold), but *Tff1* expression was considerably greater (52.7-fold) compared to the rat duodenum at day 8 (13.6-fold). At 91 days, *Tff1* induction in rat jejunum was significantly higher (10.6-fold) compared to mouse (1.8-fold), with lower EC_{50} value (25.6 in rat vs. 64.8 mg/L in mouse) and may contribute to the protection against tumor development at a later time point (Buache et al., 2011). Again, there are enough interesting significant genes in this category, without muddying the waters with distractions of uncertain reality, especially Myc, Tp53 and several of the cell cycle control genes.

Protein synthesis functions including eukaryotic translation elongation and initiation (e.g. *Eef2b2* and *Eif1ay* not in table), as well as ribosomal proteins (e.g. *Rps3*not in table, *Rps5* not in table, and *Rps27* not in table) and seryl-tRNA synthetase (*Sars*) were also over-represented at day 8 and 91, likely in support of cell growth and proliferation (**Table 1**). Confusion here

The DNA damage and modification genes *Apex1*, *Ogg1*, *Cbx3*, *Exo1*, *Fen1*, *Msh2*, and *Hmgn1* were also induced 1.6- to 3-fold at day 8 in the rat with overall low (<10 mg/L SDD) EC_{50} values. However, unlike the sustained induction of these and other DNA repair genes in mouse duodenum at 8 and 91 days (Kopec et al., submitted), maximum fold change (efficacy) expression was attenuated in the rat duodenum at 91 days (**Table 1**). Notably, no changes in 8-isoprostane or 8-OHdG were observed in the rat duodenum at day 91 (Thompson et al., In press). The DNA damage/repair genes were generally non-responsive in the jejunum with modest suppression (P1(*t*)>0.90) at low doses – despite clear signs of oxidative stress at day 91 (Thompson et al., In press). Only three gene changes are unequivocally real.

Rats exposed to SDD exhibited low serum and bone marrow iron levels at day 91 (Thompson et al., In press), and showed evidence of hypoferremia/anemia (NTP, 2008). This suggests that prolonged exposure to SDD may interfere with iron homeostasis. Although clinical analyses of blood iron were only collected at day 91, several genes involved in dietary iron absorption, transport, and export were repressed in the duodenum at day 8. Specifically, *Cybrd1* ($EC_{50\ Jejunum}$ = 4.4 mg/L SDD), *Heph* ($EC_{50\ Duodenum}$ = 51.3 mg/L SDD), *Slc40a1* ($EC_{50\ Duodenum\ and\ Jejunum}$ = 52.7 and 56.1 mg/L SDD), *and Hfe2* were repressed 2-3-fold. By day 91; however, the changes were more modest.

3.4 Comparisons of Gene Expression Changes in Rats and Mice

3.4.1 Orthologous Intestinal Differential Gene Expression at Day 8

Intestinal differential gene expression changes in rats and mice were compared in order to identify similar and divergent responses. Orthologs were identified using HomoloGene (PubMed), which relies on DNA sequence similarity among closely related species to identify orthologous genes (i.e. same gene in different species) using BLAST nucleotide sequence comparisons (Wheeler et al., 2004; Wheeler et al., 2006). Approximately 13,899 unique orthologs were identified from the 17,142 unique annotated rat and 21,307 unique annotated mouse genes, as represented on their respective 4x44K Agilent whole-genome oligonucleotide microarrays (**Supplementary Fig. 2**). Of the ~13,899 unique orthologs, 2790 and 5013 exhibited differential expression in the rat and mouse duodenum, respectively (**Fig. 4A**). Comparative

analysis revealed a significant overlap, increasing from 1986 to 3909 orthologs with reduced stringency (**Fig. 4B-C**). However, more unique orthologs (~6×) were expressed in the mouse duodenal epithelia (1649 orthologs) compared to the rat duodenal epithelia (259 orthologs). It would be more useful to stick with the stringent cut-off, since it is the genes uniquely expressed in duodenum in mouse or rat that is of greatest interest. Fortunately these genes are used in the hierarchical clustering heat maps in Fig. 5

Cross-species comparison of the jejunal gene expression identified comparable numbers of unique differentially expressed orthologs (3782 rat and 3334 mouse) (**Fig. 4D**). Like the duodenum, differentially expressed jejunal orthologs exhibited a significant overlap: increasing from 1576 to 3864 orthologs with reduced stringency (**Fig. 4E-F**). Unlike the duodenum, the number of species-specific differentially expressed genes was comparable (971 vs. 705). Hierarchical clustering of the 1986 duodenal (**Fig. 5A**) and 1576 jejunal (**Fig. 5B**) overlapping orthologs revealed that low (≤14 mg/L SDD) and high doses (≥ 60 mg/L SDD) cluster separately in a species-specific manner. Specifically, the highest 3 doses within each species clustered together, but as a group were closely linked to the other species – suggesting that the responses at ≥ 60 mg/L SDD are similar in both species. But in Fig. 5A and Suppl. Fig. S3, there are some genes that show clear difference in rat vs mouse at the two highest doses. They are relatively few in number and could be critical. These need to be listed explicitly with names, function and EC50.

This can be seen in heatmaps of the 1986 overlapping genes when only the genes are clustered, while keeping the SDD concentrations in order (**Supplementary Fig. 3**).

3.4.2 Orthologous Intestinal Differential Gene Expression at Day 91

Cross-species analysis of day 91 duodenal responses identified 1504 and 3484 differentially expressed unique orthologs for the rat and mouse, respectively (**Fig. 6A**). Comparative analysis of these differentially expressed orthologs identified that 811 (|fold change|>1.5, P1(*t*)>0.999) and 2536 (|fold change|>1.2, P1(*t*)>0.9) orthologs overlapped between the species. Again, those stringently filtered are of greatest interest. In both Fig. 4 and Fig. 6, the bolded values are for over-lap at low stringency (C and F in both figures). Instead the emphasis should be on the non-overlapping at high stringency in duodenum (B in both figures. As was observed at day 8, the mouse duodenal epithelia expressed substantially more (~5×) non-overlapping unique orthologs compared to rat following 90 days of SDD exposure (**Fig. 6B-C**). Similar comparisons of jejunal differential gene expression identified 1305 rat and 3620 mouse orthologs of which 729 were commonly expressed in both species (**Fig. 6D**). Using relaxed criteria, the overlap was comparable to the duodenal orthologs at day 91 (2772 jejunal orthologs in **Fig. 6E-F**). Hierarchical clustering of the 811 duodenal overlapping orthologs showed species-specific clustering of low and high dose groups with approximately equal numbers of induced and repressed genes (**Fig. 7A**). This should be done with the more stringent criteria and any genes identified that showed a clear distinction between mouse and rat at the two highest doses. The 729 jejunal orthologs clustered in a species-specific manner with overall more down-regulated orthologs in the rat compared to the mouse (**Fig. 7B**). Heatmaps of the 729 overlapping genes where only the genes are clustered, while keeping the SDD concentrations in order, clearly indicate differential responses between the two species in the jejunum (**Supplementary Fig. 3**). Functional annotation of these genes revealed pathways related to eukaryotic translation initiation factors, mammalian target of rapamycin (mTOR) signaling (involved in cell survival and proliferation), and polo-like kinase involvement in mitosis. Genes in these pathways were

generally up-regulated in mice at ≥ 60 mg/L SDD, and may relate to the significant increase in observed crypt cell hyperplasia in the mouse jejunum, which was not observed in rats (Thompson et al., 2011b; Thompson et al., In press).

3.4.3 Divergent Orthologous Gene Expression in Duodenum at Day 91

Correlation analysis of the overlapping orthologous microarray gene expression identified 81% of the genes were positively correlated in terms of fold change and significance in duodenum at day 91 (**Fig. 8A**, **Supplementary Fig. 4**). Where is the scatter plot for day 8?
However, examples of divergently expressed orthologs from different functional annotation clusters were also identified (**Table 2**) and verified using QRT-PCR (**Fig. 8B, Supplementary Fig. 5**). Divergently expressed orthologs were associated with the immune response (*Ccl24*, *C3*), ion transport (*Slc25a25*), and growth factor/ cytokine signaling (*Areg*). It is great that these four genes have been confirmed. However, there quite a few red dots in the A and D quadrants of Suppl. Fig. S4, and at least 150 circles in the corresponding quadrants in Fig. 8A. There are 22 genes listed in Table 2. According to Fig. 6B, there were 693 rat and 2673 mouse genes that showed unique differential expression in duodenum at day 91, using 1.5X and P>0.999. It would be good to have a full listing for the two highest doses, using a 2X cut-off? Also the legend to Table 2 is unclear; no gene name is italicized to show differential expression at 60 and 520 mg/L only.

3.4.4 Species-Specific Orthologous Gene Expression in Duodenum at Day 91

The overlap in rat and mouse duodenal genes was ~61% (2536/4177), while the number of unique orthologs in the mouse duodenum (1392) was >5 times greater than the number of unique orthologs in the rat duodenum (249) (**Fig. 6C**). Hierarchical clustering of these species-specific orthologs (disregarding fold-change and statistical cut-offs) revealed most genes exhibited different dose-response patterns (e.g., differentially expressed in mouse, non-responsive in rat) (**Supplementary Fig. 6**). This could be misleading. Use of stringent cut-off and a 2-fold change would be more likely to point the way to genes of interest. Functional annotation of the 1392 mouse orthologs identified enrichment of alanine and aspartate metabolism, FAK signaling, and the DNA damage response related to BRCA1. Genes associated with the FAK signaling pathway (involved in cell cycle, proliferation and migration) were mostly down-regulated or unaltered at various concentrations (including *Fak/Ptk2*; data not shown). Functional enrichment analysis of the 249 orthologs in the rat duodenum resulted in enrichment of intrinsic prothrombin activation (mostly down-regulated); however this may be an artifact of the relatively small number of genes (249) in this analysis. This generalized summary is not sufficient, since these are the key data in the paper.

