COMMENTS OF THE

STYRENE INFORMATION RESEARCH CENTER

ON

NOTICE OF INTENT TO LIST STYRENE

UNDER THE AUTHORITATIVE BODIES LISTING MECHANISM

HEALTH AND SAFETY CODE SECTION 25249.8(B) AND

TITLE 27, CAL. CODE OF REGS., SECTION 25902

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I. Introduction and Summary

On February 27, 2015, the California Environmental Protection Agency's Office of Environmental Health Hazard Assessment (OEHHA) published a notice of intent to list styrene as known to the State to cause cancer under the Safe Drinking Water and Toxic Enforcement Act of 1986.[1] This action was taken under the authoritative bodies listing mechanism.[2]

OEHHA based its notice on a June 2011 listing by the National Toxicology Program (NTP) of styrene as "Reasonably Anticipated to be a Human Carcinogen" in the Report on Carcinogens, Twelfth Edition (RoC). In the final substance profile, NTP explained the basis for listing as follows:

> Styrene is reasonably anticipated to be a human carcinogen based on limited evidence of carcinogenicity from studies in humans, sufficient evidence of carcinogenicity from studies in experimental animals, and supporting data on mechanisms of carcinogenesis.

Proposition 65 requires that there be sufficient evidence in humans, or sufficient evidence in animals supported by additional evidence demonstrating the relevance of the animal data to human carcinogenicity. Authoritative bodies listing proposals require OEHHA to consider new scientific data and data not considered by NTP. A listing may not proceed if it is established that the sufficiency of evidence criteria were not met.

- After briefly comparing the NTP classification and Proposition 65 listing criteria, in section III, we present the data and their regulatory significance in its traditional sequence of human, animal, and other data. This discussion supports three interrelated conclusions. First, new human studies published after the RoC listing demonstrate that the human evidence is inadequate, and not limited. Second, because NTP did not or was unable to consider scientifically valid data, the animal evidence is not sufficient, precluding a listing. Third, additional mode of action data demonstrate that the animal data are not relevant to humans, and styrene does not present a human cancer risk at anticipated exposure levels.

- The NTP listing concluded that the evidence in humans was limited. Therefore, the requirement of sufficient evidence in humans is not met by the listing of styrene in the RoC. In its Public Health Goal document for styrene, OEHHA also concluded that the human data are limited. Based upon recent updates to the major epidemiology studies of

[1] Commonly known as Proposition 65, the Safe Drinking Water and Toxic Enforcement Act of 1986 is codified in Health and Safety Code § 25249.5 et seq.

[2] *See* Health and Safety Code § 25249.8(b) and Title 27, Cal. Code of Regs. § 25902 ("Formally Required to Be Labeled or Identified").

reinforced plastics workers exposed to styrene which refute earlier proposed increases in cancer of the hematopoietic and lymphatic systems, the requirement of sufficient evidence in humans is not satisfied. Therefore, the data from human studies do not support a listing of styrene under Proposition 65.

- The styrene data in animals were described as sufficient in the RoC, based on increased lung tumors in mice by two routes of exposure – inhalation and oral. This conclusion is based on increased lung tumors in male and female mice exposed by inhalation (Cruzan 2001) and increased lung tumors in male mice from gavage administration of styrene (NCI 1979). In the NCI (1979) gavage study, the incidence of lung tumors in the treated mice were within the historical control range of mice tested at the same laboratory at about the same time; the original report concluded there was no more than suggestive evidence of increased tumors. In 2002, the International Agency for Research on Cancer (IARC) concluded the animal data were limited, not sufficient. Uncharacteristic of its established practice, NTP ignored the conclusions of the authors of the NCI 1979 study, ignored IARC's contrary evaluation, ignored previously published studies and departed from accepted science by using historical controls from another laboratory in its analysis of the oral gavage study in mice. NTP's inclusion of external historical controls led to the conclusion that sufficient evidence existed in mice following oral administration. Because NTP's misuse of external historical controls was inappropriate, NTP's sufficient evidence conclusion cannot be considered by OEHHA. Thus, the animal evidence is limited, and not sufficient. No authoritative bodies listing may be made.

- Mode of Action (MOA) research, including three later studies not considered by NTP, indicates that the mouse lung tumors are caused by mouse-specific metabolism by the enzyme CYP2F2, which does not occur in rats or humans. Thus, the animal data are irrelevant to human cancer risk.

Based on new human and mode of action studies, coupled with NTP's failure to consider scientific data and well-established scientific principles in reinterpreting NCI (1979), there is no basis for OEHHA to list styrene under the authoritative bodies listing mechanism because it is clearly established that the sufficiency of evidence criteria were not met.

II. Comparison of Proposition 65 and NTP Listing Criteria

OEHHA's authoritative body notice of intent to list is governed by § 25306 (Chemicals Formally Identified by Authoritative Bodies) in Title 27, Division 4, Chapter 1 of the California Code of Regulations. Key regulatory criteria for listing appear in the definition of causing cancer, and what additional data or factors must be considered by OEHHA in determining whether to list.

The definition for substances that may be listed under the authoritative bodies mechanism is found in § 25306(e) (Cancer Defined), which states:

> (e) For purposes of this section, "as causing cancer" means that either of the following criteria has been satisfied:
>
> > (1) sufficient evidence of carcinogenicity exists from studies in humans. For purposes of this paragraph, "sufficient evidence" means studies in humans indicate that there is a causal relationship between the chemical and cancer.
> >
> > (2) sufficient evidence of carcinogenicity exists from studies in experimental animals. For purposes of this paragraph, "sufficient evidence" means studies in experimental animals indicate that there is an increased incidence of malignant tumors or combined malignant and benign tumors in multiple species or strains, in multiple experiments (*e.g.*, with different routes of administration or using different dose levels), or, to an unusual degree, in a single experiment with regard to high incidence, site or type of tumor, or age at onset.

In addition to the threshold criteria in the definitional section, § 25306(f) describes the additional reasons that OEHHA should not list. Subsection (f) provides:

> (f) The lead agency shall find that a chemical does not satisfy the definition of "as causing cancer" if scientifically valid data which were not considered by the authoritative body clearly establish that the chemical does not satisfy the criteria of subsection (e), paragraph (1) or subsection (e), paragraph (2).

Both of the definitional provisions expressly incorporate the sufficient evidence requirement, which includes the question of whether the listing of styrene in the Report of Carcinogens is consistent with accepted scientific principles. The reasons-not-to-list provision covers two sets of data with respect to the NTP's 2011 listing, including both: (1) any information developed after the June 2011 listing, and (2) any data not considered by NTP during the listing process.

Additionally, when promulgating § 23506 in February 1990, the California Health and Welfare Agency explained that, in evaluating whether an authoritative body had formally identified chemicals as causing cancer or reproductive toxicity, the agency would determine whether the authoritative body had relied on satisfactory human or animal studies. The agency explained that:

> Where there is in fact an insufficient number of positive or studies, but the authoritative body has concluded anyway that the chemical causes cancer, the Agency will be prevented by the regulation from bringing the chemical to the list. The Agency will not completely defer to the authoritative body, and will at least determine that the body relied upon the requisite human or animal studies.

Thus, even if an authoritative body, such as NTP, has decided to identify a chemical as causing cancer or reproductive toxicity, its conclusion must be investigated for the sufficiency – including an adequate number of human or animal studies – before OEHHA decides that a substance may be listed.

III. The NTP Report on Carcinogen Listing Does Not Support Listing under Proposition 65

A. Human Data

NTP's RoC found the human data on the carcinogenicity of styrene to be limited. This does not support listing under Proposition 65.[3] Further, the RoC's evaluation was based on studies that have since been updated. These data were not available to NTP and so, reasonably, they were not considered. These new data demonstrate, however, the inadequacy of the human data that NTP considered on the carcinogenicity of styrene. This includes studies of human data based on a cohort of US reinforced plastics and composite (RPC) workers by Wong et al., (1994); a cohort of Washington state RPC workers by Ruder et al., (2004); and a combination of 8 cohorts of RPC workers in the EU by Kogevinas et al., 1994). Wong and Ruder found no styrene-related increases in cancer. Kogevinas reported no styrene-related increases in cancer based on cumulative exposure or duration of exposure, but an increase in total lymphomas based on average exposure. Part of the Kogevinas cohort was taken from a cohort of Danish workers that may have been exposed to styrene in RPC operations (Kolstad et al, 1994). Workers were divided based on companies where less than 50% of the workers were thought to be involved in RPC operations and companies where more than 50% were thought to be involved. Kolstad reported increased leukemia in the overall cohort among workers hired before 1960 and among those employed for less than 1 year. Workers from companies where more than 50% were thought to have been involved in RPC operations were included in the Kogevinas study.

After the NTP concluded its review, the Wong, Kogevinas, Ruder, and Kolstad studies have been updated. Each update shows a lack of styrene carcinogenicity, vitiating prior assessments to the contrary.

- Collins et al., (2013) updated the Wong cohort of US RPC workers. More than 85% of the cohort has been followed for more than 30 years. There were no increased incidences of leukemias or lymphomas based on cumulative exposure, average exposure, duration of exposure or peak exposures. Increased lung cancer followed an inverted dose-response pattern; *i.e.*, lowest incidence among highest exposed workers, and was attributed to smoking.

[3] *Styrene Information Research Center v. Office of Environmental Health Hazard Assessment*, 210 Cal. App. 4th 1082, 1101 (2012).

- Coggon et al., (2014) have updated their cohort of the Kogevinas study. They found no styrene-related increase in cancer.

- Ruder et al., (2014) presented an update of Washington state RPC cohort in June 2014 at the conference "Challenges for Occupational Epidemiology in the 21st Century." No styrene-related cancers were reported.

- Kolstad et al., (2014) presented update of Danish RPC workers in June 2014 at the conference "Challenges for Occupational Epidemiology in the 21st Century." No styrene-related cancers were reported.

Collectively, these new studies demonstrate the proposition that styrene is *not* carcinogenic in humans. The prior limited evidence characterization of the human data by NTP in the RoC is, then, undermined by this human data that were not available to the NTP and thus could not have been – and was not – considered by NTP. Given the number of human studies, the number of workers followed and the length of follow up, a conclusion that styrene is not a human carcinogen is well-founded.

B. Animal Data

Based on two studies, NTP found that the data from animal studies in different strains of mice were sufficient to support its decision that styrene is reasonably anticipated to be a carcinogen. Adverse findings in both studies are essential to support a finding of sufficient evidence. In concluding that the animal data constituted sufficient evidence of carcinogenicity, however, NTP did not consider scientifically valid data. Because one of the two studies does not demonstrate an increased incidence of tumors in animals, the single remaining study that NTP cited cannot serve as the basis for listing; a single study does not constitute sufficient evidence under § 23506.

One of these studies was conducted by the National Cancer Institute (NCI) in B6C3F1 mice and reported in 1979. Styrene was administered by gavage, that is, orally by a tube inserted into the stomach. NCI concluded that the data provided only "suggestive" evidence of carcinogenicity and under the conditions of the study "no convincing evidence" of carcinogenicity of styrene was obtained. NTP, despite NCI's conclusion that the evidence was not convincing, only suggestive, arrived at a contrary and scientifically inconsistent decision by using control data that were not part of the NCI study.

Animal studies involve treated animals, that is, animals exposed to the chemical being tested. Typically, chemicals will be administered to the treated animals in two or three different amounts, low and high levels with a possible medium level. The laboratory conducting the study will also involve control animals, that is, animals living in identical circumstances with the same housing, food, water, and environmental conditions, except they will not be exposed to the chemical. The effect of the exposure in the treated animals is then compared against control

animals. This comparison is used to determine whether the treated animals show a statistically significant increase in tumors (or other possible effects being studied) at the various levels of exposure than the control animals develop.

To minimize the potential effect of unusual results in the control animals in a single study that would distort the study results, historical controls are used. Historical controls are the same species and strain of animal, used in other studies conducted by the same laboratory under comparable conditions. It is imperative that the historical controls be from the same laboratory to assure that all the controls were subject to the same circumstances. Otherwise, differences in the animals' circumstances could affect the outcomes for the controls.

Using controls from the same laboratory to constitute the historical controls is a generally accepted scientific principle. Ignoring this principle, NTP used the outcome for controls from another laboratory to calculate the number of tumors developed in the controls to compare against the number of tumors in the treated mice.

NTP selected data from 12 studies conducted at a different laboratory and only two from the laboratory where NCI conducted its gavage study. Data for controls from other studies at the laboratory conducting the NCI study were available, but not selected by NTP.

The controls at the laboratory where the NCI study was conducted developed three times more tumors than the controls at the other laboratory. The use of the control data from the other laboratory exaggerated the difference between the "controls" and the treated animals. NTP used this exaggerated difference to rationalize its conclusion that the data were sufficient.

NTP did not consider sound and objective scientific practice in using animals from a laboratory different than that used in NCI (1979a). NTP justified this action as being required to obtain a sufficient number of controls for studies that used corn oil as the vehicle for administration of the test substance. In so doing, NTP did not follow its traditional practice of not engaging in additional analyses of historical controls when it chose NCI as the sole study among the hundreds referenced in the Background Document for which it chose this unusual approach.

Specifically, NTP did not consider existing data regarding (i) the effect of a corn oil vehicle for administration of the test substance, and the (ii) appropriateness of mixing controls from different labs. As to the corn oil issue, NTP's own analysis of the NTP historical control database (Haseman et al., 1985) concluded that use of corn oil vehicle in the NCI study specifically *did not* impact lung tumor incidence in the B6C3F1 mice used in NCI-NTP carcinogenesis bioassays. Importantly, NTP did not reference Haseman et al., (1985), showing that NTP did not consider scientifically valid evidence before coming to its conclusion that there is sufficient evidence of carcinogenicity of styrene in experimental animals.

In stark contrast to its treatment of NCI 1979, NTP departed from its published position that, because of significant inter-laboratory variability in the incidence of background mouse lung tumors, historical control tumor analyses for this endpoint should be restricted to tumor incidences observed within the same testing laboratory (Haseman et al., 1984). In Keenan et al., (2009) the authors, which included representatives of NTP, NIEHS, FDA, and USEPA, recommended consensus principles to guide the use of historical control data from chronic rodent bioassays. Their first consensus principle is that the "current control group is the most relevant comparator for determining treatment-related effects in a study."