3.4.5 Dose-Response Comparisons of Differential Gene Expression in Rats and Mice

3.4.5.1 EC_{50} Distribution for Over-Represented Pathways

Comparison of ToxResponse modeler results for rat and mouse (Kopec et al., submitted) datasets is summarized in **Table 3**. Compared to rat, SDD differentially dysregulated more mouse genes that met the filtering cut-offs (±2-fold at 520 mg/L SDD and P1(*t*)>0.999) resulting in a bigger subset of sigmoidal dose-responsive expression profiles for which EC_{50} values could be calculated. Overall, except for greater sensitivity in rat duodenum at day 8, the median EC_{50s} were comparable between the species.

To directly compare the EC_{50} distribution between the species, overlapping orthologous genes that met the filtering criteria in both datasets were compared for over-represented pathways (using DAVID) at each tissue and time point (**Fig. 9**). At day 8, functional categorization of 331 overlapping sigmoidal orthologs in duodenum revealed ~10 times lower median and EC_{50} range for *Translation*/*Protein Biosynthesis*, *Cell Cycle* and *Oxidoreductase* in the rat, while *Inflammatory Response* showed comparable median EC_{50s} between the species (**Fig. 9A**). These box-and-whisker plots show a lot of overlap. Which differences are of statistical significance? Comparative analysis of 195 overlapping jejunal sigmoidal orthologs at day 8 showed comparable overall median EC_{50s}, with slightly lower mouse median EC_{50s} for *Ribosome* (23.0 vs. 52.6 mg/L in rat), *Translation* (26.8 vs. 46.0 mg/L in rat), comparable changes in *Oxidoreductase* category, and significantly What is the P value? lower rat EC_{50s} for *Cell Cycle* (4.5 vs. 36.8 mg/L SDD in mouse) and *Nucleoside Binding* (6.1 vs. 52.5 mg/L SDD in mouse) (**Fig. 9B**). At day 91, the number of overlapping orthologs was decreased to 97 in duodenum, where the median EC_{50s} for *Immune Response*, *Carbohydrate Binding*, *Oxidoreductase*, *Apoptosis*, and *Proteolysis* were comparable between the species (**Fig. 9C**). Only 57 orthologs with sigmoidal dose-response profiles overlapped in jejunum at 91 days with comparable potency for *Drug Metabolism*, *Lipid Transport*, *Cell Death*, *Oxidoreductase*, and *Transport* (**Fig. 9D**).

3.4.5.2 Dose-Response Modeling Using Duodenal Tissue Concentrations of Cr(VI)

Tissue concentration of chromium were collected from a subgroup of animals in this study at day 91 in order to develop pharmacokinetic models of Cr(VI) disposition (Thompson et al., 2011a). These tissue concentrations represent chromium levels in both the proximal and distal portions of each intestinal segment. The jejunum is respectively ~18 and 67 cm long in mice and rats, and thus the jejunal chromium levels cannot be matched to the scrapings from the proximal 6 cm, which were used for genomic data collection. In contrast, full duodenal length was collected in both species and thus the measured chromium concentrations match the genomic analyses. Therefore, automated BMD modeling was performed on the day 91 duodenal gene changes in mice and rats as a function of tissue Cr levels. The BMD and BMDL values were slightly lower in rats compared to mice (**Fig. 10A**). Are these differences statistically significant? These BMD values (μg Cr/g tissue) each correspond to the 60 mg/L SDD drinking water concentration. In mice this concentration resulted in cytoplasmic vacuolization (Thompson et al., 2011b) and large increases in the total number of significant ±1.5-fold gene expression (Kopec et al., submitted; **Fig. 10B**). In rats, only histiocytic infiltration was observed in the duodenum at 60 mg/L SDD (Thompson et al., In press), along with increases in the total number of significant ±1.5-fold gene changes (**Fig. 1D** and **10B**). Although the slightly lower BMD value in rats might indicate that rats are more sensitive to SDD than mice, it is evident in **Fig. 10B** that the duodenal Cr levels in rats were much lower compared to mice at carcinogenic SDD concentrations (i.e. 170 and 520 mg/L). For example, at 60-520 mg/L SDD the duodenal Cr levels ranged from 18-32 μg Cr/g duodenum in rats, and 34-61 μg/g in mice. At greater than 40 μg Cr/g, the number of differentially expressed mouse genes exceeded 3,000 (~10% of the genome). Comparing the significant orthologous gene changes at duodenal chromium concentrations <40 μg/g tissue indicates considerable overlap using the relaxed filtering criteria (**Fig. 10C**). These are quite important results. What is accomplished by relaxing the criteria?

The differences in tissue concentration of Cr(VI) are key. Are there results for day 8? If rat and mouse are similar on day 8 with regard to tissue concentrations, it could imply that adaptive changes occur in the rat that result in enhanced clearance.

4. DISCUSSION (1679)

This is the first study to compare toxicogenomic responses in two species following 90 days of exposure to an environmental toxicant in drinking water. In Kopec et al. (submitted), SDD was shown to elicit gene changes associated with oxidative stress, DNA damage, and cell proliferation, as well as other pathways/functions (e.g. immune response) that are consistent with biochemical and histopathological findings in mice from the same study. Similarly, data herein provide evidence for gene changes associated with the aforementioned pathways and functions that are also phenotypically linked to observed biochemical and histopathological findings in rats.

For example, SDD elicited dose-dependent induction of genes involved in cell cycle/growth/proliferation such as *Myc* and *Pcna*, which is consistent with increases in crypt hyperplasia (Thompson et al., In press). The data for Pcna are convincing, while those for Myc are uncertain, especially in duodenum. Other phenotypes include histiocytic infiltration, which has been observed in the rat small intestine following 1, 13, and 104 weeks of exposure to SDD (NTP, 2007, 2008; Thompson et al., In press). In this regard, several immune-related genes were induced in the rat small intestine (e.g. *Acp5*, *Anxa5*, *C1qa*, *C3*, *Cxcl12*, *Il1rl1*). This point can be made without including the uncertain genes, yellow highlight. Inflammation and oxidative stress are intimately linked, and SDD was shown to induce oxidative stress in the small intestine of rats from this study, as evidenced by changes in GSH and GSSG (Thompson et al., In press). These findings suggest the possibility for SDD to induce oxidative DNA damage; however, drinking water exposure studies have thus far not shown an increase in 8-OHdG DNA damage in the intestine (De Flora et al., 2008; Thompson et al., 2011b; Thompson et al., In press). As noted before, the method for 8-OHdG was probably inadequate. These negative findings may relate to adaptive responses under the longer-term exposure scenarios employed in these studies. Notably, SDD altered the expression of genes involved in DNA repair (**Table 1**) – mostly at day 8 with attenuated expression at day 91. This is a useful idea. It would be worthwhile to compare day 8 with day 91 statistically, for the genes with a convincing positive response as day 8, *Fen1*, *Hmgn1*, and *Msh2*.

The health of the intestinal epithelium is influenced by redox balance (Circu and Aw, 2011). Inhibition of *de novo* GSH synthesis with buthionine sulfoximine has been shown to induce intestinal epithelial damage including loss of height of epithelial cell layer, desquamation of microvilli, mitochondrial swelling, and vacuolization in the jejunum (especially the villous tips); effects that were ameliorated with GSH supplementation or recovery time (Martensson et al., 1990). These effects are not unlike those reported for the rodent intestine following exposure to Cr(VI) concentrations that result in redox changes. In this regard, expression of oxidative stress response genes was generally comparable between both intestinal segments, including genes involved in Nrf2 signaling (see **Fig. 3**). *Gclc* and *Gpx2* were elevated at ≥ 60 mg/L in the duodenum and jejunum at day 91, with similar inductions observed in mice (Kopec et al., submitted).

Overall, the differential gene changes in rats were attenuated with increased exposure time (**Fig. 1**). Differential gene expression was also diminished in mice between days 8 and 91, albeit not as dramatically (Kopec et al., submitted). In this regard, the mg/kg bodyweight SDD doses in

the rat 520 mg/L treatment groups at day 8 and 91 were 80 and 60 mg/kg, respectively, whereas in mice the doses were 87 and 89 mg/kg (Thompson et al., in press). The decrease in gene expression is also consistent with the observation that the incidences for apoptosis and crypt hyperplasia were slightly lower in rats at day 91 than day 8 – an effect that was not observed in mice (Thompson et al., 2011b; Thompson et al., In press). Either due to inherent pharmacokinetic differences or adaptation to Cr(VI) exposure, the diminished responses observed herein are consistent with the lack of intestinal tumors in rats in the 2-year cancer bioassay (NTP, 2008). So the rats adapt better than the mice, over the time between day 8 and day 91. This is a really important observation for the MOA. It seems important to characterize the difference as fully as possible. For both rat and mouse, there should be lists of genes that are responsive at day 8 but not at day 91, and at day 91 but not day 8, particularly at the highest doses. This could be limited to those showing 2-fold change and P>0.999.

Comparative analysis of overlapping orthologous genes identified only a few orthologs that were divergently expressed between the species (**Table 2**; *C3*, *Ccl24*, *Slc25a25*, *Areg*, and *Wfdc1*). Really true that these are the only ones? As noted above, a fuller presentation of the differences is needed. *Ccl24* (eotaxin) is involved in eosinophil recruitment and chemotaxis, and was induced 3.6-fold in the mouse but repressed 2.8-fold in the rat (**Fig. 8**). Ccl24 protein expression is dependent on IL-4 stimulation in nasal polyps (Schaefer et al., 2006), and its repression in the rat duodenum may relate to the SDD-elicited (albeit minimal) decreases in IL-4 (Thompson et al., In press). Expression of *C3* is required for complement activation, and was up-regulated 2.7-fold in rats while down-regulated 5.1-fold in mice (**Fig. 8**). *C3* mRNA was shown to be induced by IL-1α (Gerritsma et al., 1996), the levels of which were elevated in the rat duodenum after 90 days of exposure to SDD (Thompson et al., In press).

Areg, which promotes intestinal epithelial regeneration after radiation injury (Shao and Sheng, 2010), was down-regulated 3.0-fold in rats, but induced ~1.9-fold in mice, although the levels did not meet statistical significance (**Fig. 8**). Up-regulation in mice is consistent with the high incidence of crypt cell hyperplasia in the mouse duodenum at day 91 (Thompson et al., 2011b); however, more modest crypt hyperplasia was also observed in the rat duodenum (Thompson et al., In press). *Wfdc1* has roles in cell adhesion, migration, proliferation and immune function (Ressler and Rowley, 2011). *Wfdc1* has anti-proliferative properties and is down-regulated in cancer cells (Madar et al., 2009; Ressler and Rowley, 2011). Up-regulation of *Wfdc1* might partially explain the milder proliferation observed in the rat duodenum. This sounds like an interesting gene, but no actual data are shown for it.

Divergent regulation was also observed in the calcium-dependent mitochondrial solute carrier *Slc25a25*. This ATP-Mg/P_i carrier, involved in mitochondrial and cytosolic adenine nucleotide (AMP, ADP, ATP) level regulation, was repressed 1.9-fold in mice but induced 2.1-fold in rats (**Fig. 8**). Studies suggest an important role for this carrier in regulating mitochondrial permeability (Hagen et al., 2003), and Cr(VI) could interfere with its function due to structural similarity with phosphate and sulfate ions (Salnikow and Zhitkovich, 2008). How does this proposed competition relate to changes in gene expression?

Having identified relatively few divergently regulated genes, we further explored species differences by examining whether known oncogenes and tumor suppressor genes were altered at 520 mg/L SDD in the duodenal mucosae of mice and rats at day 91 (**Table 4**). In both species, the tumor suppressor *Npm1* and oncogene *Pdgfra* were significantly elevated. In mice, the tumor suppressors *Apc*, *Rb1also suppressed in rat*, and *Smad4* were suppressed, whereas oncogene *Brca1* was elevated. In rats, tumor suppressor *Tp53* was elevated, whereas oncogenes *Egfr* and

Fgfr3 also suppressed in mice were suppressed. There are not any really clear differences between rats and mice in this list. A really clear difference would require a 2-fold highly significant change for one species, no change for the other. Given the suppression of *Apc* and evidence that APC inactivating mutations that lead to increased β-catenin signaling associate with increased risk of colon cancer (Giles et al., 2003), the IPA canonical pathway for Wnt/β-catenin was examined. Overall, this pathway was not significantly enriched in the duodenum of either species at day 91; however, genes in this pathway were slightly more activated in rats than mice (**Supplementary Fig. S7**). Apc results were statistically weak. What was the dose used for the data in Fig. S7? It is hard to interpret this diagram, since the significances are not shown.

Biomarkers for intestinal cancer and disease were also identified using IPA at 520 mg/L at day 91. The genes common to both species are shown in **Table 5**. Two of the 16 genes were differentially altered as previously noted in **Table 2** (*Areg* and *Tfam*). Interestingly, increased expression of *Areg* (which encodes activators of the EGF receptor pathway) in wild-type *Kras* colon cancer biopsies correlates with positive response to treatment with the anti-EGFR drug cetuximab (Baker et al., 2011). This suggests that continued EGF signaling potentiates the risk of cancer development; however, EGF signaling did not appear to be enriched *per se* in the mouse duodenum at day 91 (data not shown). How was EGF signaling evaluated? The amphiregulin findings are quite interesting, in fact possibly the most meaningful in the study. The operations of the EGFR family members and their agonists are complex. Don't downplay Areg- maybe others will pursue it.

Dose-response modeling of the genomic responses revealed the surprising observation that the median EC_{50} value in the rat duodenum at day 8 was ~10-fold lower than in the jejunum. In fact, the median EC_{50} values in all other tissues and time points in both rats and mice were ~50 mg/L SDD. Average daily doses of SDD in mice and rats were similar at each dose at day 8 (Thompson et al., In press), and thus mg/kg SDD dose does not readily explain the low EC_{50} in the rat duodenum at day 8. Interestingly, however, the GSH/GSSG ratios in the duodenum of control rats at day 91 and control mice at days 8 and 91 ranged between 71-75 (Thompson et al., 2011b; Thompson et al., In press). In contrast, the GSH/GSSG ratio in control rats at day 8 was 56 ± 2 (Thompson et al., In press). This difference might explain the shift in dose-response in rat duodenum at day 8. However, we did not detect any significant changes in redox parameters in the rat duodenum at day 8. What about induction of protective genes? How about a specific comparison of mouse and rat at day 8, with regard to EC50 of tissue-protective genes, such as anti-oxidant, DNA repair, and pro-apoptosis?

Considering duodenal dosimetry at day 91, comparable numbers of differential gene expression changes occurred in both species at similar tissue Cr levels, yet Cr levels in the rat duodenum at 520 mg/L SDD were slightly less than in the mouse at 60 mg/L SDD, and were far lower than in mice at 170 and 520 mg/L SDD. These latter concentrations were clearly associated with intestinal tumors in mice in the NTP 2-yr bioassays (NTP, 2008). As posited by Thompson et al. (2011a), the MOA of Cr(VI) in mice might be applicable to other species if the tissue Cr levels were sufficient to induce subsequent physiological effects. The data herein clearly show that the intestine is highly responsive to Cr(VI) in both species, yet the Cr levels and total number of gene changes in the mouse intestine far exceeded the rat at drinking water concentrations that were associated with intestinal tumors. The sheer number of significant differential gene expression changes (~10% of genome) at carcinogenic concentrations is likely responsible for the eventual induction of intestinal tumors. How could a sheer number of gene expression changes be carcinogenic? This is not an interpretable statement. You could say

something like “the sheer number of differential gene expression changes at carcinogenic concentrations is consistent with massive cellular effects culminating in eventual induction of intestinal tumors”. In this regard, not one intestinal tumor has been identified in two 90-day drinking water studies in rats and mice, or in rats exposed to SDD for 2 years. Moreover, no pre-neoplastic lesions have been identified in intestinal tissues except in mice exposed to SDD for 2 years (NTP, 2008).

Currently, it is unknown whether the aforementioned differences in tissue dosimetry are purely due to pharmacokinetics, or instead reflect adaptive gene expression responses in rats that result in lower tissue dosimetry. How would adaptive gene expression response result in lower tissue dosimetry? Presumably the most likely mechanism would be clearance by macrophages etc. Are these gene expression results that could pertain? Ongoing studies related to the disposition of Cr(VI) in rats and mice should provide additional important information concerning the disposition and MOA of Cr(VI) in rodents. Finally, ongoing toxicogenomic analyses of the rat and mouse oral mucosae will explore the MOA for oral mucosal tumors observed in rats.

TABLES

Table 1. Functional Categorization and Regulation of Select Rat Intestinal Genes Identified as Differentially Expressed in Response to 0.3-520 mg/L SDD after 8 and 91 Days of Exposure. Are the values given the maximum fold change at any dose?

				Duodenum (day 8)		Jejunum (day 8)		Duodenum (day 91)		Jejunum (day 91)	
Functional category	Entrez Gene ID	Gene name	Gene symbol	Fold change	EC_{50} (mg/L)	Fold change	EC_{50} (mg/L)	Fold change	EC_{50} (mg/L)	Fold change	EC_{50} (mg/L)
Nrf2-Mediated Oxidative Stress	78959	Aldo-keto reductase family 1, member A1 (aldehyde reductase)	*Akr1a1*	1.6	ND	1.5	ND	1.2	ND	1.3[a]	ND
	24185	V-akt murine thymoma viral oncogene homolog 1	*Akt1*	1.3	ND	1.6	ND	1.4	ND	1.4[a]	ND
	79255	Activating transcription factor 4 (tax-responsive enhancer element B67)	*Atf4*	1.9	ND	1.5	ND	1.5	ND	1.4	ND
	297406	Chaperonin containing Tcp1, subunit 7 (eta)	*Cct7*	2.6	4.4	2.4	4.5	1.7	ND	1.6	ND
	117030	Endoplasmic reticulum protein 29	*Erp29*	2.4	4.5	2.4	53.5	1.8	ND	1.4[a]	ND
	314322	FBJ osteosarcoma oncogene	*Fos*	-2.0	ND	-2.0	ND	-1.8	ND	NC	ND
	25283	Glutamate-cysteine ligase, catalytic subunit	*Gclc*	1.6	ND	1.9	ND	1.6	ND	1.5	ND
	29326	Glutathione peroxidase 2	*Gpx2*	2.7	ND	2.4	ND	2.3	ND	2.1	ND
	83619	Nuclear factor, erythroid derived 2, like 2	*Nfe2l2*	2.6	4.2	2.4	14.2	2.2	5.9	1.7	ND
	117254	Peroxiredoxin 1	*Prdx1*	2.2	ND	2.0	ND	1.5	ND	1.5	ND
	192277	Stress-induced phosphoprotein 1	*Stip1*	1.9	ND	1.6	ND	1.5	ND	NC	ND
	289623	Ubiquitin-conjugating enzyme E2K (UBC1 homolog, yeast)	*Ube2k*	2.0	ND	1.5	ND	1.5	ND	NC	ND
	291796	Ubiquitin specific peptidase 14	*Usp14*	2.2	4.3	1.5	ND	1.6	ND	NC	ND
	116643	Valosin-containing protein	*Vcp*	1.8	ND	1.5	ND	1.3	ND	1.3	ND
Immune Response	25732	Acid phosphatase 5, tartrate resistant	*Acp5*	2.6	182.9	1.8	ND	2.0	ND	1.6	ND
	25673	Annexin A5	*Anxa5*	2.9	6.5	3	5.2	2.1	4.8	1.8	ND
	298566	Complement component 1, q subcomponent, A chain	*C1qa*	3	4.6	2.5	4.3	2.3	82.7	1.7	ND
	24232	Complement component 3	*C3*	2.3	ND	NC	ND	2.1	17.9	1.5	ND
	288593	Chemokine (C-C motif) ligand 24	*Ccl24*	-1.5[a]	ND	-1.5	ND	-1.6	ND	-1.3[a]	ND
	25406	Cd44 molecule	*Cd44*	3.7	3.9	2.2[a]	ND	2.5	26.6	-1.6[a]	ND
	24772	Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	*Cxcl12*	1.9	ND	2.5[a]	ND	2.0	ND	-1.8	ND
	25441	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	*Fcer1g*	1.5	ND	NC	ND	1.5	ND	NC	ND
	29197	Interleukin 18	*Il18*	1.6	ND	1.3	ND	1.5[a]	ND	1.3[a]	ND
	24494	Interleukin 1 beta	*Il1b*	1.3	ND	1.3	ND	-1.6	ND	-1.6	59.4
	25556	Interleukin 1 receptor-like 1	*Il1rl1*	10.9	6.8	9.5	10.4	5.0	144.6	5.9	68.2
	361749	Interleukin 33	*Il33*	5.9	5.4	5.6	67.7	4.5	31.8	3.9	ND
	60427	KIT ligand	*Kitlg*	2.3	3.9	1.8	ND	1.6	ND	NC	ND
	297604	Lymphotoxin beta receptor (TNFR superfamily, member 3)	*Ltbr*	1.4[a]	ND	1.6	ND	1.6	ND	1.4	ND