In a 2002 review of styrene, the International Agency for Research on Cancer (IARC) considered both Cruzan, et al. (2001) and NCI (1979), in addition to other animal studies. IARC (2002). NTP did not consider that IARC reached a different conclusion, and that IARC found the animal evidence to be limited. IARC's summary of NCI (1979) explicitly referred to historical controls, meaning that IARC was aware of the issue, as the last sentence of the relevant paragraph from the IARC Monograph states (emphasis added:

> Groups of 50 male and 50 female B6C3F1 mice, six weeks of age, received daily administrations of 150 or 300 mg/kg bw styrene (purity, 99.7%) in corn oil by gavage on five days per week, for 78 weeks, and the animals were killed after a further 13 weeks. Control groups of 20 male and 20 female mice received corn oil alone. The incidence of bronchiolo-alveolar carcinomas in males was 0/20, 3/44 and 5/43, while the incidence of adenomas and carcinomas combined was 0/20, 6/44 and 9/43 (p = 0.024) for doses of 0, 150 and 300 mg/kg, respectively. There were no bronchiolo-alveolar carcinomas in female mice. The incidence of bronchiolo-alveolar adenomas in females was 0/20, 1/43 and 3/43, respectively (National Cancer Institute, 1979a). [*The Working Group noted the small number of control animals and that the incidence of both adenomas and carcinomas combined was within the historical control ranges.*]

IARC (2002) demonstrates that NTP's approach was outcome determinative and that, had NTP considered the relevant data and applied the relevant science policies, NCI (1979) would not have provided the animal evidence necessary to add styrene to the Report on Carcinogens. More importantly, OEHHA "will not completely defer to the authoritative body, and will at least determine that the body relied upon the requisite human or animal studies." "Where there is in fact an insufficient number of positive studies, but the authoritative body has concluded anyway that the chemical causes cancer, the Agency will be prevented by the regulation from bringing the chemical to the list."

That is the present situation because NTP did not consider scientifically valid data with regard to the use of historical controls and the difference in the data between the external laboratory controls it used when manipulating the comparison with historical controls. The failure to

consider these data is critical since NTP's novel analysis was necessary to support its conclusion that the animal tumorigenicity data justified the proposed "reasonably anticipated as a human carcinogen" RoC listing. NTP's analysis does not provide the needed support for listing styrene under Proposition 65. Because there is only one animal study upon which OEHHA can rely, no listing of styrene is permitted. [4]

C. Mode of Action

The second animal study upon which NTP relied was conducted by Cruzan et al., in CD-1 mice and reported in 2001. These mice were exposed by inhalation and developed lung tumors. However, the tumors resulted from a mechanism of action that does not occur in humans or even in other animal species, including rats.

- NTP hypothesized that mouse lung tumors developed as a consequence of styrene metabolizing to styrene oxide. Studies, available to but not considered by NTP, demonstrate that styrene oxide does not cause lung tumors in mice or rats.
- The mechanism of action causing lung tumors is unique to mice. Styrene is metabolized in mouse lungs by an enzyme, CYP2F2, causing cytotoxicity, that is, cell damage. The resulting regeneration of cells to repair the damage causes increased cell replication (hyperplasia) and eventually lung tumors.
- Laboratories have bred mice with the CYP2F2 enzyme "knocked out." These knockout mice do not experience cytotoxicity when they are exposed to styrene.
- Rats do not have the CYP2F2 enzyme. The rat counterpart is CYP2F4. Styrene does not cause cytotoxicity or lung tumors in rats. Humans do not have the CYP2F2 enzyme. The human counterpart is CYP2F1, and it is present at a much lower level even than the CYP2F4 enzyme in rats.
- Laboratories have developed mice with the human CYP2F1 enzyme rather than the CYP2F2 mouse enzyme. These "humanized" mice do not experience cytotoxicity when exposed to styrene.

These conclusions are bolstered by four studies not considered by NTP that were published after the Report on Carcinogens. They are:

[4] In developing a new analysis or interpretation of the original study using additional data, NTP also departed from its policy stating that it only relies on peer-reviewed studies in preparing the Background Document. The new analysis should have first been published in a peer review journal. That process would have provided the necessary scientific scrutiny and comparison with consensus practices. NTP has never done this. In any event, NTP's failure to consider Haseman (1984 and 1985) and Keenan (2009) preclude the listing of styrene by OEHHA.

1. Carlson, G.P. 2012. Modification of the metabolism and toxicity of styrene and styrene oxide in hepatic cytochrome P450 reductase deficient mice and CYP2F2 deficient mice. Toxicology 294(2-3):104-108.
2. Cruzan, G; Bus, J; Hotchkiss, J; Harkema, J; Banton, M; Sarang, S. (2012). CYP2F2-generated metabolites, not styrene oxide, are a key event mediating the mode of action of styrene-induced mouse lung tumors. Regul Toxicol Pharmacol 62: 214-220. http://dx.doi.org/10.1016/j.yrtph.2011.10.007
3. Cruzan, G; Bus, J; Hotchkiss, J; Sura, R; Moore, C; Yost, G; Banton, M; Sarang, S. (2013). Studies of styrene, styrene oxide and 4-hydroxystyrene toxicity in CYP2F2 knockout and CYP2F1 humanized mice support lack of human relevance for mouse lung tumors. Regul Toxicol Pharmacol 66: 24-29. http://dx.doi.org/10.1016/j.yrtph.2013.02.008
4. Shen, S; Li, L; Ding, X; Zheng, J. (2014). Metabolism of styrene to styrene oxide and vinylphenols in cytochrome P450 2F2- and P450 2E1-knockout mouse liver and lung microsomes. Chem Res Toxicol 27: 27-33. http://dx.doi.org/10.1021/tx400305w

Further elaboration of these points follows, but because NTP did not consider these data, an authoritative bodies listing may not proceed.

1. Mode of Action of Mouse Lung Tumors Requires CYP2F2 Metabolism

Do the mouse lung tumors provide evidence of human cancer? This can be examined by determining the Mode of Action (MOA) by which styrene induces lung tumors in mice. Facets examined include toxic effects, cells affected, metabolic considerations, and gene mutations. Cruzan et al., (2015) examined mouse lung genomic responses in styrene treated wild-type CYP2F2 knockout and CYP2F1 humanized mice. The evidence supports the conclusion that the mouse-specific lung toxicity and tumorigenicity are not relevant to humans. The metabolism of styrene by mouse lung CYP2F2 to cytotoxic metabolite(s) has been postulated as an essential step for mouse lung toxicity and mouse lung specific tumorigenicity (Cruzan et al., RTP, 2012, 2013). The purpose of this study was to use whole-lung genomic analysis to further investigate potential MOAs of styrene in C57BL/6 wild-type, CYP2F2 knockout (-/-; KO) and CYP2F21 humanized (2F2-KO + 2F1,2A13,2B6-transgenic, TG) male mice. Mice were exposed to 0, 40, or 120 ppm styrene for 6 hours a day, 5 days a week, for either 1 or 4 weeks. Five biological replicates for each treatment group were analyzed for relative gene expression using Affymetrix whole genome HT_MG430_PM Titan arrays. 287 genes were significantly differentially expressed in wild-type mice at both styrene concentrations. Gene ontology enrichment showed a strong dominance of cell cycle regulatory pathways consistent with cell proliferation. No genes were significantly differentially expressed in knockout mice. Only a single gene was significantly differentially expressed at 120 ppm in transgenic mice after 1 week and a different single gene at 40 ppm after 4 weeks. This study supports the conclusion that the MOA of styrene mouse lung toxicity requires CYP2F2 metabolism (but not by human CYP2F1) as a key gateway

event, and also evidences that alternative MOAs mediated by either parent styrene or non-CYP2F2 generated styrene metabolites (*e.g.*, styrene oxide) are unlikely.

2. Toxic Effects Are Limited to Lung Bronchiolar Epithelium in Mice Only

Toxic effects in the mouse lung from styrene have been demonstrated following inhalation, oral, or intraperitoneal (IP) administration. In studies with up to 2 weeks of exposure, increased cells and protein is found in broncho-alveolar lavage fluid (BALF), cell replication is increased (as measured by bromodeoxyuridine (BrdU) incorporation) in lung bronchiolar epithelium but not in alveolar cells, the intensity of staining of the endoplasmic reticulum in bronchiolar cells is diminished, and hyperplasia is observed in terminal bronchioles. Longer exposures indicate continued increased cell replication causing hyperplasia, which eventually extends into alveolar ducts.

No toxic effects are seen in alveolar cells at any time point from styrene exposure, even up to 2 years.

Using whole lung homogenates from C57BL/6 mice exposed to 40 or 120 ppm styrene by inhalation for 1 or 4 weeks (6 hrs/day for 5 days/week), full genomic evaluation indicated that styrene dramatically increases the expression of genes controlling cell cycle and replication.

In rat lungs, no cells are affected even at inhalation concentrations that are 8-fold higher – up to 1,000 ppm styrene 6 hrs/day 5 days/week – for 2 years. (Cruzan et al., 1998) In mice, Club (formerly called Clara) cells are affected. Increased BrdU labeling occurs only in bronchiolar tissue. Hyperplasia is found only in terminal bronchiolar tissues. Using enriched cell fractions from mouse lungs, Carlson reported that metabolism of styrene occurred only in Club (Clara) cells (Hynes et al., 1999).

Some have suggested that styrene is metabolized in Club cells and the metabolites cause events in nearby alveolar cells that lead to tumors. There is no evidence of toxicity or mutagenicity in alveolar cells in mouse lung. In fact using RNA from whole lung of C57BL/6 mice exposed to styrene at 40 or 160 ppm 5 days/week for 1 or 4 weeks, there was no indication of mutagenic events in the lung.

3. While Styrene Has Some Genotoxic Potential *In Vitro*, There Is No Convincing Evidence That Styrene Is Genotoxic *In Vivo*.

The overall picture presented by the available *in vitro* assay results available is that at least in some test systems (including tests from *in vitro* chromosome aberration studies in mammalian cells), styrene has some genotoxic potential *in vitro*. However, based on standard *in vivo* regulatory tests, arguably the more relevant testing environment, there is no convincing evidence that styrene is mutagenic/clastogenic.

In vitro mutagenicity assays (Ames) of styrene are negative (IARC, 1994). Micronucleus and chromosomal aberration assays in rats and mice are negative (IARC, 1994, 2002). Since styrene causes increased tumors only in mouse lung, assessment of genotoxicity in mouse lung is most relevant. Limited assays of genotoxic potential have been conducted in the lungs of mice exposed to styrene. There were no increases in chromosomal aberrations in the lungs of B6C3F1 mice exposed to 125, 250 or 500 ppm styrene for 2 weeks (Kligerman et al. 1993). Using A/J mice, a strain very susceptible to lung tumor formation, in an initiation/promotion assay, styrene administered for 7 weeks by IP injection did not initiate lung tumor formation (Brunnemann et al., 1992).

Other data are often cited to support a genotoxic MOA for styrene. *In vitro* mutagenicity assays of styrene-7,8-oxide (SO) are generally positive when epoxide hydrolase is inhibited (IARC, 1994). *In vivo* assays of micronucleus formation and chromosomal aberrations following exposure to SO were of mixed results, about half positive and half negative. However as described later, lung toxicity and tumor formation in mice from styrene exposure does not appear to be related to SO.

Studies in workers exposed to styrene and *in vitro* studies using human cells provide conflicting results. Most *in vitro* studies of micronucleus formation and chromosomal aberrations using human lymphocytes are positive (IARC, 1994, 2002). (This is in contrast to the *in vivo* studies in rats and mice, which are uniformly negative.) About 30 studies of workers in industries where workers are exposed to styrene have exhibited micronucleus and/or chromosomal aberrations. There does not seem to be any correlation between styrene exposure and micronucleus formation. However, about half of the chromosomal aberration studies are positive.

The one assay that seems to be consistently positive across *in vitro*, animal, and human studies is sister chromatid exchange (SCE). However, the Organisation for Economic Cooperation and Development (OECD 2014) recently removed SCE from the list of acceptable assays for genotoxicity because there is no functional connection between SCE and tumor formation.

4. DNA Adducts from Styrene Are Not Sufficient to Cause Tumors

Exposure to styrene by mice, rats and humans results in N-7-SO DNA adducts, as well as other adducts. Some authors have taken the findings of DNA adducts in mouse lung as an indication of a mutagenic MOA for styrene-induced mouse lung tumors and SO as the tumorigenic metabolite. Exposure of mice to 40 ppm styrene vapors for 2 years results in increased lung tumors; short-term exposures to this level of styrene in mice results in DNA adducts in lung. The lung DNA adducts are not an indication of a mutagenic MOA in lung since there are a greater number of adducts/gram tissue in liver, but no increase in liver tumors. *I.e.*, DNA adducts from styrene are not sufficient to cause tumors. Furthermore, rats exposed to 500 ppm styrene vapor develop more

DNA adducts in lung than do mice exposed to 40 ppm styrene, yet rats do not develop lung tumors. *I.e.*, DNA adducts from styrene are not sufficient to cause tumors.

The role of DNA adducts, in general, for causing mutations that lead to tumors as a linear, non-threshold MOA is questioned by recent research on aflatoxin. Johnson et al., (2014) recently published a set of experiments on the MOA of aflatoxin-induced liver cancer and chemoprevention. Aflatoxin B1 (AFB1) forms N-7-guanine adducts, produces GST-P-positive foci and eventually hepatocellular carcinoma. A specific gene signature is produced by AFB1 exposure. Administration of 200 μg/kg AFB1 via daily gavage for 4 weeks to F344 rats resulted in 100 pmol N-7AF-Guanine adducts/mg creatinine. Lifetime exposure to this dose resulted in a 96% incidence of hepatocellular carcinoma. Simultaneous exposure to 1-[2-cyano-3-,12-dioxooleana-1,9(11)-dien-28-oyl]imidazole (CDDO-Im) reduced the DNA adducts by 66% (*i.e.*, DNA adducts in the AFB1/CDDO-Im treated rats was 34 pmol N-7AF-Guanine adducts/mg creatinine). The genomic signature was also altered. The incidence of hepatocellular carcinoma was 0.

This study demonstrates that even for a genotoxic carcinogen such as aflatoxin, there is a threshold of DNA damage before cancer occurs.

A commentary by Drs. Olden and Vulimiri (2014) noted:

> They showed that AFB1 is a classic genotoxic substance in that it binds covalently to DNA and induces mutations. In fact, DNA adduct formation exhibits a characteristic linear dose–response curve over a wide range. But, further analysis demonstrated a threshold mode of action, with respect to internal dose of active metabolite and hepatocarcinogenesis. That is, there was substantial adduct formation and DNA damage without having any affect on development of hepatocellular carcinoma.

Although a genotoxicity MOA is the NTP's default assumption and limited genotoxicity data are found for styrene, there is no evidence that styrene induces mouse lung tumors through a genotoxic MOA, and these are the only tumors found in animals.

5. Metabolic Activation is Essential to Mouse Lung Toxicity

Metabolism by CYP2F2 is absolutely essential for toxic effects in mouse lung from styrene exposure. Although CYP2E1 readily metabolizes styrene to styrene-7,8-oxide (SO), elimination of CYP2E1 (CYP2E1-null mice) had no impact on styrene-induced lung toxicity. Styrene lung toxicity is reduced in CYP2E1-null mice.

Preliminary studies demonstrated that inhibition of CYP2F2 by 5-phenyl-1-pentyne reduced the lung toxicity of styrene (Green et al., 2001). More recent studies using CYP2F2-null mice demonstrated a complete loss of lung toxicity from styrene in the absence of CYP2F2

metabolism (Cruzan et al, 2012 and Carlson 2012). These studies also indicate that SO is not the toxic agent from styrene, because there was no toxicity from SO in the absence of further metabolism by CYP2F2 (Cruzan et al., 2012).