Protein Synthesis	64530	CCHC-type zinc finger, nucleic acid binding protein	*Cnbp*	2.1	ND	1.9	ND	1.4	ND	1.5	ND
	288923	Deoxyhypusine synthase	*Dhps*	1.8	ND	1.9	ND	1.6	ND	1.4	ND
	363241	Eukaryotic translation elongation factor 1 beta 2	*Eef1b2*	2.1	4.0	1.7	ND	1.6	ND	NC	ND
	300033	Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)	*Eef1d*	1.9	ND	2.1	72.7	1.5[a]	ND	NC	ND
	303746	Mitochondrial ribosomal protein L12	*Mrpl12*	1.6	ND	1.6	ND	1.4	ND	NC	ND
	299938	Mitochondrial ribosomal protein L13	*Mrpl13*	1.8	ND	1.7	ND	1 4[a]	ND	NC	ND
	81765	Ribosomal protein L13	*Rpl13*	2.0	4.5	2.1	22.1	1.7	ND	NC	ND
	245981	Ribosomal protein L15	*Rpl15*	2.6	56.5	2.4	56.6	1.9	ND	1.4[a]	ND
	81766	Ribosomal protein L18	*Rpl18*	2.1	ND	2.1	37.5	1.7	ND	NC	ND
	290641	Ribosomal protein L18A	*Rpl18a*	2.0	ND	1.7	ND	1.6[a]	ND	NC	ND
	81773	Ribosomal protein S10	*Rps10*	2.3	4.0	2.4	48.0	1.8	ND	NC	ND
	29287	Ribosomal protein S19	*Rps19*	2.3	54.3	2.1	3.9	1.8	ND	NC	ND
	122799	Ribosomal protein s25	*Rps25*	2.3	143.9	2.1	3.3	1.7	ND	NC	ND
	266975	Seryl-tRNA synthetase	*Sars*	2.0	ND	2.1	173.5	1.5	ND	1.4[a]	ND
Cell Cycle/Cell Growth and Proliferation	114512	Apoptosis antagonizing transcription factor	*Aatf*	2.3	ND	2.0	ND	1.8[a]	ND	NC	ND
	25203	Cyclin B1	*Ccnb1*	3.2	ND	3.1	4.0	1.9[a]	ND	NC	ND
	64515	Cell division cycle 20 homolog (S. cerevisiae)	*Cdc20*	2.7	54.9	3.2	5.2	1 7[a]	ND	NC	ND
	366381	Cell division cycle 26	*Cdc26*	1.8	ND	1 3[a]	ND	1.3	ND	NC	ND
	114562	Cell division cycle 37 homolog (S. cerevisiae)	*Cdc37*	1.9	ND	1.6	ND	1.5	ND	1.4	ND
	171456	CDK105 protein	*Cdk105*	2.4	67.0	2.0	4.7	1.6	ND	1.4	ND
	362817	Cyclin dependent kinase 2	*Cdk2*	2.0	ND	1.7[a]	ND	1.7[a]	ND	NC	ND
	688405	CDK2-associated protein 2	*Cdk2ap2*	2.1	4.4	2.1	115.2	1.7	ND	1.4	ND
	94201	Cyclin-dependent kinase 4	*Cdk4*	2.3	ND	2.0	3.7	1 7[a]	ND	NC	ND
	24330	Early growth response 1	*Egr1*	1.7	ND	-1.7	ND	1.6	ND	NC	ND
	24482	Insulin-like growth factor 1	*Igf1*	2.9	3.9	2.9	67.0	2.7	34.2	1.4[a]	ND
	24577	Myelocytomatosis oncogene	*Myc*	1.6	ND	2.0	ND	1.5	ND	-1.8	ND
	25737	Proliferating cell nuclear antigen	*Pcna*	2.1	4.1	2.1	5.0	1.5	ND	1.7	ND
	117270	Trefoil factor 1	*Tff1*	13.6	4.2	11.1	35.3	NC	ND	10.6	25.6
	24842	Tumor protein p53	*Tp53*	1.7	ND	1.3	ND	1.6	ND	NC	ND
DNA Damage and Repair	79116	APEX nuclease (multifunctional DNA repair enzyme) 1	*Apex1*	2.6	ND	2.7	ND	NC	ND	-2.0[a*]	ND
	297093	Chromobox homolog 3 (HP1 gamma homolog, Drosophila)	*Cbx3*	1.8	ND	2.1	ND	NC	ND	-1.5[a*]	ND
	305000	Exonuclease 1	*Exo1*	2.1	ND	2.4	6.4	1.5[a]	ND	-1.7[a*]	ND
	84490	Flap structure-specific endonuclease 1	*Fen1*	2.6	4.2	2.5	107.5	NC	ND	-2.2[a*]	ND
	360704	High-mobility group nucleosome binding domain 1	*Hmgn1*	2.8	4.3	3.0	3.9	1.7[a]	ND	-1.9[a*]	ND
	81709	MutS homolog 2 (E. coli)	*Msh2*	2.9	3.7	3.0	5.0	2.2	5.0	1.4	ND
	81528	8-oxoguanine DNA glycosylase	*Ogg1*	1.8	ND	1.6	ND	1.3[a]	ND	NC	ND

[a]Maximum absolute fold change, P1(*t*) >0.90.
*Single low-dose (0.3 mg/L SDD) significant (P1(*t*)>0.90) suppression.
NC- no change; genes did not meet an absolute fold change of at least 1.2 and P1(*t*) >0.90.
EC_{50} values were calculated using ToxResponse Modeler (Burgoon *et al*., 2008).
ND - not detected, EC_{50} values were not available for genes that did not meet the |fold change|>2.0 and P1(*t*)>0.999 cut-off in the 520 mg/L SDD group, or if the modeling returned a Gaussian best fit model. Many of the genes are ND and so are not convincing. It might be well to distinguish between those that did not meet stringent cut-off, from those with a Gaussian profile. Among the latter, some could be of interest if peaking at 170 mg/L.
Also, each gene was assessed with 3 different pairs of treated/control rats. The data could be more convincing if an SD or SE was given for each fold-change result.

Reviewer 3 Comments

ABSTRACT (250)
Continuous exposure to high concentrations of hexavalent chromium [Cr(VI)] in drinking water results in intestinal tumors in mice but not rats. Concentration-dependent gene expression effects were evaluated in female F344 rat duodenal and jejunal epithelia following 7 and 90 days of exposure to 0.3-520 mg/L $Na_2Cr_2O_7 \bullet H_2O$ (SDD) in drinking water. Whole-genome microarrays identified 3269 and 1815 duodenal, and 4557 and 1534 jejunal differentially expressed genes at 8 and 91 days, respectively, with significant overlaps between the intestinal segments. Functional annotation identified gene expression changes associated with oxidative stress, cell cycle, cell death, and immune response that were consistent with reported changes in redox status and histopathology. Comparative analysis with B6C3F1 mouse data from a similarly designed study identified 2790 rat orthologs differentially expressed in the duodenum compared to 5013 mouse orthologs at day 8. Comparable numbers of orthologs were altered in the jejunum of both species (~3500). Approximately 40% fewer orthologs were altered at day 91. Automated dose-response modeling resulted in similar median EC_{50s} in the rodent duodenal and jejunal mucosae at day 91 (39-55 mg/L SDD). Comparative evaluation of the small number of divergently regulated orthologs, and several oncogenes and tumor suppressors revealed few differences that would likely explain the disparate intestinal tumor outcomes. Comparable numbers of differentially expressed genes were observed at equivalent Cr concentrations (μg Cr/g duodenum); however, at ≥170 mg/L SDD mice accumulated higher Cr levels than rats, resulting in a dramatic ~2-fold increase in the number of differentially expressed genes (~10% of the genome).