Genomics analysis demonstrated altered expression of genes related to control of cell cycle in the lungs of mice exposed to styrene. There was no altered expression in the lungs of CYP2F2-null mice (Cruzan et al., 2015).[5]

The fourth study that NTP did not have an opportunity to consider is Shen et al., (2014), which demonstrated that toxic naphthalene metabolites are generated in the lung by CYP2F2 and not CYP2A5, while in the nose it is CYP2A5, not CYP2F2, that generates toxic naphthalene metabolites.

MOA Conclusions

Metabolism of styrene or styrene oxide in the mouse lung by CYP2F2 is required for toxicity. In the absence of CYP2F2, there is no increase in cells, lactate dehydrogenase (LDH), or protein in broncho-alveolar lavage fluid (BALF), no increase in bromodeoxyuridine (BrdU) incorporation, and no differential expression of cell cycle genes in lung. Based on the lung tumor initiation assay, lung chromosomal aberration assay and lung genomic analysis, there is no indication of a genotoxic MOA. Using mice with the human CYP2F1 gene in place of the normal mouse CYP2F2 indicates that humans are incapable of producing sufficient metabolites to cause the lung effects seen in mice.

Mode of Action (MOA) research evidences that the mouse lung tumors are caused by mouse-specific metabolism by CYP2F2, which does not occur in humans. Thus, the animal data in Cruzan et al., (2001) are irrelevant to human cancer risk. Thus, the animal data are less than sufficient and does not support the listing of styrene under Proposition 65.

D. Inadequate Numbers of Human or Animal Studies Preclude Listing

Even if Cruzan et al., (2001) did support listing, it would be the only animal study in support of listing because the NCI (1979) study does not support listing as it did not show sufficient evidence of tumorigenicity in the study mice. As the California Health and Welfare Agency explained in promulgating § 23506, in determining whether to list a chemical, "[t]he Agency will look to determine whether the authoritative body relied upon animal or human data in an amount sufficient to satisfy the criteria." A single study does not satisfy this criterion. As such, there are insufficient animal data to support the listing of styrene under Proposition 65.

[5] Bolstering this conclusion is a SIRC-sponsored 4-week styrene inhalation lung toxicity study in KO, WT, and TG mice that supported the conclusion that CYP2F2 metabolism was a key event in the production of lung toxicity in mice following short-term exposure of inhaled styrene vapor. The final report from this study is provided as an attachment to these comments.

IV. Conclusion

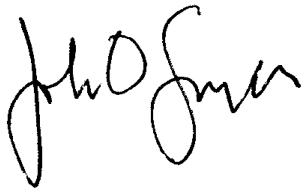
As detailed in these comments:

- Based on data that NTP did not consider, the human data do not support an association between exposure to styrene and human cancer, which precludes a listing.
- Based on data and scientific practices that NTP did not consider, the animal data are limited, not sufficient, which precludes a listing.
- Recent mode of action studies that NTP did not consider demonstrate that the mouse lung tumors identified in Cruzan et al., (2001) are not relevant to human cancer risk, which precludes a listing.

We recognize that OEHHA was obligated to consider an authoritative bodies listing for styrene based on a settlement agreement involving a number of chemicals. Based on the scientific record confronting OEHHA today, there is no basis for listing styrene under the authoritative bodies mechanism. Standing alone, the new human and mode of action studies that NTP did not consider preclude OEHHA from proceeding with an authoritative bodies listing. The impropriety of listing is further established by the absence of sufficient evidence of carcinogenicity in animal studies.

For these reasons, the NTP listing did not satisfy the sufficiency of evidence criteria to support an authoritative bodies listing and OEHHA should withdraw its Notice of Intent to List styrene.

Respectfully submitted,



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Protocol Number: DEDSTY131-1
(QPS Code: 409)

Sponsor:

Styrene Information and Research Center
910 17th Street NW, #500-B
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STUDY PROTOCOL: 4-WEEK STYRENE INHALATION LUNG TOXICITY STUDY IN MICE

FINAL REPORT

AUTHOR

Darol E. Dodd, PhD, DABT

STUDY INITIATION DATE

October 1, 2013

STUDY COMPLETION DATE

February 26, 2014

Approved by:

Study Director: Darol E. Dodd, PhD, DABT

Darol E Dodd 2/26/14
Signature/Date

Sponsor Representative: George Cruzan, PhD, DABT

George Cruzan 02/24/2014
Signature/Date

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A. Executive Summary

Male C57Bl/6 mice (wild-type; WT), male CYP2F2(-/-) mice (knock-out; KO), and male CYP2F2(-/-) 2F1,2A13,2B6-tg mice (transgenic; TG), 6-9 weeks of age, were exposed by whole-body inhalation to one of three styrene concentrations; 0 ppm (air-only control), 40 ppm, or 120 ppm. Exposure duration was 6 hours/day, 5 days/week, for either 1- or 4-weeks. Group size for gene expression profiling was eight mice per strain per concentration per time point. For cell proliferation evaluation, WT mice (6/group) were implanted with BrdU minipumps and exposed to 0 ppm or 120 ppm styrene for one week. In-life evaluations included clinical observations, body weights, and food consumption. At necropsy, lungs were collected for gene expression analysis, cell proliferation analysis, and histopathology evaluation.

For the 1-week duration, the analytical means (± standard deviations) for the three styrene concentration groups were 118.9 (±1.2), 40.9 (±2.0), and 0.0 (±0.0) ppm for the target exposure concentrations of 120, 40, and 0 ppm, respectively. For the 4-week duration, the analytical means (± standard deviations) for the three styrene concentration groups were 119.1 (±2.5), 39.6 (±1.6), and 0.0 (±0.0) ppm. Exposure temperature and relative humidity means were 72 °F and 49-52%, respectively, for all exposure groups throughout the study.

In-Life Observations. There were no clinical signs of toxicity in all study animals throughout the 4-week study. There were no statistically significant differences in mean body weights between control and styrene-exposed animals throughout the study for all three strains. Decreases in mean body weight gain were observed in the 40 ppm and 120 ppm KO mice during study week 4, but these decreases did not result in differences in mean absolute body weights. The only statistically significant decreases in mean food consumption (relative to body weight) were in the 40 ppm and 120 ppm WT mice during study week 1. At necropsy, there were no gross lesions observed in all study animals.

Cell Proliferation and Histopathology. In the WT mice, inhalation of 120 ppm styrene for 1 week resulted in an approximate 7-fold increase compared to the control group in the mean BrdU labeling index of epithelial cells lining the lung terminal bronchioles. WT mice receiving 40 or 120 ppm styrene for either 1- or 4-weeks had microscopic lesions in the lungs consisting of decreased eosinophilia of terminal bronchiolar epithelium; degeneration, necrosis, exfoliation, and/or attenuation of individual terminal bronchiolar epithelial cells (most likely Clara cells); and/or hyperplasia of the terminal bronchiolar epithelium that sometimes extended into the distally connected alveolar duct. No styrene-induced microscopic lesions were observed in the KO and TG mice after either 1- or 4-weeks of exposure. Overall, the microscopic findings in the WT mice did not appear to be concentration-dependent except for the degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium at the 4-week time point, which appeared slightly more severe in mice receiving 120 ppm styrene compared to control mice. The mean severity scores for most microscopic lesions in the WT mice were greater at the 1-week time point compared with WT mice from the 4-week time point, except for the mean severity score for hyperplasia of the bronchiolar epithelium which was greater in the WT mice from the 4-week time point compared with WT mice from the 1-week time point.

Gene Expression. There were almost no significantly differentially expressed (DE) genes in either the KO or the TG mice at any time point for any styrene concentration. A single significant gene was detected for TG mice at 120 ppm following 1 week exposure (Npas2), and a single gene at 40 ppm following 4 weeks exposure (Dcaf4). Significant differential gene expression was observed in WT mice at 1 week with 378 DE genes in the 40 ppm exposure group (relative to WT controls), and 516 DE genes in the 120 ppm group. Of these, 287 genes were significantly DE at both styrene concentrations. However, by 4 weeks, there were fewer DE genes detected: 19 at 40 ppm and 69 at 120 ppm with 17 of these significant at both concentrations. A total of ten genes were significantly DE at all concentrations and both time points. Gene ontology enrichment of the 1 week WT DE genes showed a strong dominance of cell cycle regulatory pathways. Pathways for cholesterol synthesis were also enriched at the 40 ppm, 1 week exposures using either Metacore or GO ontologies. The greatest significant enrichment was observed with the 287 genes that were DE at both 40 ppm and 120 ppm at 1 week. At 4 weeks, significant enrichment was only observed for a single pathway due to the limited numbers of DE genes detected, and this pathway was also significantly enriched at 1 week.

Conclusion. Cell proliferation and histopathology results of this study support the implication of CYP2F2 metabolism as a key event in the production of lung toxicity in mice following short-term exposure of inhaled styrene vapor. Results of gene expression analysis showed marked differences in the number of significantly DE genes between styrene-exposed WT mice and styrene-exposed KO or TG mice void of CYP2F2 enzyme. A temporal relationship of significantly DE genes was observed in the styrene-exposed WT mice, and cell cycle regulatory pathways were predominantly enriched.

B. Test Facility and Key Personnel

The Hamner Institutes for Health Sciences

Study Director/Test Site Manager:	Darol E. Dodd, PhD
Co-Investigator (genomics):	Melvin E. Andersen, PhD
Inhalation Exposure:	Kay C. Roberts, AS
Animal Care and Surgery Oversight:	Carol A. Bobbitt, AAS, VMT, LATg
Animal Necropsy and Tissue Histology:	Nigel A. Edgerton, BS
Gene Expression Analysis:	Linda J. Pluta, BS
Gene Expression Statistics/ Bioinformatics:	Michael B. Black, PhD
Health and Safety:	Richard Cravener, Jr., MS, CIH

Mispro Biotech Services Corporation

Animal Care Supervisor/ Test Site Manager:	Amanda Pacio, LAT
Attending Veterinarian (contractor):	Coralie Z. Cannon, DVM, DACLAM

QPS, LLC

Quality Assurance:	Brenda Bourn, BS, MBA

Charles River Pathology Associates

Histopathology and Cell Proliferation/ Principal Investigator:	James T Raymond DVM, DACVP

Funding Source (Sponsor):
Styrene Information and Research Center

Representative:	George Cruzan, PhD

C. Statement of Compliance

The study was conducted in accordance with U.S. Environmental Protection Agency's Good Laboratory Practice Standards (GLPs; 40 CFR, Part 792) from animal receipt through euthanasia of the animals. Lung histology, histopathology, and cell proliferation adhered to GLPs. Gene expression analysis was conducted non-GLP and to the highest standards of research practice, but was not audited by a Quality Assurance Unit (QAU). All applicable Standard Operating Procedures (SOPs) of The Hamner Institutes (The Hamner), Mispro Biotech Services Corporation (Mispro), or QPS, LLC (QPS) were followed and a list of SOPs used was placed in the study file. Study specific SOPs were part of the study protocol. GLP-compliant data were quality control reviewed.

The study protocol was reviewed by the QPS QAU before final approval by the Sponsor. A quality assurance inspection was conducted per an audit plan to assure the quality and integrity of the study results. An audit of the final report and associated study records was conducted to verify the consistency between the reported study results and the raw data. A Quality Assurance Statement of inspections and audits follows.

Quality Assurance Statement

This study has been audited to assure the integrity of the study in accordance with Good Laboratory Practices. Based on the audit findings, the study protocol and standard operating procedures have been made available to study personnel and were followed. A review of the final report confirms that the report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study. The following table summarizes the inspections and audits performed and reported throughout the course of this study per the study audit plan defined in protocol DEDSTY131-1.

Audit Description (QPS Audit Tracking No.)	Auditor(s)/Date(s)	Date(s) Reported to SD/TFM
Draft Protocol (409-DEDSTY131-1-01)	B. Bourn, 10SEP2013	11SEP2013
Protocol Review	B. Bourn, 01OCT2013	01OCT2013
Draft Protocol Amendment 01 (409-DEDSTY131-1-02)	B. Bourn, 10OCT2013	10OCT2013
Amendment 01 Review	B. Bourn, 16OCT2013	16OCT2013
In-Life Inspection (409-DEDSTY131-1-03)	B. Bourn, 28-29OCT2013	30OCT2013
In-Life and Analysis Related Inspection (409-DEDSTY131-1-04)	B. Bourn, 2NOV2013, 5-7NOV2013	12NOV2013
Process Audit – Animal Husbandry (409-DEDSTY131-1-05)	B. Bourn, 13NOV2013	**13NOV2013**
Draft Final Report and Study Records Audit (409-DEDSTY131-1-06)	B. Bourn, 28-29JAN2014, 4-6FEB2014	11FEB2014

The undersigned has assured that the final report accurately reflects the source data.

Brenda Bourn
QPS Quality Assurance

/ 26 Feb 2014
Date

D. Introduction - Background

Styrene caused increased lung tumors in mice, but not in rats. The lung tumors were found mostly at the conclusion of a life-time (104 weeks for males) exposure study and most were benign. Styrene was largely negative in genotoxicity assays. Styrene metabolism by CYP2F2 produced a different metabolite pattern in mouse lung than in liver or in rats or humans. Short-term exposure of styrene produced lung toxicity including degeneration and exfoliation of Clara cells, regenerative cell replication, and reduced production of Clara cell secretory protein (CCSP, CC10, or CC16). Longer term exposure resulted in cellular crowding, hyperplasia and hyperplasia extending into alveolar ducts. In the styrene bioassay in mice (Cruzan et al., 2001), lung pathology at 12 months included decreased staining of Clara cells and hyperplasia of terminal bronchioles with extension into alveolar ducts. These observations were present in nearly all mice examined that were exposed to 80 or 160 ppm. Decreased staining was seen in some mice at 20 and 40 ppm, but hyperplasia was only occasionally found in mice in these two groups.

Recent studies have shown elimination of the Clara cell toxicity from exposure to styrene in mice genetically engineered to not produce CYP2F2 (CYP2F2(-/-) mice) [called CYP2F2-knockout or CYP2F2KO]. Furthermore, in lung Clara cells of wild type (WT) mice, there is benzene ring oxidation of styrene; this ring-oxidation of styrene in lung is completely eliminated in CYP2F2(-/-) mice. Levels of other CYPs are not changed in these mice, so major changes in styrene metabolites are not expected in these mice, except elimination of metabolites produced by CYP2F2.

In humanized mice (2F2KO/2F1tg) styrene did not induce lung toxicity, indicating humans are not likely to develop lung toxicity from styrene exposure. CYP2F1 is present in these mice at about 2.5% of the amount of CYP2F2 in the lungs of WT mice. This level is at least as great as the level of CYP2F1 in human lungs.

To date, styrene experiments have focused on short-term exposures and showed a consistent pattern implicating CYP2F2 metabolism as the key event in the production of mouse lung cytotoxicity and tumors.

E. Study Objective

The objective of this study was to evaluate the mode-of-action for styrene-induced lung toxicity by comparing gene expression and cell proliferation effects following inhalation exposure (0, 40, 120 ppm) in three male mouse strains with different genotypes. Specific enzymes of styrene metabolism aligned with specific genotypes may be identified as important contributors to producing lung toxicity. Understanding which metabolic pathways are involved with lung toxicity in mice following styrene exposure may provide a prediction of potential lung toxicity in humans exposed to styrene.