Key words: chromium, Cr(VI), microarray, intestine, toxicogenomics, phenotypic anchoring

1. INTRODUCTION (353)

Hexavalent chromium [Cr(VI)] is a recognized lung carcinogen (IARC, 1990). In contrast, oral exposure to Cr(VI) at environmentally relevant exposure levels is thought not to pose a cancer risk due to reduction of Cr(VI) to Cr(III) by bodily fluids and cellular constituents (U.S. EPA, 1991; Proctor et al., 2002). However, chronic exposure to high concentrations of Cr(VI), in the form of sodium dichromate dihydrate (SDD), results in alimentary canal tumors in rodents (NTP, 2008). Oral mucosa tumors were reported in Fisher 344 rats at SDD concentrations ≥172 mg/L, and duodenal tumors in B6C3F1 mice at ≥57 mg/L (NTP, 2008). Notably, these concentrations were associated with significant reductions in water intake in both species that was attributed, in part, to unpalatability (NTP, 2008).

To further elucidate the key events involved in the mode of action (MOA) of intestinal tumor development, a complementary series of dose-dependent comparative drinking water studies were conducted in female F344 rats and B6C3F1 mice (Thompson et al., 2011a; Thompson et al., 2011b; Thompson et al., In press). In both species, intestinal lesions included evidence of oxidative stress, villous cytotoxicity, and crypt hyperplasia. Given the similar phenotypic (or apical) responses in our rat and mice studies, it is critical to compare genomic responses to Cr(VI) in order to assess whether there are species-specific responses that may explain the different tumor outcomes.

Messenger RNA (mRNA) was extracted from rat duodenal and jejunal epithelial scrapings and analyzed using whole-genome Agilent oligonucleotide microarrays following continuous exposure to SDD in drinking water for 7 and 90 days. Differential gene expression was analyzed for over-represented functions and phenotypically anchored to published gross physiology, histopathology, and biochemistry data from complementary studies (Thompson et al., In press). In addition, the rat toxicogenomic data were compared to B6C3F1 mouse gene expression data collected using the same study design, exposure regimen, tissue collection, and gene expression analysis methods (Kopec et al., submitted). Significant qualitative and quantitative differences in the number and types of differentially expressed gene were identified in rat and mouse toxicogenomic dataset comparisons. These results support a proposed MOA involving oxidative stress, cytotoxicity, cell proliferation, and DNA modification.

2. MATERIALS & METHODS

2.1 Animal Husbandry and Study Design

Detailed description of the test substance, animal husbandry, and study design has been previously described (Thompson et al., 2011b; Thompson et al., In press). Briefly, Southern Research Institute (Birmingham, AL) obtained 4-5 week old female Fischer rats (Charles Rivers Laboratories International, Stone Ridge, NY), which were acclimated for a minimum of 7 days and fed *ad libitum* with irradiated NTP-200 wafers (Zeigler Bros, Gardners, PA). Animals were continuously exposed to sodium dichromate dihydrate (SDD) dissolved in tap water at 0, 0.3, 4, 60, 170 and 520 mg/L, corresponding to 0, 0.1, 1.4, 20.9, 59.3, and 181 mg/L Cr(VI) for 7 and 90 days (referred to as day 8 and day 91). Rodents were then euthanized using CO_2 and intestinal sections were collected and flushed with ice-cold phosphate buffered saline. Duodenal and jejunal sections were cut longitudinally and the epithelium was scraped using disposable sterile plastic spatulas (VWR International) into vials containing ~1 ml of TRIzol (Invitrogen, Carlsbad, CA) and snap-frozen in liquid nitrogen. The samples were stored at –80°C and shipped

on dry ice to Michigan State University for gene expression analysis. All procedures were carried out with the approval of the Institutional Animal Care and Use Committee at Southern Research Institute.

2.2 RNA Isolation

Frozen rat intestinal samples were homogenized using a Mixer Mill 300 tissue homogenizer (Retsch, Germany). Total RNA was isolated according to the manufacturer's protocol with an additional acid phenol:chloroform extraction. Isolated RNA was resuspended in RNA storage solution (Ambion Inc., Austin, TX), quantified (A_{260}), and quality was assessed by evaluation of the A_{260}/A_{280} ratio and by visual inspection of 1 μg total RNA on a denaturing gel.

2.3 Microarray Analysis

Dose-dependent changes in gene expression were examined using rat 4x44 K Agilent whole-genome oligonucleotide microarrays (version 1, Agilent Technologies, Inc., Santa Clara, CA). Treated samples were co-hybridized with vehicle controls to individual arrays according to manufacturer's protocol (Agilent Manual: G4140-90050 v. 5.0.1). All hybridizations were performed with three independent biological replicates for treated and control tissues (i.e., RNA samples were not pooled) and independent labeling of each sample (Cy3 and Cy5, including dye swap) for each treatment group at each time point (8 and 91 days). Microarray slides were scanned at 532 nm (Cy3) and 635 nm (Cy5) on a GenePix 4000B scanner (Molecular Devices, Union City, CA). Images were analyzed for feature and background intensities using GenePix Pro 6.0 software (Molecular Devices). All data passed our laboratory quality assurance protocol (Burgoon et al., 2005) and were deposited in TIMS dbZach data management system (Burgoon and Zacharewski, 2007). Microarray data were normalized using a semi-parametric approach (Eckel et al., 2005) and the posterior probabilities were calculated using an empirical Bayes method based on a per gene and dose basis using model-based *t* values (Eckel et al., 2004). Gene expression data were ranked and prioritized using |fold change|>1.5 and statistical P1(*t*) value >0.999 criteria to identify differentially expressed genes.

2.4 Dose-Response Modeling

Dose-response modeling was performed using the ToxResponse Modeler, which identifies the best-fit between five different mathematical models (linear, exponential, Gaussian, sigmoidal, quadratic) (Burgoon and Zacharewski, 2008). The algorithm then identifies the best-fit from the five best in-class models for subsequent EC_{50} calculations. Microarray data sets were first sorted using more stringent criteria (|fold change|>2 and P1(*t*)>0.999 cut-off in the 520 mg/L SDD group), and then modeled to identify genes exhibiting sigmoidal dose-response profiles. EC_{50} values were only determined for genes exhibiting a sigmoidal dose-response curve.

BMDExpress was also used to model individual gene responses at day 91 via benchmark dose (BMD) modeling using a modified version of a previously published procedure (Thomas et al., 2007; Kopec et al., submitted). Hill, power, linear and 2° polynomial models were fit assuming constant variance and the benchmark response (BMR) factor was set to 1.349; the best fitting models for each probe were collated and probes with poor model fits or BMD values outside of experimental dose range were excluded.

2.5 Quantitative Real-Time PCR (QRT-PCR)

QRT-PCR was used to confirm the differential expression of selected genes identified in the microarray analysis (Kopec et al., submitted). Briefly, total RNA was reverse transcribed to cDNA and PCR amplified on an Applied Biosystems PRISM 7500 Sequence Detection. **Supplementary Table S1** provides the names, gene symbols, accession numbers, forward and reverse primer sequences, and amplicon sizes. cDNAs were quantified using a standard curve approach and the copy number of each sample was standardized to 3 housekeeping genes (mouse: *Actb*, *Gapdh*, *Hprt;* rat: *ActB, Hprt, Rpl13a*) to control for differences in RNA loading, quality, and cDNA synthesis (Vandesompele et al., 2002). For graphing purposes (GraphPad Prism 5.0), the relative expression levels were scaled such that the expression level of the time-matched control group was equal to one.

2.6 Functional Gene Annotation and Statistical Analysis

Annotation and functional categorization of differentially regulated genes was performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis et al., 2003) and Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, CA). For cross-species comparisons, HomologeneID was used to identify differentially expressed orthologous genes. Hierarchical clustering (average linkage method; Pearson correlation) was performed using MultiExperiment Viewer (MeV v. 4.6.0) implemented in the TM4 microarray software suite (Saeed et al., 2003). QRT-PCR statistical analyses were performed with SAS 9.2 (SAS Institute, Cary, NC). Unless stated otherwise, all data were analyzed by analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. Differences between treatment groups were considered significant when $p<0.05$.

3. RESULTS

3.1 Effects of SDD on Gene Expression in Rat Small Intestine

3.1.1 Rat Intestinal Differential Gene Expression at Day 8

Rat intestinal gene expression was evaluated using 4x44K Agilent oligonucleotide microarrays containing 17,142 unique annotated genes. Microarray analysis (|fold change|>1.5, P1(*t*)>0.999) identified 3269 duodenal and 4557 jejunal differentially expressed genes, identified at one or more doses, at day 8. The number of differentially expressed duodenal and jejunal genes increased with dose (**Fig. 1A**). Comparative analysis identified 2312 genes that were differentially expressed (|fold change|>1.5, P1(*t*)>0.999) in both intestinal sections following SDD exposure (**Fig. 1B**). Relaxing the filtering criteria to |fold change|>1.2, and P1(*t*)>0.9 to avoid exclusion of genes bordering the stringent cut-offs, increased the number of overlapping genes to 4240 (**Fig. 1C**). This suggests that SDD elicited the differential expression of the same genes in the rat duodenum and jejunum. However, duodenal differential gene expression exhibited greater fold changes (-31.2- to 54.5-fold) compared to the jejunum (-41.7- to 16.6-fold). The top 10 most induced and repressed genes for duodenum and jejunum at each concentration are shown in **Supplementary Tables S2A-B**.

3.1.2 Rat Intestinal Differential Gene Expression at Day 91

Microarray analysis (|fold change|>1.5, P1(*t*)>0.999) identified 1815 duodenal and 1534 jejunal differentially expressed genes at day 91 (**Fig. 1D**). This represents a 56% and 66% decrease in unique differentially expressed genes, respectively, compared to day 8. Approximately 765 genes overlapped between the duodenal and jejunal epithelia, which increased to 2151 genes when the criteria were relaxed to |fold change|>1.2, and P1(*t*)>0.9 (**Fig. 1E-F**). Relative fold induction at the highest dose was comparable for both tissues (up to 19.4-fold), but duodenal epithelium showed greater suppression (-26.5-fold) of gene expression relative to jejunum (-12.4-fold). The top 10 most induced and repressed genes for duodenum and jejunum at each concentration are shown in **Supplementary Tables S3A-B**.

3.2 Dose-Response Modeling of Rat Intestinal Differential Gene Expression

Differentially expressed probes meeting the criteria of |fold change|>2 and P1(*t*)>0.999 at 520 mg/L SDD were selected for dose-response analysis using ToxResponse modeler (Burgoon and Zacharewski, 2008). Of the 1572 probes (943 unique genes) meeting the criteria at day 8, 1269 (744 unique genes) exhibited a sigmoidal-dose response profile with ~67% of all EC_{50s} between 0.3 and 10 mg/L SDD (**Fig. 2A**). Jejunum analysis identified 1934 sigmoidal probes (1021 unique genes) with 65% having EC_{50s} between 10-100 mg/L SDD (**Fig. 2B**). DAVID analysis of the 858 duodenal genes with EC_{50s} <10 mg/L SDD identified over-represented functions associated with protein synthesis, translation and ribosome-related genes. Sustained induction (~2-fold) across 4-520 mg/L SDD was observed for eukaryotic translation elongation and initiation factors (*Eef1b2, Eef1e1*, *Eif2b3, Eif2s1*, and *Eif2s2)* and the ribosomal proteins (*Rpl13, Rps3, Rps5, Rps10*, and *Rps27*) that exhibited EC_{50s} <10 mg/L. At day 91, only 310 duodenal and 167 jejunal genes exhibited sigmoidal dose-response profiles with >72% having EC_{50s} between 10 and 100 mg/L SDD (**Supplementary Fig. 1**). Overall, the day 8 median duodenal EC_{50} value was ~10 times lower compared to the median jejunal EC_{50} value (5 vs. 52 mg/L SDD), while at day 91, the median EC_{50s} were comparable for rat duodenum and jejunum (49 vs. 52 mg/L SDD).

3.3 Phenotypic Anchoring of Rat Differential Gene Expression Responses

DAVID and IPA were used to associate functions with differential gene expression and phenotypically anchor significantly expressed genes to complementary histopathology and biochemical data (**Table 1**). For example, reductions in the GSH/GSSG ratio suggested that the rat intestinal epithelium experienced oxidative stress (Thompson et al., In press). Induction of *Nrf2* (*Nfe2l2*) ~2.6-fold and subsequent induction (up to 2.7-fold) of downstream targets are also suggestive of oxidative stress. For example, ubiquitination and proteasomal degradation of proteins (*Vcp*, *Usp14* and *Ube2k)*, chaperone and stress proteins (*Stip1*, *Cct7*, *Erp29*), and antioxidant proteins (*Atf4*, *Gpx2*, and *Prdx1*) are all suggestive of oxidative stress. Induction of *Nrf2* and its target genes between the duodenum and jejunum was comparable at day 8. Interestingly, the calculated EC_{50} values for *Nrf2* were 4.2 and 14.2 mg/L SDD in the duodenum and jejunum at day 8, respectively. The ToxResponse modeler also calculated EC_{50} values below 5.0 mg/L SDD for *Nrf2*-regulated *Usp14*, *Cct7,* and *Erp29* at day 8. The *Nrf2*-mediated oxidative stress response was also observed at day 91 and select genes were verified by QRT-PCR, including induction of *Nrf2*, *Gclc* and *Gpx2* (**Fig. 3**). Compared to day 8, the induction was more modest, but overall the efficacy (maximum fold change) in the jejunum was slightly higher, in agreement with more oxidative stress and lower GSH/GSSG levels (Thompson et al., In press).

Genes associated with immune response (e.g., *Acp5*, *Anxa2*, *Blnk*, *Ccl24 Cxcl12*, *Kitlg*, *Il1rl1*, *Il33* and *C1qa*) were also differentially expressed (**Table 1**), consistent with the mild to marked histiocytic infiltration at days 8 and 91. Interestingly, *Il1rl1* (5- to 10.9- fold) and *Il33* (4.5- to 5.9-fold) exhibited the greatest fold expression with EC_{50} values of 6.8 and 5.4 mg/L SDD, respectively in the duodenum at day 8. The same genes were also among the highest induced immune response in the mouse, although their maximum fold induction (efficacy) was lower with higher EC_{50s} (Kopec et al., submitted).

Differentially expressed genes involved in cell cycle, growth and proliferation exhibited dose-dependent induction in the rat including *Myc*, *Tp53* and their downstream regulated genes. SDD also induced cyclin-dependent kinases and cell division associated proteins including *Cdc20*, *Cdc26*, *Cdc37*, *Ccnb1*, *Cdk2*, *Cdk4*, and *Cdk105* up to 3.2-fold with EC_{50} values between 3.9 and 115 mg/L SDD. Moreover, insulin-like growth factor 1 (*Igf1*), trefoil factor 1 (*Tff1*; $EC_{50\ \text{Duodenum and Jejunum Day 8}}$ = 4.2 and 35.3 mg/L SDD) and proliferating cell nuclear antigen (*Pcna*; $EC_{50\ \text{Duodenum and Jejunum Day 8}}$ = 4.1 and 5.0 mg/L SDD) were also significantly induced in the rat intestine. The mouse intestinal epithelium showed comparable *Pcna* induction (~2-fold), but *Tff1* expression was considerably greater (52.7-fold) compared to the rat duodenum at day 8 (13.6-fold). At 91 days, *Tff1* induction in rat jejunum was significantly higher (10.6-fold) compared to mouse (1.8-fold), with lower EC_{50} value (25.6 in rat vs. 64.8 mg/L in mouse) and may contribute to the protection against tumor development at a later time point (Buache et al., 2011). Protein synthesis functions including eukaryotic translation elongation and initiation (e.g. *Eef2b2* and *Eif1ay*), as well as ribosomal proteins (e.g. *Rps3*, *Rps5*, and *Rps27*) and seryl-tRNA synthetase (*Sars*) were also over-represented at day 8 and 91, likely in support of cell growth and proliferation (**Table 1**).

The DNA damage and modification genes *Apex1*, *Ogg1*, *Cbx3*, *Exo1*, *Fen1*, *Msh2*, and *Hmgn1* were also induced 1.6- to 3-fold at day 8 in the rat with overall low (<10 mg/L SDD) EC_{50} values. However, unlike the sustained induction of these and other DNA repair genes in mouse duodenum at 8 and 91 days (Kopec et al., submitted), maximum fold change (efficacy) expression was attenuated in the rat duodenum at 91 days (**Table 1**). Notably, no changes in 8-isoprostane or 8-OHdG were observed in the rat duodenum at day 91 (Thompson et al., In press). The DNA damage/repair genes were generally non-responsive in the jejunum with modest suppression ($P1(t)>0.90$) at low doses – despite clear signs of oxidative stress at day 91 (Thompson et al., In press).

Rats exposed to SDD exhibited low serum and bone marrow iron levels at day 91 (Thompson et al., In press), and showed evidence of hypoferremia/anemia (NTP, 2008). This suggests that prolonged exposure to SDD may interfere with iron homeostasis. Although clinical analyses of blood iron were only collected at day 91, several genes involved in dietary iron absorption, transport, and export were repressed in the duodenum at day 8. Specifically, *Cybrd1* ($EC_{50\ \text{Jejunum}}$ = 4.4 mg/L SDD), *Heph* ($EC_{50\ \text{Duodenum}}$ = 51.3 mg/L SDD), *Slc40a1* ($EC_{50\ \text{Duodenum and Jejunum}}$ = 52.7 and 56.1 mg/L SDD), *and Hfe2* were repressed 2-3-fold. By day 91; however, the changes were more modest.

3.4 Comparisons of Gene Expression Changes in Rats and Mice

3.4.1 Orthologous Intestinal Differential Gene Expression at Day 8

Intestinal differential gene expression changes in rats and mice were compared in order to identify similar and divergent responses. Orthologs were identified using HomoloGene (PubMed), which relies on DNA sequence similarity among closely related species to identify

orthologous genes (i.e. same gene in different species) using BLAST nucleotide sequence comparisons (Wheeler et al., 2004; Wheeler et al., 2006). Approximately 13,899 unique orthologs were identified from the 17,142 unique annotated rat and 21,307 unique annotated mouse genes, as represented on their respective 4x44K Agilent whole-genome oligonucleotide microarrays (**Supplementary Fig. 2**). Of the ~13,899 unique orthologs, 2790 and 5013 exhibited differential expression in the rat and mouse duodenum, respectively (**Fig. 4A**). Comparative analysis revealed a significant overlap, increasing from 1986 to 3909 orthologs with reduced stringency (**Fig. 4B-C**). However, more unique orthologs (~6×) were expressed in the mouse duodenal epithelia (1649 orthologs) compared to the rat duodenal epithelia (259 orthologs).

Cross-species comparison of the jejunal gene expression identified comparable numbers of unique differentially expressed orthologs (3782 rat and 3334 mouse) (**Fig. 4D**). Like the duodenum, differentially expressed jejunal orthologs exhibited a significant overlap: increasing from 1576 to 3864 orthologs with reduced stringency (**Fig. 4E-F**). Unlike the duodenum, the number of species-specific differentially expressed genes was comparable (971 vs. 705). Hierarchical clustering of the 1986 duodenal (**Fig. 5A**) and 1576 jejunal (**Fig. 5B**) overlapping orthologs revealed that low (≤14 mg/L SDD) and high doses (≥ 60 mg/L SDD) cluster separately in a species-specific manner. Specifically, the highest 3 doses within each species clustered together, but as a group were closely linked to the other species – suggesting that the responses at ≥ 60 mg/L SDD are similar in both species. This can be seen in heatmaps of the 1986 overlapping genes when only the genes are clustered, while keeping the SDD concentrations in order (**Supplementary Fig. 3**).