F. Study Schedule

October 16, 2013	Animal arrival
October 27, 2013	BrdU implant surgery
October 28, 2013	1st inhalation exposure
November 2, 2013	1-week necropsy
November 22, 2013	Final inhalation exposure
November 23, 2013	4-week necropsy

G. Critical Materials

Test Substance: Styrene monomer, PO-11 Grade bulk (CAS Number 100-42-5) was received from Lyondell Chemical Company (LCC), a division of LyondellBasell, Houston, TX, on August 13, 2013. The styrene test substance (Lot Number 130807.6008) was shipped in sixteen 1-liter containers and appeared as a colorless liquid. A material safety and data sheet (MSDS) was provided by LCC and archived with the study records. A Certificate of Analysis (COA) was provided by LCC. The COA (see Attachment 1 in Appendix 2) indicated the test substance to be 99.95% (weight) styrene monomer. An inhibitor of styrene polymer formation, t-butyl catechol (13 ppm), had been added to the test substance by LCC. Per storage

instructions, the styrene test substance was maintained under climate controlled ambient conditions at a slightly cool temperature (e.g., approximately 68°F). The test substance was stored in Hamner Room 200U during the inhalation study. A reserve sample of the test substance was shipped by LCC to EPL Archives (Sterling, VA) for archiving. EPL Archives received the sample on August 13, 2013 (Sponsor communication).

The Sponsor communicated that the test substance would remain stable, except for potential trace amounts of polymer formation and would be suitable for use for the inhalation study. All further documentations of the test substance, such as method of synthesis and other Good Laboratory Practice (GLP) compliance requirements were the responsibility of the Sponsor.

H. Methods

Additional details of materials and methods are described in the study protocol (Appendix 1). One protocol amendment (Amendment 01) was recorded pertaining to the number of animals purchased and a change in diet supplier. A few deviations were documented during the study regarding embedding the duodenum in paraffin blocks (Deviation 1), disruption of electrical power on Study Day 19 (Deviations 2 and 3), and mean body weights for group assignments (Deviation 4), animal room conditions (Deviation 5), and body weights prior to euthanasia (Deviation 6). The Study Director indicated there was no impact on the integrity of the study due to protocol amendments or deviations.

<u>Test System</u>

Species:	Mouse, *Mus musculus*
Strain/Sex:	Male C57Bl/6 (wild-type; WT) Male CYP2F2(-/-) (knock-out; KO) Male CYP2F2(-/-) 2F1,2A13,2B6-tg (transgenic; TG)
Source:	Taconic Farms (Germantown, NY)
Number Purchased:	63 WT, 48 KO, and 51 TG
Acclimation:	12 days
Age at arrival:	4-7 weeks of age
Age at administration:	6-9 weeks of age
Weight at initiation of exposure:	Approximately 20-30 grams.
Identification:	Each animal was uniquely identified by an ear tag at the time of randomization and group assignment. Cages holding the animals had cage cards and cage labels associated with assigned animal numbers.
Justification:	There were no *in vitro* or other alternative models to specifically study styrene vapor-induced lung effects, such as gene expression and cell proliferation, between these three mouse genotypes. The mouse was the most appropriate animal species because the mouse had been shown to be responsive to styrene inhalation toxicity and had established transgenic lines (via breeders) for scientific investigation. Previous mouse toxicity studies with styrene indicated no significant sex difference in lung cytotoxicity, but male mice had a higher lung tumor incidence then female mice in a life-time inhalation exposure bioassay, thus male mice were selected for study.

Animal Allocation and Group Assignment

The animals were stratified randomly and assigned by body weight to a styrene exposure group. Animals were clinically healthy at the time of group assignment and individual animal body weights were within 25% of the mean group body weights (Deviation 4, Appendix 1). Prior to the start of the study (during housing acclimation period), one KO mouse (#148) was found dead with no obvious cause of death. Additional KO animals were not available to replace this animal on the study (Amendment 01, Appendix 1). Unused animals were humanely euthanized and death confirmed by exsanguinations.

Group	Endpoint	Number and Strain	Animal Identification	Time Point
Air only - 0 ppm (control)	Genomics	8 WT 8 KO 8 TG	1-8 101-108 201-208	1-week
Air only - 0 ppm (control)	Cell proliferation	6 WT	49-54	1-week
Styrene – 40 ppm	Genomics	8 WT 8 KO 8 TG	9-16 109-116 209-216	1-week
Styrene – 120 ppm	Genomics	8 WT 8 KO 8 TG	17-24 117-124 217-224	1-week
Styrene - 120 ppm	Cell proliferation	6 WT	55-60	1-week
Air only - 0 ppm (control)	Genomics	8 WT 8 KO 8 TG	25-32 125-132 225-232	4-week
Styrene – 40 ppm	Genomics	8 WT 8 KO 8 TG	33-40 133-140 233-240	4-week
Styrene – 120 ppm	Genomics	8 WT 7 KO 8 TG	41-48 141-147 241-248	4-week

Animal Husbandry

Current acceptable practices of good animal husbandry were followed per National Research Council's *Guide for the Care and Use of Laboratory Animals* (2011) and were in compliance with all appropriate parts of the Animal Welfare Act (1996). An animal use protocol was approved by an Institutional Animal Care and Use Committee (IACUC) prior to the initiation of this study.

Housing : 1 per cage

Cage Type: During Non-exposure Period (18 hr/day, 5 days/week or 24 hr/day, 2 days/week): Polycarbonate shoebox cages with micro-isolator lids, bedding, food and water *ad libitum*, and enrichment products in an animal housing area

During Exposure Period (6 hr/day, 5 days/week): Hanging stainless steel wire-mesh cages in an $8m^3$ inhalation exposure chamber. To allow the animals to acclimate to the exposure cages, animals were placed in exposure cages (air-only exposure) for approximately 3-4 hours per day

for at least two days during the week prior to the initiation of styrene exposures.

Cage Type: Shoebox cage: AN75HT
Exposure cage: Hazelton M-48

Bedding: Shoebox cage: Alpha-dri cellulose bedding (Shepard Specialty Papers, Kalamazoo, MI). Vendor analysis and dates of autoclaving are in the study data files.
Exposure cage: None. Stainless steel pans were placed under cages to catch urine and feces. Pans were changed daily.

Cage Changes: Once per week

Diet: Non-exposure Period: LabDiet 5002 Certified Rodent Diet (PMI Nutrition, St. Louis, MO) *ad libitum* (Amendment 01, Appendix 1)
Exposure Period: None.

Analysis: The manufacturer's composition and certification data sheets are in the study data files. There were no known contaminants in the feed expected to interfere with the results of the study.

Water: Filtered reverse osmosis (RO) treated tap water (City of Durham, NC) *ad libitum*.

Supplied: Non-exposure Period: Polycarbonate bottle with stainless steel sipper tube
Exposure Period: Automatic stainless steel water lixits built into inhalation exposure cage unit system

Analysis: The results of current chemical analysis of in-house RO water (conducted approximately quarterly) including RO water sample from an inhalation exposure chamber were obtained from National Testing Laboratories, Inc. (Cleveland, OH) and are in the study data files. There were no known contaminants in the RO water expected to interfere with the results of the study.

Animal Room Conditions:
Temperature: 20-25°C (Deviation 5, Appendix 1)
Humidity 35-65% (Deviation 5, Appendix 1)
Lighting: 12/12 hour light/dark cycle

Enrichment: Non-exposure Period: Shepard Shacks, Nestlets, or Enviro-dri
Analysis: The manufacturer's report of mouse enrichment products is in the study data files.

<u>In-life Measurements</u>
Mortality/Moribundity: Twice daily on weekdays (morning and afternoon) and once daily on weekends (morning)

Clinical Observations: Within 3 days of arrival, at group allocation, prior to initial exposure, weekly (morning coinciding with body weights), and prior to euthanasia

Body Weights: Within 3 days of arrival, at group allocation, prior to initial exposure, weekly (morning), and prior to euthanasia (Deviation 6, Appendix 1)

Food Consumption: During non-exposure period, measurement began 1st exposure day and then weekly measurements (morning coinciding with body weights)

Clinical observations, body weights, and food consumption were recorded in Provantis8 (NT2000 version 8.4.2.0, Instem, Conshohocken, PA).

Inhalation Exposure Administration

Justification for Route of Administration and Selection of Exposure Concentrations: Styrene is volatile and inhalation is the most likely route of human exposure occupationally or environmentally. Lung tumors were observed in male and female mice following 104 weeks inhalation exposure. Previous inhalation toxicity studies in mice have shown 80 to 160 ppm styrene to be an effect concentration for lung cytotoxicity and tumorigenicity. Thus, 120 ppm was selected as a high concentration in this study. Marginal, if any, lung cytoxicity effects were expected at 40 ppm styrene following 4 weeks exposure, though 40 ppm was an effect concentration following 104 weeks exposure.

Exposure Type/Duration: Whole-body inhalation exposure, 6 hours/day, 5 days/week for either 1-week or 4-weeks duration.

Exposure Chamber System And Operation: 8-m^3 stainless steel and glass chambers operated at approximately 2000 L/min airflow rate (~15 air changes/hour). Details of exposure chamber system and operation are described in Appendix 2. Protocol deviations 2 and 3 (Appendix 1) describe minor alterations due to disruption of electrical power on Study Day 19.

Styrene Vapor Generation And Analysis: The styrene vapor was generated by metering liquid styrene into a J-tube generator filled with glass beads. For each exposure chamber, styrene vapor concentration was monitored using an infrared spectrophotometer (MIRAN 1A, Foxboro Co., Norwalk, CT). Details of styrene vapor generation and analysis are described in Appendix 2.

Chamber Distribution: Styrene exposure chambers were checked for uniformity of distribution of the styrene vapor/air mix by measuring the concentration of styrene at various locations within the animal breathing zone of the chamber. Details of styrene vapor chamber distribution are described in Appendix 2.

Osmotic Pump Implantation

Twelve WT mice (six control and six 120 ppm styrene groups) had an osmotic minipump (1.0 µL/hour flow rate) filled with 20 mg/mL of BrdU in sterile saline implanted subcutaneously one day prior to the first inhalation exposure. Details of the surgical procedure are described in the study protocol (Appendix 1).

Termination/Necropsy

Moribunds/Unscheduled: There were no moribund animals or unscheduled animal necropsies during the study. Animal #148 died prior to the start of the study (during housing acclimation period) and did not undergo necropsy following evaluation by study personnel.

Scheduled (1-week or 4-week lung genomics/ histopathology animals): On the morning following the last inhalation exposure, mice assigned to lung genomics/histopathology evaluation were observed, weighed and humanely euthanized by sodium pentobarbital (Euthasol®), 50 mg/kg, via intraperitoneal injection and exsanguinated from the abdominal aorta to confirm death. Details of the necropsy procedure, collection of lung tissue, tissue processing, and tissue storage are described in the study protocol (Appendix 1).

Scheduled (1-week lung cell proliferation/histopathology animals): On the morning following the last inhalation exposure, mice assigned to lung cell proliferation/histopathology evaluation were observed, weighed and humanely euthanized by sodium pentobarbital (Euthasol®), 50 mg/kg, via intraperitoneal injection and exsanguinated from the abdominal aorta to confirm death. Details of the necropsy procedure, collection of lung and duodenum tissues, tissue processing, and tissue storage are described in the study protocol (Appendix 1).

Lung RNA and Microarray Analysis

Total RNA was isolated from the lung tissue of all animals assigned to genomics evaluation using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The isolated RNA was further purified using the QIAcube automated system and RNeasy columns (Qiagen, Valencia, CA). The integrity of the RNA was verified spectrophotometrically and with the Agilent 2100 Bioanalyzer (Palo Alto, CA).

Five of eight samples/strain/exposure group/time point were selected as samples with the best quantity of RNA. Double-stranded cDNA was synthesized from 150ng of total RNA, then transcribed to biotin-labeled cRNA using the 3' Express IVT kit (Affymetrix, Santa Clara, CA). Fifteen µg of labeled cRNA was fragmented and prepped for hybridization. The Affymetrix HT MG430 PM peg arrays were used with the Gene Titan system. Gene Titan robotic instrumentation was used to perform the hybridization, washing and scanning of the peg arrays.

Lung Histology and Histopathology

The left lobes removed from animals assigned for genomics evaluation were embedded in paraffin and 5-µm sections were cut, mounted on slides, and stained with hematoxylin and eosin (H&E). Slides were evaluated microscopically by a board-certified pathologist (Appendix 3).

Lung Cell Proliferation Evaluation

Sections of the left lobe removed from animals assigned for cell proliferation evaluation were embedded in paraffin, and the blocks were processed for both cell proliferation and light microscopic evaluations (Appendix 3). The duodenum was also removed from these animals, embedded in the same paraffin blocks (left lung lobes) (Deviation 1, Appendix 1), and used as a positive control in staining for consistency across all staining runs.

For the lung cell proliferation evaluation, 300-650 stained and unstained epithelial cells lining the terminal bronchioles (e.g., 8-12 terminal bronchioles) were counted per animal (Appendix 3). Data were expressed as the cumulative BrdU labeling index (LI); LI was defined as the percentage of the cells counted that was positively stained for the presence of BrdU.

I. Data Analysis

Body weight and food consumption data were analyzed using the statistical tests provided by the Provantis software system (NT2000 version 8.4.2.0, Instem, Conshohocken, PA). Details of the statistical procedures are described in the study protocol (Appendix 1). A result of $p<0.05$ was considered statistically significant. Details of analysis of the gene expression microarray data are described in Appendix 4.

J. Results and Discussion

Inhalation Exposure Concentrations

Results of inhalation exposure concentrations and exposure chamber environmental conditions (temperature, relative humidity, air flow, and static pressure) throughout the study are shown in Appendix 2. For the 1-week duration, the analytical means (± standard deviations) for the three styrene concentration groups were 118.9 (±1.2), 40.9 (±2.0), and 0.0 (±0.0) ppm for the target exposure concentrations of 120, 40, and 0 ppm, respectively. For the 4-week duration, the analytical means (± standard deviations) for the three styrene concentration groups were 119.1 (±2.5), 39.6 (±1.6), and 0.0 (±0.0) ppm. The disruption of electrical power on Study Day 19 (Deviations 2 and 3, Appendix 1) did not impact the integrity of the study. Exposure temperature and relative humidity means were 72 °F and 49-52%, respectively, for all exposure groups throughout the study.

Body Weights

Results, including statistical analysis, of mean body weight and body weight gains are shown in Appendix 5a. Styrene exposure groups (40 and 120 ppm) were compared to the control group (0 ppm) for each strain of mice (WT, KO, and TG). There were no statistically significant differences in mean body weights between control and styrene-exposed animals throughout the study for all three strains. There was a mean body weight gain increase in the 40 ppm WT mice and in the 40 ppm TG mice during study week 4, but these observations were considered spurious. Decreases in mean body weight gain were observed in the 40 ppm and 120 ppm KO mice during study week 4, but these decreases did not result in differences in mean absolute body weights.