3.4.2 Orthologous Intestinal Differential Gene Expression at Day 91

Cross-species analysis of day 91 duodenal responses identified 1504 and 3484 differentially expressed unique orthologs for the rat and mouse, respectively (**Fig. 6A**). Comparative analysis of these differentially expressed orthologs identified that 811 (|fold change|>1.5, P1(*t*)>0.999) and 2536 (|fold change|>1.2, P1(*t*)>0.9) orthologs overlapped between the species. As was observed at day 8, the mouse duodenal epithelia expressed substantially more (~5×) non-overlapping unique orthologs compared to rat following 90 days of SDD exposure (**Fig. 6B-C**). Similar comparisons of jejunal differential gene expression identified 1305 rat and 3620 mouse orthologs of which 729 were commonly expressed in both species (**Fig. 6D**). Using relaxed criteria, the overlap was comparable to the duodenal orthologs at day 91 (2772 jejunal orthologs in **Fig. 6E-F**). Hierarchical clustering of the 811 duodenal overlapping orthologs showed species-specific clustering of low and high dose groups with approximately equal numbers of induced and repressed genes (**Fig. 7A**). The 729 jejunal orthologs clustered in a species-specific manner with overall more down-regulated orthologs in the rat compared to the mouse (**Fig. 7B**). Heatmaps of the 729 overlapping genes where only the genes are clustered, while keeping the SDD concentrations in order, clearly indicate differential responses between the two species in the jejunum (**Supplementary Fig. 3**). Functional annotation of these genes revealed pathways related to eukaryotic translation initiation factors, mammalian target of rapamycin (mTOR) signaling (involved in cell survival and proliferation), and polo-like kinase involvement in mitosis. Genes in these pathways were generally up-regulated in mice at ≥ 60 mg/L SDD, and may relate to the significant increase in observed crypt cell hyperplasia in the mouse jejunum, which was not observed in rats (Thompson et al., 2011b; Thompson et al., In press).

3.4.3 Divergent Orthologous Gene Expression at Day 91

Correlation analysis of the overlapping orthologous microarray gene expression identified 81% of the genes were positively correlated in terms of fold change and significance in duodenum at day 91 (**Fig. 8A**, **Supplementary Fig. 4**). However, examples of divergently expressed orthologs from different functional annotation clusters were also identified (**Table 2**) and verified using QRT-PCR (**Fig. 8B, Supplementary Fig. 5**). Divergently expressed orthologs were associated with the immune response (*Ccl24*, *C3*), ion transport (*Slc25a25*), and growth factor/ cytokine signaling (*Areg*).

3.4.4 Species-Specific Orthologous Gene Expression in Duodenum at Day 91

The overlap in rat and mouse duodenal genes was ~61% (2536/4177), while the number of unique orthologs in the mouse duodenum (1392) was >5 times greater than the number of unique orthologs in the rat duodenum (249) (**Fig. 6C**). Hierarchical clustering of ~~these~~non-overlapping species-specific orthologs (disregarding fold-change and statistical cut-offs) revealed most genes exhibited different dose-response patterns (e.g., differentially expressed in mouse, non-responsive in rat) (**Supplementary Fig. 6**). Functional annotation of the 1392 mouse orthologs identified enrichment of alanine and aspartate metabolism, FAK signaling, and the DNA damage response related to BRCA1. Genes associated with the FAK signaling pathway (involved in cell cycle, proliferation and migration) were mostly down-regulated or unaltered at various concentrations (including *Fak/Ptk2*; data not shown). Functional enrichment analysis of the 249 orthologs in the rat duodenum resulted in enrichment of intrinsic prothrombin activation (mostly down-regulated); however this may be an artifact of the relatively small number of genes (249) in this analysis.

3.4.5 Dose-Response Comparisons of Differential Gene Expression in Rats and Mice

3.4.5.1 EC_{50} Distribution for Over-Represented Pathways

Comparison of ToxResponse Modeler results for rat and mouse (Kopec et al., submitted) datasets is summarized in **Table 3**. Compared to rat, SDD differentially dysregulated more mouse genes that met the filtering cut-offs (±2-fold at 520 mg/L SDD and P1(*t*)>0.999) resulting in a bigger subset of sigmoidal dose-responsive expression profiles for which EC_{50} values could be calculated. Overall, except for greater sensitivity in rat duodenum at day 8, the median EC_{50s} were comparable between the species.

To directly compare the EC_{50} distribution between the species, overlapping orthologous genes that met the filtering criteria in both datasets were compared for over-represented pathways (using DAVID) at each tissue and time point (**Fig. 9**). At day 8, functional categorization of 331 overlapping sigmoidal orthologs in duodenum revealed ~10 times lower median and EC_{50} range for *Translation*/*Protein Biosynthesis*, *Cell Cycle* and *Oxidoreductase* in the rat, while *Inflammatory Response* showed comparable median EC_{50s} between the species (**Fig. 9A**). Comparative analysis of 195 overlapping jejunal sigmoidal orthologs at day 8 showed comparable overall median EC_{50s}, with slightly lower mouse median EC_{50s} for *Ribosome* (23.0 vs. 52.6 mg/L in rat), *Translation* (26.8 vs. 46.0 mg/L in rat), comparable changes in *Oxidoreductase* category, and significantly lower rat EC_{50s} for *Cell Cycle* (4.5 vs. 36.8 mg/L SDD in mouse) and *Nucleoside Binding* (6.1 vs. 52.5 mg/L SDD in mouse) (**Fig. 9B**). At day 91, the number of overlapping orthologs was decreased to 97 in duodenum, where the median EC_{50s} for *Immune Response*, *Carbohydrate Binding*, *Oxidoreductase*, *Apoptosis*, and *Proteolysis* were comparable between the species (**Fig. 9C**). Only 57 orthologs with sigmoidal dose-response

profiles overlapped in jejunum at 91 days with comparable potency for *Drug Metabolism*, *Lipid Transport*, *Cell Death*, *Oxidoreductase*, and *Transport* (**Fig. 9D**).

3.4.5.2 Dose-Response Modeling Using Duodenal Tissue Concentrations

Tissue concentration of chromium were collected from a subgroup of animals in this study at day 91 in order to develop pharmacokinetic models of Cr(VI) disposition (Thompson et al., 2011a). These tissue concentrations represent chromium levels in both the proximal and distal portions of each intestinal segment. The jejunum is respectively ~18 and 67 cm long in mice and rats, and thus the jejunal chromium levels cannot be matched to the scrapings from the proximal 6 cm, which were used for genomic data collection. I don't understand the argument the authors are making here. Why can't concentration and genomic data be matched in the jejunum, but can be in the duodenum. It sounds like a portion of the jej was used for genomic analysis, but then what about the distal section. Why does this not also apply to the duo?

In contrast, full duodenal length was collected in both species and thus the measured chromium concentrations match the genomic analyses. Therefore, automated BMD modeling was performed on the day 91 duodenal gene changes in mice and rats as a function of tissue Cr levels. The BMD and BMDL values were slightly lower in rats compared to mice (**Fig. 10A**). These BMD values (μg Cr/g tissue) each correspond to the 60 mg/L SDD drinking water concentration. In mice this concentration resulted in cytoplasmic vacuolization (Thompson et al., 2011b) and large increases in the total number of significant ±1.5-fold gene expression (Kopec et al., submitted; **Fig. 10B**). In rats, only histiocytic infiltration was observed in the duodenum at 60 mg/L SDD (Thompson et al., In press), along with increases in the total number of significant ±1.5-fold gene changes (**Fig. 1D** and **10B**). Although the slightly lower BMD value in rats might indicate that rats are more sensitive to SDD than mice, it is evident in **Fig. 10B** that the duodenal Cr levels in rats were much lower compared to mice at carcinogenic SDD concentrations (i.e. 170 and 520 mg/L). For example, at 60-520 mg/L SDD the duodenal Cr levels ranged from 18-32 μg Cr/g duodenum in rats, and 34-61 μg/g in mice. At greater than 40 μg Cr/g, the number of differentially expressed mouse genes exceeded 3,000 (~10% of the genome). Comparing the significant orthologous gene changes at duodenal chromium concentrations <40 μg/g tissue indicates considerable overlap using the relaxed filtering criteria (**Fig. 10C**).

4. DISCUSSION (1679)

This is the first study to compare toxicogenomic responses in two species following 90 days of exposure to an environmental toxicant in drinking water. In Kopec et al. (submitted), SDD was shown to elicit gene changes associated with oxidative stress, DNA damage, and cell proliferation, as well as other pathways/functions (e.g. immune response) that are consistent with biochemical and histopathological findings in mice from the same study. Similarly, our data ~~herein provide evidence for~~links ~~-~~gene changes associated with the aforementioned pathways and functions with ~~that are also phenotypically linked to observed~~ biochemical and histopathological findings in rats.