Food Consumption

Results, including statistical analysis, of mean food consumption relative to body weights are shown in Appendix 5b. The only statistically significant decreases were in the 40 ppm and 120 ppm WT mice during study week 1. By study week 2, relative food consumption means were similar between air control and styrene-exposed WT mice. Increases in mean relative food consumption (120 ppm WT mice, 40 ppm KO mice, and 120 ppm KO mice during study week 4) were considered spurious and therefore were not considered toxicologically important.

Clinical Observations

There were no clinical signs of toxicity in any of the study animals throughout the 4-week study (Appendix 5c). At necropsy, there were no gross lesions observed in any of the study animals.

Lung Cell Proliferation and Histopathology

Results of lung cell proliferation and histopathology are provided in Appendix 3. Inhalation of 120 ppm styrene six hours/day, five days/week for 1 week resulted in an approximate 7-fold increase compared to the control group in the mean BrdU labeling index of epithelial cells lining the lung terminal bronchioles (WT mice).

WT mice receiving 40 or 120 ppm styrene for either 1- or 4-weeks had microscopic lesions in the lungs consisting of decreased eosinophilia of bronchiolar epithelium mainly lining the terminal bronchioles; degeneration, necrosis, exfoliation, and/or attenuation of individual bronchiolar epithelial cells (most likely Clara cells) mainly lining the terminal bronchioles; and/or hyperplasia of the bronchiolar epithelium mainly lining the terminal bronchioles that sometimes extended into the distally connected alveolar duct. No styrene-induced microscopic lesions were observed in the KO and TG mice after either 1- or 4-weeks of exposure. Overall, the microscopic findings in the WT mice did not appear to be concentration-

dependent except for the degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium at the 4-week time point, which appeared slightly more severe in mice receiving 120 ppm styrene compared to control mice.

The mean severity scores for most microscopic lesions in the WT mice were greater at the 1-week time point compared with WT mice from the 4-week time point, except for the mean severity score for hyperplasia of the bronchiolar epithelium which was greater in the WT mice from the 4-week time point compared with WT mice from the 1-week time point.

Genomics Analyses

A genomics analyses summary report is provided in Appendix 4. There were almost no significantly differentially expressed (DE) genes in either the KO or the TG mice at any time point for any styrene concentration. A single significant gene was detected for TG mice at 120 ppm following 1 week exposure (Npas2), and a single gene at 40 ppm following 4 weeks exposure (Dcaf4). Significant differential gene expression was observed in WT mice at 1 week with 378 DE genes in the 40 ppm exposure group (relative to WT controls), and 516 DE genes in the 120 ppm group. Of these, 287 genes were significantly DE at both styrene concentrations. However, by 4 weeks, there were fewer DE genes detected: 19 at 40 ppm and 69 at 120 ppm with 17 of these significant at both concentrations. A total of ten genes were significantly DE at all concentrations and both time points. Gene ontology enrichment of the 1 week WT DE genes showed a strong dominance of cell cycle regulatory pathways. The greatest significant enrichment was observed with the 287 genes that were DE at both 40 ppm and 120 ppm at 1 week. At 4 weeks, significant enrichment was only observed for a single pathway due to the limited numbers of DE genes detected, and this pathway was also significantly enriched at 1 week.

K. Conclusion

Cell proliferation and histopathology results of this study support the implication of CYP2F2 metabolism as a key event in the production of lung toxicity in mice following short-term exposure of inhaled styrene vapor. Results of gene expression analysis showed marked differences in the number of significantly DE genes between styrene-exposed WT mice and styrene-exposed KO or TG mice void of CYP2F2 enzyme. A temporal relationship of significantly DE genes was observed in the styrene-exposed WT mice, and cell cycle regulatory pathways were predominantly enriched.

L. References

Animal Welfare Act: Public Law 89-544, 1966, as amended (P.L. 91-579, P.L. 94-279 and P.L. 99-198) 7 U.S.C. 2131 et seq. Code of Federal Regulations, Title 9, Chapter 1, Subchapter A, Parts 1, 2, and 3.

NRC (National Research Council). 2011. Guide for the Care and Use of Laboratory Animals. Eighth Edition. *The National Academies Press,* Washington, D.C.

U.S. Environmental Protection Agency's Good Laboratory Practice Standards. 40 CFR Ch. I (7-1-12 Edition), Part 792.

M. Retention of Records and Specimens

The study's GLP data/records/samples/specimens collected at the Test Facility or at the Test Sites, and the Final Report will be archived by QPS, LLC at 6 Davis Drive, RTP, NC, for at least 6 months following the study completion date. Non-GLP data (e.g., gene expression) generated at the Test Facility will be archived by The Hamner's Institute for Chemical Safety Sciences at 6 Davis Drive, RTP, NC, for at least 6 months following the study completion date. At that time, The Hamner and QPS will coordinate with the Sponsor the transfer of the GLP data/records/samples/specimens and/or non-GLP data to a location designated by the Sponsor.

N. Appendices

Appendix 1 – Study Protocol, Protocol Amendments, and Protocol Deviations – 29 pages
Appendix 2 – Inhalation Exposure Summary Report – 24 pages
Appendix 3 – Histopathology and Cell Proliferation Report – 44 pages
Appendix 4 – Genomics Analyses Summary Report – 17 pages
Appendix 5a – Mean Body Weight and Mean Body Weight Gain Tables – 10 pages
Appendix 5b – Mean Food Consumption Relative to Body Weight Tables – 3 pages
Appendix 5c – Clinical Observations – 6 pages

Appendix 1

Study Protocol, Protocol Amendments, and Protocol Deviations

29 pages

The Hamner Institutes for Health Science
Post Office Box 12137
6 Davis Drive
Research Triangle Park, NC 27709

Protocol Number: DEDSTY131-1
(QPS Code: 409)

Sponsor:

Styrene Information and Research Center
910 17th Street NW, #500-B
Washington, DC 20006

Test Facility:

The Hamner Institutes for Health Sciences
6 Davis Drive
Research Triangle Park, NC 27709

STUDY PROTOCOL: 4-WEEK STYRENE INHALATION LUNG TOXICITY STUDY IN MICE

Approved By:

Study Director and Test Facility Manager: Darol E. Dodd, PhD

Darol E. Dodd 10/1/13
Signature/Date

Co-Investigator (Genomics): Melvin E. Andersen, PhD

Signature/Date

Principal Investigator at PAI Test Site: James T. Raymond, DVM, DACVP

Signature/Date

Sponsor Representative: George Cruzan, PhD

Signature/Date

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Approved By:

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Darol E. Dodd 10/1/13
Signature/Date

Co-Investigator (Genomics): Melvin E. Andersen, PhD

Melvin E. Andersen 10/7/2013
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Principal Investigator at PAI Test Site: James T. Raymond, DVM, DACVP

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Sponsor Representative: George Cruzan, PhD

Signature/Date

Reviewed By:

Health and Safety: Richard Cravener, Jr., MS

Richard Cravener Jr 02 OCT 13

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B Bourn 01 OCT 2013

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A. Background

Styrene causes increased lung tumors in mice, but not in rats. The lung tumors are found mostly at the conclusion of a life-time (104 weeks for males) exposure study and most are benign. Styrene is largely negative in genotoxicity assays. Styrene metabolism by CYP2F2 produced a different metabolite pattern in mouse lung than in liver or in rats or humans. Short-term exposure of styrene produced lung toxicity (including degeneration and exfoliation of Clara cells, regenerative cell replication, and reduced production of Clara cell secretory protein (CCSP, CC10, or CC16). Longer term exposure resulted in cellular crowding, hyperplasia and hyperplasia extending into alveolar ducts. In the styrene bioassay in mice (Cruzan et al., 2001), lung pathology at 12 months included decreased staining of Clara cells and hyperplasia of terminal bronchioles with extension into alveolar ducts. These observations were present in nearly all mice examined that were exposed to 80 or 160 ppm. Decreased staining was seen in some mice at 20 and 40 ppm, but hyperplasia was only occasionally found in mice in these two groups.

Recent studies have shown elimination of the Clara cell toxicity from exposure to styrene in mice genetically engineered to not produce CYP2F2 (CYP2F2(-/-) mice) [called CYP2F2-knockout or CYP2F2KO]. Furthermore, in lung Clara cells of wild type (WT) mice, there is benzene ring oxidation of styrene; this ring-oxidation of styrene in lung is completely eliminated in CYP2F2(-/-) mice. Levels of other CYPs are not changed in these mice, so major changes in styrene metabolites are not expected in these mice, except elimination of metabolites produced by CYP2F2.

In a humanized mouse (2F2KO/2F1tg) styrene did not induce lung toxicity, indicating humans are not likely to develop lung toxicity from styrene exposure. CYP2F1 is present in these mice at about 2.5% of the amount of CYP2F2 in the lungs of WT mice. This level is at least as great as the level of CYP2F1 in human lungs.

To date, styrene experiments have focused on short-term exposures and show a consistent pattern implicating CYP2F2 metabolism as the key event in the production of mouse lung cytotoxicity and tumors. No studies have been conducted to ascertain if these effects are also eliminated in the absence of CYP2F2 metabolism.

B. Objective

The objective of this study is to evaluate the mode-of-action for styrene-induced lung toxicity by comparing gene expression and cell proliferation effects following inhalation exposure (0, 40, 120 ppm) in 3 male mouse strains with different genotypes. Specific enzymes of styrene metabolism aligned with specific genotypes may be identified as important contributors to producing lung toxicity. Understanding which metabolic pathways are involved with lung toxicity in mice following styrene exposure may provide a prediction of potential lung toxicity in humans exposed to styrene.

C. Experimental Design/Approach

Male C57Bl/6 mice (wild-type; WT), male CYP2F2(-/-) mice (knock-out; KO), and male CYP2F2(-/-) 2F1,2A13,2B6-tg mice (transgenic; TG) will be received at 4-7 weeks of age from Taconic, Germantown, NY. For gene expression profiling, eight mice per strain (WT, KO, and TG) per time point (1-week and 4-week) will be randomly assigned to one of three styrene concentration groups as follows:

- 0 ppm (air-only control)
- 40 ppm styrene
- 120 ppm styrene

For cell proliferation evaluation, six WT mice will be randomly assigned to an air-only control or to a 120 ppm styrene group and evaluated following one week of exposure.

All groups will receive inhalation exposure six hours/day, five days/week, for time points of 1 week or 4 weeks. In-life evaluations include clinical observations, body weights, and food consumption. For post-exposure lung cell proliferation evaluation, mice will have an osmotic minipump containing BrdU implanted subcutaneously one day prior to first styrene inhalation exposure. At necropsy, mice will be humanely euthanized with sodium pentobarbital (intraperitoneal administration) and exsanguinated from the abdominal aorta. Lungs will be collected for gene expression analysis, cell proliferation analysis, and histopathology evaluation.

D. Proposed Study Schedule

October 15-16, 2013	Animal arrival
October 27, 2013	BrdU implant surgery
October 28, 2013	1st inhalation exposure
November 2, 2013	1-week necropsy
November 22, 2013	Final inhalation exposure
November 23, 2013	4-week necropsy
February 28, 2014	Draft report

E. Regulatory Compliance and Standard Operating Procedures

The study will be conducted in accordance with U.S. Environmental Protection Agency's Good Laboratory Practice Standards (GLPs; 40 CFR, Part 792) from animal receipt through euthanasia of the animals. Lung histology, histopathology, and cell proliferation will adhere to GLPs. Gene expression analysis will be conducted non-GLP and to the highest standards of research practice, but will not be audited by a Quality Assurance Unit (QAU). All applicable Standard Operating Procedures (SOPs) of The Hamner Institutes (The Hamner), Mispro Biotech Services Corporation (Mispro), or QPS, LLC (QPS) will be followed and a list of SOPs used will be placed in the study file. Study specific SOPs will be part of the study protocol. GLP-compliant data will undergo quality control review.

The study protocol will be reviewed by the QPS QAU before final approval by the Sponsor. A quality assurance inspection will be conducted per the audit plan (below) to assure the quality and integrity of the study results. An audit of the final report and associated study records will be conducted to verify the consistency between the reported study results and the raw data. A Quality Assurance Statement of inspections and audits completed will be included in the final toxicology study report.

Study Audit Plan

In-life

Dosing Animals (Inhalation Exposure Administration)
Animal Sample collection and Processing (Termination/Necropsy)

Analysis Related

Sample Preparation (Lung Histopathology/Cell Proliferation)

Additional

Process audits, as necessary

Select endpoints of the study (lung histopathology and cell proliferation) will be conducted at Test Sites (see Resources section) and shall follow their respective quality standards (e.g., GLPs). Each Test Site's QAU (Charles River Pathology Associates) shall conduct quality assurance inspections and audits according to their Standard Operating Procedures and will verify that their portion of the study has been conducted in accordance with the protocol. Each Test Site will provide an audited final test site report which includes a Quality Assurance Statement signed by their QAU.

F. Critical Materials

Test Substance: Styrene monomer, PO-11 Grade bulk (CAS Number 100-42-5) was received from Lyondell Chemical Company (LCC), Houston, TX, on August 13, 2013. The styrene test substance (Lot Number 130807.6008) was shipped in sixteen 1-liter containers and appeared as a colorless liquid. A material safety and data sheet (MSDS) was provided by LCC and will be archived with the study records. A Certificate of Analysis (COA) was provided by LCC. The COA indicated the test substance to be 99.95% (weight) styrene monomer. An inhibitor of styrene polymer formation, t-butyl catechol (13 ppm), had been added to the test substance by LCC. Storage instructions were to maintain the test substance under climate controlled ambient conditions, preferably at a slightly cool temperature (e.g., approximately 68ºF). The test substance will be kept in Hamner Room 200U for storage and use during the inhalation study. A reserve sample of the test substance was shipped by LCC to EPL Archives (Sterling, VA) for archiving. EPL Archives received the sample on August 13, 2013 (Sponsor communication).

The Sponsor instructed that the test substance will remain stable, except for potential trace amounts of polymer formation, and will be suitable for use for this inhalation study. All further documentation of the test substance, such as method of synthesis and other Good Laboratory Practice (GLP) compliance requirements are the responsibility of the Sponsor.

G. Methods

Test System

Species:	Mouse, *Mus musculus*
Strain/Sex:	Male C57Bl/6 (wild-type; WT) Male CYP2F2(-/-) (knock-out; KO) Male CYP2F2(-/-) 2F1,2A13,2B6-tg (transgenic; TG)
Source:	Taconic Farms (Germantown, NY)
Number:	63 WT, 51 KO, and 51 TG (to be purchased)
Acclimation:	Approximately 12-13 days
Age at arrival:	4-7 weeks of age

Age at administration:	6-9 weeks of age
Weight at initiation of exposure:	Approximately 20-30 grams.
Identification:	Each animal will be uniquely identified by an ear tag at the time of randomization and group assignment. Cages holding the animals will have cage cards and cage labels associated with assigned animal numbers.
Justification:	There are no *in vitro* or other alternative models to specifically study styrene vapor-induced lung effects, such as gene expression and cell proliferation, between these three mouse genotypes. The mouse is the most appropriate animal species because the mouse has been shown to be responsive to styrene inhalation toxicity and has established transgenic lines (via breeders) for scientific investigation. Previous mouse toxicity studies with styrene indicate no significant sex difference in lung cytotoxicity, but male mice had a higher lung tumor incidence then female mice in a life-time inhalation exposure bioassay, thus male mice were selected for study.

Animal Allocation and Group Assignment

The animals will be stratified randomly and assigned by body weight to a styrene exposure group. Individual animal body weights will be within 20% of the mean group body weights. Only clinically healthy animals will be used for allocation. Unused animals will be humanely euthanized and death confirmed by exsanguinations.

Group	Endpoint	Number and Strain	Animal Identification	Time Point
Air only - 0 ppm (control)	Genomics	8 WT 8 KO 8 TG	1-8 101-108 201-208	1-week
Air only - 0 ppm (control)	Cell proliferation	6 WT	49-54	1-week
Styrene – 40 ppm	Genomics	8 WT 8 KO 8 TG	9-16 109-116 209-216	1-week
Styrene – 120 ppm	Genomics	8 WT 8 KO 8 TG	17-24 117-124 217-224	1-week
Styrene - 120 ppm	Cell proliferation	6 WT	55-60	1-week
Air only - 0 ppm (control)	Genomics	8 WT 8 KO 8 TG	25-32 125-132 225-232	4-week
Styrene – 40 ppm	Genomics	8 WT 8 KO 8 TG	33-40 133-140 233-240	4-week
Styrene – 120 ppm	Genomics	8 WT 8 KO 8 TG	41-48 141-148 241-248	4-week

Animal Husbandry

The Hamner has an accredited Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) animal facility operated by trained animal care personnel employed by Mispro Biotech Services Corporation (Mispro). Current acceptable practices of good animal husbandry will be followed per National Research Council's *Guide for the Care and Use of Laboratory Animals* (2011) and will be in compliance with all appropriate parts of the Animal Welfare Act (1996). In addition, a study design and animal use protocol will be approved by Mispro's Institutional Animal Care and Use Committee (IACUC) prior to the initiation of this study. A copy of the approved IACUC protocol (Mispro #: 2013-08-27-TH11-01, approved 10/01/13) will be placed in the study file.

Housing : 1 per cage

Cage Type: During Non-exposure Period (18 hr/day, 5 days/week or 24 hr/day, 2 days/week): Polycarbonate shoebox cages with micro-isolator lids, bedding, food and water *ad libitum*, and enrichment products in an animal housing area

During Exposure Period (6 hr/day, 5 days/week): Hanging stainless steel wire-mesh cages in an $8m^3$ inhalation exposure chamber. To allow the animals to acclimate to the exposure cages, animals will be placed in exposure cages (air-only exposure) for approximately 3-4 hours per day

for at least two days during the week prior to the initiation of styrene exposures.

Cage Type: Shoebox cage: AN75HT
Exposure cage: Hazelton M-48

Bedding: Shoebox cage: Alpha-dri cellulose bedding (Shepard Specialty Papers, Kalamazoo, MI). Vendor analysis and dates of autoclaving will be included in the study data files.
Exposure cage: None. Stainless steel pans will be placed under cages to catch urine and feces. Pans are changed daily.

Cage Changes: Once per week (documented in study data files)

Diet: Non-exposure Period: Certified PicoLab Rodent Diet 20 (LabDiet, Richmond, IN) *ad libitum*.
Exposure Period: None.

Analysis: The manufacturer's composition and certification data sheets will be included in the study data files. There are no known contaminants in the feed that are expected to interfere with the results of the study.

Archival: A sample (~200 g) will be retained and stored between 0 and -30°C until approval of the final report.

Water: Filtered reverse osmosis (RO) treated tap water (City of Durham, NC) *ad libitum*.

Supplied: Non-exposure Period: Polycarbonate bottle with stainless steel sipper tube
Exposure Period: Automatic stainless steel water lixits built into inhalation exposure cage unit system

Analysis: The results of current chemical analysis of in-house RO water (conducted approximately quarterly) including RO water sample from an inhalation exposure chamber will be obtained from National Testing Laboratories, Inc. (Cleveland, OH) and included in the study data files. There are no known contaminants in the RO water that are expected to interfere with the results of the study.

Animal Room Conditions:
Temperature: 20-25°C
Humidity 35-65%
Lighting: 12/12 hour light/dark cycle

Enrichment: Non-exposure Period: Shepard Shacks, Nestlets, or Enviro-dri
Analysis: The manufacturer's report of mouse enrichment products will be included in the study data files.

<u>In-life Measurements</u>
Mortality/Moribundity: Twice daily on weekdays (morning and afternoon) and once daily on weekends (morning)

Clinical Observations: Within 3 days of arrival, at group allocation, prior to initial exposure, weekly (morning coinciding with body weights), and prior to euthanasia

Body Weights:	Within 3 days of arrival, at group allocation, prior to initial exposure, weekly (morning), and prior to euthanasia
Food Consumption:	During non-exposure period, measurement begins 1st exposure day and then weekly measurements (morning coinciding with body weights)

Clinical observations, body weights, and food consumption will be recorded in Provantis8 (NT2000 version 8.4.2.0, Instem, Conshohocken, PA).

Inhalation Exposure Administration

Justification for Route of Administration and Selection of Exposure Concentrations:	Styrene is volatile and inhalation is the most likely route of human exposure occupationally or environmentally. Lung tumors were observed in male and female mice following 104 weeks inhalation exposure. Previous inhalation toxicity studies in mice have shown 80 to 160 ppm styrene to be an effect concentration for lung cytotoxicity and tumorigenicity. Thus, 120 ppm was selected as a high concentration in this study. Marginal, if any, lung cytoxicity effects are expected at 40 ppm styrene following 4 weeks exposure, though 40 ppm was an effect concentration following 104 weeks exposure.
Exposure Type/Duration:	Whole-body inhalation exposure, 6 hours/day, 5 days/week for either 1-week or 4-weeks duration.
Exposure Chamber System And Operation:	8-m^3 stainless steel and glass chambers operated at approximately 2000 L/min airflow rate (~15 air changes/hour). One chamber per exposure concentration of 0, 40 and 120 ppm styrene. Animals will be individually caged in stainless steel wire mesh cages with metal discs placed on the cage floor to support the feet during exposure. Stainless steel pans will be placed under cages to catch urine and feces. The supply air flow through each chamber will be controlled by a Continuum Building Automation System (CBAS; Andover Controls Corporation, Andover, MA). Temperature and humidity in each chamber will also be controlled and recorded by the CBAS. To the extent possible, temperature will be maintained approximately 66 to 77 °F with a target of 72 °F. Relative humidity will be maintained at approximately 30 to 70% with a target of 50%. Static pressure within each styrene exposure chamber will be maintained at a slightly negative value relative to the room housing the chamber. Static pressure within the control chamber will be maintained at a slightly positive value.
Styrene Vapor Generation:	The styrene vapor will be generated by metering liquid styrene (FMI pump) into a J-tube generator filled with glass beads. The FMI pump will be calibrated and set to the nominal flow rate expected for target exposure concentrations of 40 or 120 ppm styrene. Nitrogen will flow upward through the J-tube at a flow rate of approximately 25 L/min. The styrene will be pumped into the upper portion of the J-tube and allowed to flow downward through glass beads, counter-current to the nitrogen flow. A heating jacket (not to exceed 145 deg C) will be used to warm the J-tube. The vaporized styrene/nitrogen will be mixed countercurrent with HEPA-filtered air approximately 5 feet upstream of the exposure

chamber inlet. The vapor generation system will be monitored by the CBAS.

Styrene Vapor Analysis: For each exposure chamber, styrene vapor concentrations will be monitored using an infrared spectrophotometer (MIRAN 1A, Foxboro Co., Norwalk, CT). The Miran analyzer will be connected to the CBAS, so that continuous monitoring of each chamber concentration will be achieved during each six hour daily exposure. The Miran analyzer will be calibrated using a closed loop method by injecting known volumes of liquid styrene. Calibrations will then be verified by analyzing styrene vapor concentrations from certified gas cylinders. Adjustments during daily exposures will be made, if necessary, to attain a daily mean exposure concentration within +/- 10% of the target exposure concentration (40 or 120 ppm).

Chamber Distribution: Styrene exposure chambers will be checked for uniformity of distribution of the styrene vapor/air mix by measuring the concentration of styrene at various locations within the animal breathing zone of the chamber. The chamber distribution will be performed prior to the start of the inhalation study.

Osmotic Pump Implantation

Twelve WT mice will have an osmotic minipump (1.0 µL/hour flow rate) filled with 20 mg/mL of BrdU in sterile saline implanted subcutaneously one day prior to the first inhalation exposure.

Animals will be anesthetized with 3-5% isoflurane in oxygen at a flow rate of 1-5 liters per minute in a chamber. Once recumbent, the animal will be removed, placed on a heated pad, and a nose cone will be placed over the mouth and nose to continue anesthesia. The surgical site (dorsal thorax) will be shaved, cleaned of hair and debris, and then wiped with povidone iodine scrub solution (10%) with attention to not wet the surrounding fur. The area will then be wiped with sterile saline to remove any povidone iodine scrub.

The animal will be observed until a surgical anesthesia plane has been reached (no reflex at toe pinch), and administered buprenorphine at 0.05-0.1 mg/kg subcutaneously away from surgical site to prevent leakage of the pump and documented. If necessary (animal appears to be in pain, not eating, not drinking, unkempt hair coat), buprenorphine may be administered at this dose twice daily for up to 48 hours. A transverse incision (~ 1 cm or less) will be made through the skin at the neck. The posterior edge of the skin will be lifted and a forcep will be used to form a subcutaneous pouch by blunt dissection. The sterile osmotic minipump will be inserted into the pouch. The incision will be closed using sterile metal wound clips. Following surgery, the animal is removed from the anesthesia gas and the heating pad and hand-held until showing signs of beginning to right itself. The animal is then returned to its domiciliary cage and observed until it is fully ambulatory, which is usually 5 to 10 minutes following the removal of anesthesia gas. Since the animal will be necropsied within 1 week of minipump implantation, wound clips will not be removed.

Termination/Necropsy

Moribunds/Unscheduled: Moribund animals will be humanely euthanized by sodium pentobarbital, 50 mg/kg, via intraperitoneal injection and exsanguinated from the abdominal aorta to confirm death. Lungs will be inflated with neutral-buffered formalin (NBF) and preserved in NBF.

Scheduled (1-week or 4-week lung genomics/ histopathology animals): On the morning following the last inhalation exposure, mice assigned to lung genomics/histopathology evaluation will be observed and weighed

and humanely euthanized by sodium pentobarbital, 50 mg/kg, via intraperitoneal injection and exsanguinated from the abdominal aorta to confirm death. The thoracic cavity will be opened and examined for gross lesions. Lung samples will be obtained by first placing loosely a suture around the trachea. A second suture will be placed around the right bronchus and pulled tightly to tie off the right lung lobes. The right lung lobes will be removed, diced finely, and placed in RNAlater. [If volume of tissue is too great for the RNAlater vial, the excess tissue will be placed in a freezing vial, and frozen in liquid nitrogen. After freezing, samples will be stored at approximately -80°C.] Samples placed in RNAlater will be stored in a 4°C refrigerator overnight and then frozen at approximately -80°C. The trachea will be cannulated below the larynx and the left lung infused under hydrostatic pressure with 10% neutral buffered formalin. After the left lung is filled with fixative, the suture around the trachea will be pulled tightly to tie off the expanded left lobe. The left lung lobe with trachea attached will be removed from the carcass and transferred to a cup of 10% neutral buffered formalin (NBF) for histopathology. All protocol required necropsy activities will be documented on individual animal necropsy records and retained in a study notebook.

Scheduled (1-week lung cell proliferation/histopathology animals):

On the morning following the last inhalation exposure, mice assigned to lung cell proliferation/histopathology evaluation will be observed and weighed and humanely euthanized by sodium pentobarbital, 50 mg/kg, via intraperitoneal injection and exsanguinated from the abdominal aorta to confirm death. The thoracic cavity will be opened and examined for gross lesions. A suture will be placed loosely around the trachea. The trachea will then be cannulated below the larynx and the whole lung will be infused under hydrostatic pressure with 10% neutral buffered formalin. After the lung is filled with fixative, the suture around the trachea will be pulled tightly to tie off the expanded lung lobes. The lung lobes with trachea attached will be removed from the carcass and transferred to a cup of NBF. A small piece of the proximal duodenum (2-3 cm) will also be removed, cut transversely, and placed in NBF. The entire lung and duodenum will be fixed in NBF for approximately 48 hours, then rinsed and placed in 70% ethanol until processed. Prior to processing, a section of the left lobe will be cut and placed in a labeled cassette with the duodenum and used for lung cell proliferation assay.

Lung RNA and Microarray Analysis

Total RNA will be isolated from the lung tissue of all animals assigned to genomics evaluation using Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The isolated RNA will be further purified using the QIAcube automated system and RNeasy columns (Qiagen, Valencia, CA). The integrity of the RNA will be verified spectrophotometrically and with the Agilent 2100 Bioanalyzer (Palo Alto, CA).

Five of eight samples/strain/exposure group/time point will be selected as samples with the best quantity of RNA. Double-stranded cDNA will be synthesized from 50-150ng (dependent on RNA yield) of total RNA, then transcribed to biotin-labeled cRNA using the 3' Express IVT kit (Affymetrix, Santa Clara, CA). Fifteen µg of labeled cRNA will be fragmented and prepped for hybridization. The Affymetrix HT MG430 PM peg arrays will be used with the Gene Titan system. Gene Titan robotic instrumentation will perform the hybridization, washing and scanning of the peg arrays.

Lung Histology and Histopathology

The left lobes removed from animals assigned for genomics evaluation will be embedded in paraffin and 5-µm sections will be cut, mounted on slides, and stained with hematoxylin and eosin (H&E). Slides will be shipped to the following address at Charles River Pathology Associates for light microscopic evaluation.

Sean Bowman, BS
Charles River Pathology Associates, Maryland
15 Worman's Mill Court Suite I
Frederick, Maryland 21701
Telephone No.: 301-360-1744
Email: sean.bowman@crl.com

Emphasis of evaluation will be on the terminal bronchiolar and alveolar regions. Once the evaluation is complete, the slides will be returned to The Hamner Institutes for Health Sciences at the following address:

Nigel Edgerton, BS
The Hamner Institutes for Health Sciences
6 Davis Drive
Research Triangle Park, NC 27709
Telephone No.: 919-558-1321
Email: nedgerton@thehamner.org

Lung Cell Proliferation Evaluation

Sections of the left lobe removed from animals assigned for cell proliferation evaluation will be embedded in paraffin and the blocks will be shipped for both cell proliferation and light microscopic evaluations to Charles River Pathology Associates at the following address:

Sean Bowman, BS
Charles River Pathology Associates, Maryland
15 Worman's Mill Court Suite I
Frederick, Maryland 21701
Telephone No.: 301-360-1744
Email: sean.bowman@crl.com

In addition, the duodenum removed from these animals will be embedded in separate paraffin blocks (from the left lung lobes) and sent to Charles River Pathology Associates for use as a positive control in staining for consistency across all staining runs.