For example, Structurally, this is not a good way to begin a paragraph. I'm not sure what the point of this paragraph is; where are you going? A paragraph should begin with an introductory topic sentence that establishes the theme of the paragrap(See Strunk & White, *The elements of*

style or *The Bedford handbook for writers.)*SDD elicited dose-dependent induction of genes involved in cell cycle/growth/proliferation such as *Myc* and *Pcna*, which is consistent with increases in crypt hyperplasia (Thompson et al., In press). Another example includes ~~Other phenotypes include~~ histiocytic infiltration, which has been observed in the rat small intestine following 1, 13, and 104 weeks of exposure to SDD (NTP, 2007, 2008; Thompson et al., In press). In this regard, several immune-related genes were induced in the rat small intestine (e.g. *Acp5*, *Anxa5*, *C1qa*, *C3*, *Cxcl12*, *Il1rl1*). Inflammation and oxidative stress are intimately linked, and SDD was shown to induce oxidative stress in the small intestine of rats from this study, as evidenced by changes in GSH and GSSG (Thompson et al., In press). These findings suggest that ~~the possibility for~~ Don't double hedge what you are saying. "Suggest possibilities"? Just go with either suggest or possibilities – you don't need both. SDD ~~to~~ induces oxidative DNA damage; however, drinking water exposure studies ~~have~~thus far have not shown an increase in 8-OHdG DNA damage in the intestine (De Flora et al., 2008; Thompson et al., 2011b; Thompson et al., In press). These negative findings may relate to adaptive responses under the longer-term exposure scenarios employed in these studies. Notably, SDD altered the expression of genes involved in DNA repair (**Table 1**) – mostly at day 8 with attenuated expression at day 91.

The health of the intestinal epithelium is influenced by redox balance (Circu and Aw, 2011). Inhibition of *de novo* GSH synthesis with buthionine sulfoximine has been shown to induce intestinal epithelial damage including loss of height of epithelial cell layer, desquamation of microvilli, mitochondrial swelling, and vacuolization in the jejunum (especially the villous tips); effects that were ameliorated with GSH supplementation or recovery time (Martensson et al., 1990). These effects are ~~not unlike~~similar to those reported for the rodent intestine following exposure to Cr(VI) concentrations that result in redox changes. In this regard, expression of oxidative stress response genes was generally comparable between both intestinal segments, including genes involved in Nrf2 signaling (see **Fig. 3**). *Gclc* and *Gpx2* were elevated at ≥ 60 mg/L in the duodenum and jejunum at day 91, with similar inductions observed in mice (Kopec et al., submitted).
Overall, the differential gene changes in rats were attenuated with increased exposure time (**Fig. 1**). Differential gene expression was also diminished in mice between days 8 and 91, albeit not as dramatically (Kopec et al., submitted). In this regard, the mg/kg bodyweight SDD doses in the rat 520 mg/L treatment groups at day 8 and 91 were 80 and 60 mg/kg Presumably this is mg/kg/day, respectively, whereas in mice the doses were 87 and 89 mg/kg (Thompson et al., in press). The decrease in gene expression is also consistent with the observation that the incidences for apoptosis and crypt hyperplasia were slightly lower in rats at day 91 than day 8 – an effect that was not observed in mice (Thompson et al., 2011b; Thompson et al., In press). Either due to inherent pharmacokinetic differences or adaptation to Cr(VI) exposure, the diminished gene responses observed in rats ~~herein~~ are consistent with the lack of intestinal tumors ~~in rats~~ in the 2-year cancer bioassay (NTP, 2008).

Comparative analysis of overlapping orthologous genes identified only a few orthologs that were divergently expressed between the species (**Table 2**; *C3*, *Ccl24*, *Slc25a25*, *Areg*, and *Wfdc1*). *Ccl24* (eotaxin) is involved in eosinophil recruitment and chemotaxis, and was induced 3.6-fold in the mouse but repressed 2.8-fold in the rat (**Fig. 8**). Ccl24 protein expression is dependent on IL-4 stimulation in nasal polyps (Schaefer et al., 2006), and its repression in the rat duodenum may relate to the SDD-elicited (albeit minimal) decreases in IL-4 (Thompson et al., In press). Expression of *C3* is required for complement activation, and was up-regulated 2.7-fold in

rats while down-regulated 5.1-fold in mice (**Fig. 8**). *C3* mRNA was shown to be induced by IL-1α (Gerritsma et al., 1996), the levels of which were elevated in the rat duodenum after 90 days of exposure to SDD (Thompson et al., In press). Likewise, *Areg*, which promotes intestinal This does not appear to be a separate paragraph. Rather, it appears to be a continuation of the same theme from the previous paragraph. epithelial regeneration after radiation injury (Shao and Sheng, 2010), was down-regulated 3.0-fold in rats but induced ~1.9-fold in mice, although the levels did not meet statistical significance (**Fig. 8**). Up-regulation of *Areg* in mice is consistent with the high incidence of crypt cell hyperplasia in the mouse duodenum at day 91 (Thompson et al., 2011b); however, more modest crypt hyperplasia was also observed in the rat duodenum (Thompson et al., In press). *Wfdc1* has roles in cell adhesion, migration, proliferation and immune function (Ressler and Rowley, 2011). *Wfdc1* has anti-proliferative properties and is down-regulated in cancer cells (Madar et al., 2009; Ressler and Rowley, 2011). Up-regulation of *Wfdc1* might partially explain the milder proliferation observed in the rat duodenum.

Divergent regulation was also observed in the calcium-dependent mitochondrial solute carrier *Slc25a25*. This ATP-Mg/P_i carrier, involved in mitochondrial and cytosolic adenine nucleotide (AMP, ADP, ATP) level regulation, was repressed 1.9-fold in mice but induced 2.1-fold in rats (**Fig. 8**). Studies suggest an important role for this carrier in regulating mitochondrial permeability (Hagen et al., 2003), and Cr(VI) could interfere with its function due to structural similarity with phosphate and sulfate ions (Salnikow and Zhitkovich, 2008).

Having identified relatively few divergently regulated genes, we further explored species differences by examining whether known oncogenes and tumor suppressor genes were altered at 520 mg/L SDD in the duodenal mucosae of mice and rats at day 91 (**Table 4**). In both species, the tumor suppressor *Npm1* and oncogene *Pdgfra* were significantly elevated. In mice, the tumor suppressors *Apc*, *Rb1*, and *Smad4* were suppressed, whereas oncogene *Brca1* was elevated. In rats, tumor suppressor *Tp53* was elevated, whereas oncogenes *Egfr* and *Fgfr3* were suppressed. Given the suppression of *Apc* and evidence that APC inactivating mutations ~~that~~ lead to increased β-catenin signaling associate with increased risk of colon cancer (Giles et al., 2003), the IPA canonical pathway for Wnt/β-catenin was examined. Overall, this pathway was not significantly enriched in the duodenum of either species at day 91; however, genes in this pathway were slightly more activated in rats than mice (**Supplementary Fig. S7**).

Biomarkers for intestinal cancer and disease were also identified using IPA at 520 mg/L at day 91. The genes common to both species are shown in **Table 5**. Two of ~~the~~ 16 genes were differentially altered as previously noted in **Table 2** (*Areg* and *Tfam*). Interestingly, increased expression of *Areg* (which encodes activators of the EGF receptor pathway) in wild-type *Kras* colon cancer biopsies correlates with positive response to treatment with the anti-EGFR drug cetuximab (Baker et al., 2011). This suggests that continued EGF signaling potentiates the risk of cancer development; however, EGF signaling did not appear to be enriched *per se* in the mouse duodenum at day 91 (data not shown).

Dose-response modeling of the genomic responses revealed the surprising observation that the median EC_{50} value in the rat duodenum at day 8 was ~10-fold lower than in the jejunum. In fact, the median EC_{50} values in all other tissues and time points in both rats and mice were ~50 mg/L SDD. Average daily doses of SDD in mice and rats were similar at each dose at day 8 (Thompson et al., In press), and thus mg/kg SDD dose does not readily explain the low EC_{50} in the rat duodenum at day 8. Interestingly, however, the GSH/GSSG ratios in the duodenum of control rats at day 91 and control mice at days 8 and 91 ranged between 71-75 (Thompson et al., 2011b; Thompson et al., In press). In contrast, the GSH/GSSG ratio in control rats at day 8 was

56 ± 2 (Thompson et al., In press). This difference might explain the shift in dose-response in rat duodenum at day 8. However, we did not detect any significant changes in redox parameters in the rat duodenum at day 8.

Considering duodenal dosimetry at day 91, comparable numbers of differential gene expression changes occurred in both species at similar tissue Cr levels, yet Cr levels in the rat duodenum at 520 mg/L SDD were slightly less than in the mouse at 60 mg/L SDD, and were far lower than in mice at 170 and 520 mg/L SDD. These latter concentrations were clearly associated with intestinal tumors in mice in the NTP 2-yr bioassays (NTP, 2008). As posited by Thompson et al. (2011a), the MOA of Cr(VI) in mice might be applicable to other species if the tissue Cr levels were sufficient to induce subsequent physiological effects. The data herein clearly show that the intestine is highly responsive to Cr(VI) in both species, yet the Cr levels and total number of gene changes in the mouse intestine far exceeded the rat at drinking water concentrations that were associated with intestinal tumors. The shear number of significant differential gene expression changes (~10% of genome) at carcinogenic concentrations is likely responsible for the eventual induction of intestinal tumors. In this regard, not one intestinal tumor has been identified in two 90-day drinking water studies in rats and mice, or in rats exposed to SDD for 2 years. Moreover, no pre-neoplastic lesions have been identified in intestinal tissues except in mice exposed to SDD for 2 years (NTP, 2008).

Currently, it is unknown whether the aforementioned differences in tissue dosimetry are purely due to pharmacokinetics, or instead reflect adaptive gene expression responses in rats that result in lower tissue dosimetry. Ongoing studies related to the disposition of Cr(VI) in rats and mice should provide additional important information concerning the disposition and MOA of Cr(VI) in rodents. Finally, ongoing toxicogenomic analyses of the rat and mouse oral mucosae will explore the MOA for oral mucosal tumors observed in rats.