Slides from each lung block will be prepared and stained appropriately for BrdU cell counting or light microscopy, and evaluated. A limited number of duodenum blocks will be used only for BrdU staining controls and will not be evaluated for BrdU cell counting or light microscopy. For the lung cell proliferation evaluation, 350-400 stained and unstained epithelial cells lining the terminal bronchioles (e.g., 8-12 terminal bronchioles) will be counted per animal. Data will be expressed as the labeling index (LI); LI is defined as the percentage of the cells counted that is positively stained for the presence of BrdU.

A report containing the results from the lung histopathology and lung cell proliferation evaluations will be provided to the Study Director. Once the evaluation is complete, any remaining blocks will be returned to The Hamner Institutes for Health Sciences at the following address:

Nigel Edgerton, BS
The Hamner Institutes for Health Sciences
6 Davis Drive
Research Triangle Park, NC 27709

Telephone No.: 919-558-1321
Email: nedgerton@thehamner.org

H. Health and Safety

No specific hazards are posed by the study. Standard operating procedures (SOPs) for the conduct of inhalation studies will be followed (SOP list will be placed in the study file). The procedures include safety checks on when to open and close inhalation chamber doors housing the study animals. Procedures for cleaning inhalation exposure cages are also defined in the SOPs. No special donning of face respirators is necessary. Appropriate personal protection equipment will be used and precautions stated in the MSDS for styrene will be followed. As indicated previously, the test substance will be stored under climate controlled ambient conditions, preferably at a slightly cool temperature (e.g., approximately 68°F).

I. Data Analysis

Body weight and food consumption data will be analyzed using the statistical tests provided by the Provantis software system (NT2000 version 8.4.2.0, Instem, Conshohocken, PA). A one-way analysis of variance (ANOVA) will be used (per time point). If significant, the ANOVA will be followed by a Dunnett's test to compare styrene concentration groups with the control group. Cell proliferation labeling indices will be tested for normality (Shapiro-Wilk, $p<0.01$) and homogeneity of variances (Levene's, $p<0.05$) using JMP 9.0.0 software (SAS Institute, Inc., Cary, NC). For non-normal distributions or unequal variances, a Welch ANOVA followed by Steel's test will be used. A result of $p<0.05$ will be considered statistically significant.

The analysis of the gene expression microarray data will be performed as described previously (Thomas *et al.* 2007) with modification. The microarray data will be processed using Robust Multi-Array Average (RMA) normalization with a $\log_2$ transformation (Irizarry *et al.* 2003). All probe sets will be fit as continuous data to a series of four different dose-response models — linear, 2° polynomial, 3° polynomial, and power models. Each model will be run assuming constant variance and the benchmark response (BMR) factor will be set to 1.349 multiplied by the standard deviation in the control animals to estimate a benchmark dose with a 10% increase in tail area (BMD_{10})(see Thomas et al., 2007 for details on its derivation). For model selection, a nested likelihood ratio test will be performed on the linear, 2° polynomial, and 3° polynomial models. If the more complex model provides a significantly improved fit ($p < 0.05$), then the more complex model will be selected. If the more complex model does not provide a significantly improved fit ($p \geq 0.05$), the simpler model will be selected (Posada and Buckley 2004). The Akaike information criterion (AIC) for the selected polynomial model will then be compared with the AIC for the power model. The model with the lowest AIC (Akaike 1973) will be selected as the final model and will be used to calculate a BMD_{10} and $BMDL_{10}$. To avoid model extrapolation and any potential bias from poor fitting genes, probe sets with a BMD_{10} value greater than the highest dose or a goodness of fit p-value < 0.1 will be removed from further analysis.

The gene annotations and gene ontology (GO) classifications for all probe sets on the Affymetrix Rat Genome 230 2.0 array will be downloaded from the Affymetrix web site. Affymetrix probe sets that had a BMD_{10} less than the highest dose and a goodness of fit p-value > 0.1 will be converted into unique genes based on their NCBI Entrez Gene ID. When two or more probe sets are associated with a single gene, the BMD_{10} and $BMDL_{10}$ values for the individual probe sets will be averaged to obtain a single BMD_{10} and $BMDL_{10}$ value. The Entrez Gene identifiers will then be matched to their corresponding biological process GO categories. GO categories with < 5 genes will be removed from the analysis. The median BMD_{10} and $BMDL_{10}$ values will be used to summarize benchmark dose for each GO category.

Additionally, gene lists for those Entrez gene IDs with a BMD less than the highest dose and a goodness of fit p-value > 0.1 will be searched against MetaCore (Thomson Reuter's proprietary, curated gene ontology database) to augment the public GO category analysis. MetaCore pathways will be summarized by median BMD_{10} and $BMDL_{10}$ for those pathways with 5 or more genes.

J. Reports (Deliverables)

- Inhalation exposure report with methodology and results (e.g., styrene concentrations and chamber environmental conditions)
- Animal toxicity report with clinical observations, body weight, food consumption, and gross lesions at necropsy; also feed and water analysis reports
- Genomics report
- Integrated histopathology and cell proliferation report

K. Records/Specimens/Archives

Records will be kept in bound notebooks and/or 3-ring binders, and will be maintained by the Study Director per The Hamner Institutes SOP QUA-005, and per Study Director responsibilities defined in QPS SOP GN-008 for GLP studies. Data/records/samples collected at Test Sites will be transferred to the Test Facility following the completion of Test Site activities/responsibilities (e.g., submission of a final report). Records (including computer/machine output and other study activity documentations) and formalin-fixed tissues will be archived at the Test Facility for a period of 6 months following the submission of a final report. At that time, The Hamner and QPS will coordinate with the Sponsor transfer of the records and samples back to the Sponsor, or a location designated by the Sponsor.

Unused styrene test substance will be either returned to the Sponsor after issuance of the final report or disposed of, per the Sponsor's instruction. Records of shipment or disposition will be maintained in the study file.

L. Resources

Test Facility and Key Personnel

The Hamner Institutes for Health Sciences
Six Davis Drive
Research Triangle Park, NC 27709

Study Director/Test Site Manager:	Darol E. Dodd, PhD Tel: 919-558-1317; ddodd@thehamner.org
Co-Investigator (genomics):	Melvin E. Andersen, PhD
Inhalation Exposure:	Kay C. Roberts, AS
Animal Care and Surgery Oversight:	Carol A. Bobbitt, AAS, VMT, LATg
Animal Necropsy and Tissue Histology:	Nigel A. Edgerton, BS
Gene Expression Analysis:	Linda J. Pluta, BS
Gene Expression Statistics:	Michael B. Black, PhD
Health and Safety:	Richard Cravener, Jr., MS, CIH

Test Sites and Key Personnel

Mispro Biotech Services Corporation
(co-located at The Hamner Institutes)
Six Davis Drive
Research Triangle Park, NC 27709

Animal Care Supervisor/ Test Site Manager:	Amanda Pacio, LAT Tel: 919-281-2470; amanda.pacio@misprobiotech.com
Attending Veterinarian (contractor):	Coralie Z. Cannon, DVM, DACLAM Tel: 919-622-0873; cazvet@yahoo.com

QPS, LLC
(co-located at The Hamner Institutes)
Six Davis Drive
Research Triangle Park, NC 27709
Quality Assurance: Brenda Bourn, MBA
Tel: 919-246-7636; brenda.bourn@qps.com

Charles River Pathology Associates
15 Worman's Mill Court, Suite I
Frederick, MD 21701
Histopathology and Cell Proliferation/
Principal Investigator: James T Raymond DVM, DACVP
Tel: 301-360-1741; james.raymond@crl.com

Funding Source (Sponsor)

Styrene Information and Research Center
910 17th Street NW, #500-B
Washington, DC 20006
Reprensentative: George Cruzan, PhD
Tel: 856-453-3478; toxworks@aol.com

M. References

Akaike, H. (1973). Information theory and an extension of the maximum likelihood principle. In *Second International Symposium on Information Theory* (B. N. Petrov and F. Csaki, Eds.). Budapest, Hungary: Academiai Kiado, 267–281.

Animal Welfare Act: Public Law 89-544, 1966, as amended (P.L. 91-579, P.L. 94-279 and P.L. 99-198) 7 U.S.C. 2131 et seq. Code of Federal Regulations, Title 9, Chapter 1, Subchapter A, Parts 1, 2, and 3.

Irizarry, R. A., Bolstad, B. M., Collin, F., Cope, L. M., Hobbs, B., and Speed, T. P. (2003). Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* **31,** e15.

NRC (National Research Council). 2011. Guide for the Care and Use of Laboratory Animals. Eighth Edition. *The National Academies Press,* Washington, D.C.

Posada, D., and Buckley, T. R. (2004). Model selection and model averaging in phylogenetics: Advantages of akaike information criterion and Bayesian approaches over likelihood ratio tests. *Syst. Biol.* **53,** 793–808.

Thomas, R. S., Allen, B. C., Nong, A., Yang, L., Bermudez, E., Clewell, H. J., 3rd, and Andersen, M. E. (2007). A method to integrate benchmark dose estimates with genomic data to assess the functional effects of chemical exposure. *Toxicol. Sci.* **98,** 240–248.

U.S. Environmental Protection Agency's Good Laboratory Practice Standards. 40 CFR Ch. I (7-1-12 Edition), Part 792.

THI Protocol Amendment

Protocol Number:	DEDSTY131-1	**Amendment Number:**	01
Protocol Title:	4-Week Styrene Inhalation Lung Toxicity Study In Mice		

Section(s)	**Original Text**	**Amended Text**
Section G (Methods/Test Animals), Page 6	Number: 63 WT, 51 KO, and 51 TG (to be purchased)	Number: 63 WT, 48 KO, and 51 TG (to be purchased)
	Justification/Rationale: Only 48 of the 51 KO mice requested are available for delivery, so only 48 KO mice will be purchased. This change does not Impact the study design, because 3 extra KO mice had been requested in case of poor health status upon receipt. **Effective Date:** Upon final approval of this amendment **IACUC Approval Date:** October 10, 2013	
	Attachments? ☐ Yes ☒ No	
Section G (Methods/ Animal Husbandry), Page 9	Diet: Non-exposure Period: Certified PicoLab Rodent Diet 20 (LabDiet, Richmond, IN) *ad libitum*.	Diet: Non-exposure Period: LabDiet 5002 Certified Rodent Diet (PMI Nutrition, St. Louis, MO) *ad libitum*.
	Justification/Rationale: Certified PicoLab diet was not available at LabDiet (Richmond, IN) to meet the study schedule. Another certified diet and supplier were selected. The certified diets from both suppliers are comparable. No impact on the study is expected due to this change in diet. **Effective Date:** Upon final approval of this amendment **IACUC Approval Date:** October 10, 2013	
	Attachments? ☐ Yes ☒ No	

Approved By:

Study Director and Test Facility Manager: Darol E. Dodd, PhD

Darol E. Dodd 10/11/13

Signature/Date

Sponsor Representative: George Cruzan, PhD

George Cruzan 10/14/13
Signature/Date

Co-Investigator (Genomics): Melvin E. Andersen, PhD

Signature/Date

Principal Investigator at PAI Test Site: James T. Raymond, DVM, DACVP

Signature/Date

Reviewed By:

Quality Assurance (QPS): Brenda Bourn, MBA

Signature/Date

Health and Safety: Richard Cravener, Jr., MS

Signature/Date

Sponsor Representative: George Cruzan, PhD

Signature/Date

Co-Investigator (Genomics): Melvin E. Andersen, PhD

Signature/Date

Principal Investigator at PAI Test Site: James T. Raymond, DVM, DACVP

14-OCT-2013

Signature/Date

Reviewed By:

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Signature/Date

Health and Safety: Richard Cravener, Jr., MS

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Sponsor Representative: George Cruzan, PhD

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Co-Investigator (Genomics): Melvin E. Andersen, PhD

Melvin E. Andersen 10/16/13

Signature/Date

Principal Investigator at PAI Test Site: James T. Raymond, DVM, DACVP

Signature/Date

Reviewed By:

Quality Assurance (QPS): Brenda Bourn, MBA

BBourn 16 OCT 2013

Signature/Date

Health and Safety: Richard Cravener, Jr., MS

Richard Cravener Jr 16 OCT 13

Signature/Date

THI Protocol Deviation Form

Protocol Number:	DEDSTY131-1	**Deviation Number:**	1
Protocol Title:	4 Week Styrene Inhalation Lung Toxicity Study in Mice		

Section(s)	**Protocol Requirement**	**Deviation/Departure**
G (Lung Cell Proliferation Evaluation) – page 13	The duodenum was instructed to be embedded in separate paraffin blocks from the left lung lobe.	The duodenum was embedded in the same block as the left lung lobe for cell proliferation evaluation.

Root Cause: It is routine procedure to embed the duodenum in the same paraffin block as other tissues collected for cell proliferation evaluation; not separate blocks.

Impact Assessment (check one):

☒ No impact on the integrity of study

☐ Impact or potential impact on the integrity of the study (explain below)

Corrective/Preventive Action:

☒ None required

Approved By:

Study Director/Principle Investigator: Darol Dodd, PhD

Darol E. Dodd 11/13/13

Signature/Date

THI Protocol Deviation Form

Protocol Number:	DEDSTY131-1	**Deviation Number:**	2
Protocol Title:	4-Week Styrene Inhalation Lung Toxicity Study in Mice		

Section(s)	**Protocol Requirement**	**Deviation/Departure**
Exposure Chamber System and Operation – page 10	8-m3 stainless steel and glass chambers operated at approximately 2000 L/min airflow rate (~15 air changes/hour).	On Study Day 19 (15-Nov-2013) between 9:15 am and 10:20 am airflow rates in the exposure chambers were Chamber 297: 731.8 L/min Chamber 299: 398.3 – 404.4 L/min Chamber 305: 405.0 L/min

Root Cause:

An interruption of power caused damage to a controller element which resulted in a loss of primary and redundant fans which provide both Supply and Exhaust airflow to the Inhalation Chambers.

Impact Assessment (check one):

- [x] No impact on the integrity of study
- [ ] Impact or potential impact on the integrity of the study (explain below)

Corrective/Preventive Action:

During the period of time when the exposure chambers were not operating at the approximate 2000 L/min airflow, the delivery of Styrene test material to Chamber 297 and Chamber 299 was stopped. The oxygen content inside each exposure chamber was checked and the animals were checked for signs of stress by Mispro Biotech Services and documented in Animal Room Notebook.

The Hamner's maintenance department immediately started working to correct the equipment failure caused by the interruption of power.

Styrene exposure concentrations deviated from target exposure concentrations of approximately 30 minutes. To make up this difference, the exposures were extended 30 minutes from the start time, thus providing a 6 hour daily exposure to target styrene concentrations.

- [ ] None required

Approved By:

Study Director/Principle Investigator: Darol Dodd

Darol E. Dodd 11/22/13

Signature/Date

THI Protocol Deviation Form

Protocol Number:	DEDSTY131-1	**Deviation Number:**	3
Protocol Title:	4-Week Styrene Inhalation Lung Toxicity Study in Mice		

Section(s)	**Protocol Requirement**	**Deviation/Departure**
Exposure Chamber System and Operation – page 10	Static pressure within each styrene exposure chamber will be maintained at a slightly negative value relative to the room housing the chamber.	On Study Day 19 (15-Nov-2013) between 9:15 am and 10:20 am static pressure within the exposure chambers were Chamber 297: 0.012 – 0.009 in H_2O Chamber 299: 0.042 – 0.012 in H_2O

Root Cause:

An interruption of power caused damage to a controller element which resulted in a loss of primary and redundant fans which provide both Supply and Exhaust airflow to the Inhalation Chambers ultimately resulting in the chamber static pressure equalizing with the static pressure of the room housing the chamber.

Impact Assessment (check one):

- [x] No impact on the integrity of study
- [ ] Impact or potential impact on the integrity of the study (explain below)

Corrective/Preventive Action:

The Hamner's maintenance department immediately started working to correct the equipment failure caused by the interruption of power.

Styrene exposure concentrations deviated from target exposure concentrations of approximately 30 minutes. To make up this difference, the exposures were extended 30 minutes from the start time, thus providing a 6 hour daily exposure to target styrene concentrations.

- [ ] None required

Approved By:

Study Director/Principle Investigator: Darol Dodd

Darol E Dodd 11/22/13

Signature/Date

THI Protocol Deviation Form

Protocol Number:	DEDSTY131-1	**Deviation Number:**	4
Protocol Title:	4 Week Styrene Inhalation Lung Toxicity Study in Mice		

Section(s)	**Protocol Requirement**	**Deviation/Departure**
G (Animal Allocation and Group Assignment) – page 8	Individual animal body weights will be within 20% of the mean group body weights.	Individual animal body weights were within 25% of the mean group body weights.

Root Cause: The 20% requirement was met for the WT and KO strains, but with the TG strain, individual animal body weights were within 25% of the mean group body weight. Due to the small group sizes, the 25% range was the best we could achieve.

Impact Assessment (check one):

☒ No impact on the integrity of study

☐ Impact or potential impact on the integrity of the study (explain below)

Corrective/Preventive Action:

☒ None required

Approved By:

Study Director/Principle Investigator: Darol Dodd, PhD

Darol E. Dodd 1/20/14

Signature/Date

THI Protocol Deviation Form

Protocol Number:	DEDSTY131-1	**Deviation Number:**	5
Protocol Title:	4 Week Styrene Inhalation Lung Toxicity Study in Mice		

Section(s)	**Protocol Requirement**	**Deviation/Departure**
G (Animal Room Conditions) – page 9	Animal Room Conditions: Temperature: 20-25°C Humidity 35-65%	There were temporary minor excursions in the protocol-defined range of animal room temperature and humidity during the study.

Root Cause: The normal thermostat ranges of temperature and relative humidity in The Hamner's animal facility are 64-79ºF and 30-70%, respectively. These ranges are slightly greater than the protocol requested ranges of 20-25ºC (68-77ºF) and 35-65%, respectively. The thermostat ranges of the heating and air conditioning units used to control environmental conditions of the animal rooms used in the study were inadvertently not reset to the protocol requested ranges.

Impact Assessment (check one):

☒ No impact on the integrity of study

☐ Impact or potential impact on the integrity of the study (explain below)

Corrective/Preventive Action:

☒ None required

Approved By:

Study Director/Principle Investigator: Darol Dodd, PhD

Darol Dodd 2/19/14

Signature/Date

THI Protocol Deviation Form

Protocol Number:	DEDSTY131-1	**Deviation Number:**	6
Protocol Title:	4 Week Styrene Inhalation Lung Toxicity Study in Mice		

Section(s)	**Protocol Requirement**	**Deviation/Departure**
G (Body Weights) – page 10	Body Weights: Within 3 days of arrival, at group allocation, prior to initial exposure, weekly (morning), and prior to euthanasia	Prior to euthanasia, body weight for animal #129 was measured but inadvertently not recorded.

Root Cause: At necropsy, the person in charge of weighing animals prior to euthanasia weighed animal #129, but inadvertently failed to record the weight on the animal necropsy sheet.

Impact Assessment (check one):

☒ No impact on the integrity of study

☐ Impact or potential impact on the integrity of the study (explain below)

Corrective/Preventive Action:

☒ None required

Approved By:

Study Director/Principle Investigator: Darol Dodd, PhD

Darol E. Dodd 2/19/14

Signature/Date

Appendix 2

Inhalation Exposure Summary Report

24 pages

Report Prepared by:

K. Roberts 21. Feb. 2014

Kay C. Roberts, A. S. Date

Research Associate

Reviewed by:

2/21/14

Carl Parkinson, LATg Date

Research Associate

Report Approved by:

2/21/14

Darol E. Dodd, Ph.D., DABT Date

Study Director and Test Facility Manager

Senior Research Toxicologist and Manager,

Research Support Services

Protocol Title

4-Week Styrene Inhalation Lung Toxicity Study in Mice

Appendix Title

Inhalation Exposure Summary Report

Study Protocol

DEDSTY131-1

Author

Kay C. Roberts

Performing Laboratory

The Hamner Institutes for Health Sciences

6 Davis Drive

Research Triangle Park, NC 27709-2137

Study Sponsor

Styrene Information and Research Center

910 17th Street NW, #500-B

Washington, DC 20006

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Introduction

The purpose of the study was to evaluate the mode-of-action of styrene-induced lung toxicity by comparing gene expression and cell proliferation effects following inhalation exposure (0, 40, 120 ppm) in 3 male mouse strains with different genotypes. Specific enzymes of styrene metabolism aligned with specific genotypes may be identified as important contributors to producing lung toxicity. Understanding which metabolic pathways are involved with lung toxicity in mice via specific genotypes following styrene exposure may provide a prediction of potential lung toxicity in humans exposed to styrene.

Summary

Male mice were exposed via whole-body inhalation to styrene vapor at target concentrations of 0 (air-only control), 40, or 120 ppm, 6 hours per day, 5 days per week for either 1 week or 4 weeks duration. The concentration of styrene in each exposure chamber was monitored continuously using calibrated infrared spectrophotometers and recorded approximately every 30 minutes during each exposure period.

For the 1-week duration, the analytical means (± standard deviations) for the three styrene concentration groups were 118.9 (±1.2), 40.9 (±2.0), and 0.0 (±0.0) ppm for the target exposure concentrations of 120, 40, and 0 ppm, respectively. For the 4-week duration, the analytical means (± standard deviations) for the three styrene concentration groups were 119.1 (±2.5), 39.6 (±1.6), and 0.0 (±0.0) ppm. Exposure temperature and relative humidity means were 72 °F and 49-52%, respectively, for all exposure groups throughout the study.

Materials and Methods

Chemical

Styrene monomer, PO-11 Grade bulk (CAS No. 100-42-5) was received from Lyondell Chemical Company (LCC), a division of LyondellBasell, Houston TX, on 13-Aug-2013. Chemical and physical properties of styrene are listed in Table 1. A Certificate of Analysis (COA) was provided by LCC (Attachment 1). The COA indicated the test substance to be 99.95% (weight) styrene monomer. An

inhibitor of styrene polymer formation, t-butyl catechol (13 ppm), had been added to the test substance by LCC.

The Styrene test substance (Lot No. 130807.6008) was received in 16 glass bottles; each bottle contained approximately 800 mL. Upon receipt the styrene test substance was stored in a ventilated hood in Hallway 200U at ambient room temperature. When needed for pre-study work or to conduct animal exposures, individual bottles were moved to the generation hood (Hallway 200U) for each exposure chamber.

Generation System

Styrene exposure concentrations were generated by metering liquid styrene test substance from a secondary glass container into a stainless-steel J-tube generator using an FMI pump (QVG-50, FMI, Fluid Metering Inc., Syosset, NY). Each FMI pump was calibrated and set to a nominal flow rate calculated for each exposure concentration. The styrene was pumped into the upper portion of the J-tube generator and allowed to flow downward through glass beads, counter-current to a nitrogen gas flow of approximately 25 L/min. A heat jacket warmed the J-tube generator to approximately 100 to 110 °C as measured between the heat jacket and the outside of the J-tube. The heat and the increased surface area from the glass beads facilitated the vaporization of the styrene test substance. A sight gage at the bottom of the J-tube verified the total vaporization of the test substance. The vaporized styrene/nitrogen gas mixture was diluted counter-current with HEPA-filtered chamber supply air that flowed into the inhalation exposure chamber.

Exposure System

Whole-body animal inhalation exposures were conducted in $8m^3$ stainless-steel and glass exposure chambers. One chamber was used for each target exposure concentration (Figures 1 and 2). A supply fan, an exhaust fan and two butterfly dampers were used to control air flow through the chambers. Air flow was monitored and controlled with a Continuum Building Automation System (Andover Controls Corporation, Andover, MA) that uses flow stations to detect velocity pressure in the chamber exhaust duct. The air flow was calibrated with a hot-wire anemometer (Sierra Steel-Mass In-Line Mass Flow Meter, Model No. 760-N8-V4-PV1-V1-EO, Sierra Instruments Co., Monterey, CA). Each $8m^3$ exposure chamber was operated at an air flow rate of approximately 2000 L/min, providing approximately 15 air changes per hour.

Chamber temperature was measured near the center top of the chamber by a Pre-Con All Purpose Thermister Sensor (Model ST-A, Kele & Associates, Memphis, TN) connected to the Continuum Building Automation System. The thermister sensor was calibrated by comparing the thermister probe to a certified mercury thermometer. Chamber temperature was maintained at approximately 66 to 77 °F with a target temperature of 72 °F.

Chamber relative humidity was measured near the center top of the chamber by a Rotronic Humidity Sensor (Series 1200, Rotronic Instrument Corp., Huntington, NY) connected to the Continuum Building Automation System. The relative humidity sensor was calibrated by immersing the sensor probe in an atmosphere of known humidity generated from saturated salt solutions. Chamber relative humidity was maintained at approximately 30 to 70% with a target relative humidity of 50%.

Static pressure within each styrene exposure chamber was maintained at a slightly negative value relative to the room where the chamber was located by controlling the supply air flow into each chamber. Static pressure within the control chamber was maintained at a slightly positive value relative to the room where the chamber was located. Chamber static pressure was measured using a transducer (MODUS T30, Modus Instruments, Inc., Northboro, MA).

Chamber Concentration Analysis

Styrene exposure atmospheres within each $8m^3$ chamber were analyzed continuously during the exposure period using calibrated MIRAN infrared spectrophotometers (MIRAN 1A, The Foxboro Co., Foxboro, MA) with one MIRAN sampling system per exposure chamber. The operating conditions for each MIRAN are listed in Table 2. Voltage output from each MIRAN corresponding to the styrene exposure concentration was recorded continuously and logged by the Continuum Building Automation System (Andover Controls Corporation, Andover, MA). Styrene exposure concentrations were averaged at 5-minute intervals and these averages were used to calculate a 30-minute average.

Analytical Instrument Calibration

Each MIRAN was calibrated by injecting measured volumes of liquid styrene test substance into a closed loop of known volume. A metal bellows pump circulated the vaporized test substance in the closed loop. The data for the calibration curves were plotted using the output voltage of each MIRAN on the X-axis and the concentration (ppm) on the Y-axis. Calibration procedures were completed prior to animal inhalation exposures.

MIRAN calibrations were verified by analyzing styrene vapor concentrations from certified gas standard cylinders obtained from Airgas (Airgas Specialty Gases, Port Allen, LA).

Estimated Limit of Detection

The Estimated Limit of Detection (ELOD) was calculated during the calibration process using the standard deviation of the voltage output of the MIRAN at the lowest calibration point other than zero, and then multiplying by three. The lowest voltage detectable by the Continuum Building Automation System is 0.005 volt. ELODs are shown in Table 2.

Study Day Numbering

Study day numbering began with the first day of exposure and continued sequentially up to and including the day when the last animal was necropsied.

An animal exposure period began at approximately 8:00 am and continued for six hours until approximately 2:00 pm. The six-hour exposure was followed by a 30 minute clearance period to ensure that the test substance had cleared the exposure chamber prior to the animals being removed from the exposure chamber. The light cycle for animal exposure and non-exposure housing was 12 hours of light (7:00 am to 7:00 pm) followed by 12 hours of darkness and was controlled by the Continuum Building Automation System. The six-hour styrene inhalation exposure periods were conducted for 5 continuous days per week for either 1-week or 4-weeks duration from October 28, 2013 to November 22, 2013.

Environmental Parameters

Exposure Chambers

The chamber temperature, relative humidity, air flow, and static pressure data were recorded at 30 minute intervals by the Continuum Building Automation System. For each six-hour exposure period, the 30-minute recordings were reported on the Daily Exposure Report for each exposure chamber.

Domiciliary Housing

Animals were received and acclimated in Room 305 prior to the first day of styrene or air (control) exposure. Animals were housed during non-exposure periods in polycarbonate shoebox cages with micro-isolator lids in the anteroom of each $8m^3$ exposure chamber.

Statistical Procedures

Daily Exposure Reports included inhalation chamber temperature, relative humidity, air flow, static pressure, FMI pump flow rate (styrene), generator temperature (J-tube) and analytical chamber concentration of styrene. Daily statistics of exposure chamber parameters, including mean, standard deviation, and maximum and minimum values were calculated from each Daily Exposure Report. Grand average statistics (mean, standard deviation, maximum and minimum values) for these parameters were calculated from the daily mean values for each exposure group.

Project Personnel

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	Senior Research Toxicologist and Manager, Research Support Services
Kay C. Roberts, A. S.	Research Associate
Carl U. Parkinson, LATg	Research Associate

Results

Test Substance

The identity, purity, stability and method of synthesis, fabrication or derivation of the styrene test substance were the responsibility of the Sponsor. A certificate of analysis received from the Sponsor indicated that the test substance was 99.95% styrene monomer. Further communication from the test substance manufacturer (Lyondell Chemical Company, a division of LyondellBasell) indicated that the test substance would be stable throughout the study period.

Chamber Distribution

Results of the styrene vapor chamber distribution measurements are shown in Tables 3a – 3b. The total port, within-port, and between-port variations were less than 5% for each exposure chamber used, indicating that the test substance was uniformly distributed throughout each exposure chamber. The chamber exposure distributions were performed during pre-study testing, prior to the beginning of the animal exposures.

Vapor Generation, Chamber Sytrene Concentrations, and Environmental Exposure Conditions

Table 4a shows the 1-week summary exposure data for styrene vapor generation, styrene vapor characterization, and environmental exposure conditions for each of the exposure groups. Table 4b shows the 4-week summary exposure data. The grand mean and standard deviation of the average daily means for analytical chamber concentration (ACC), air flow rate, temperature, relative humidity, static pressure, FMI pump flow rate, and J-tube generator temperature are reported. The minimum and maximum daily mean values are also shown.

The grand means (± standard deviations) of analytical styrene concentrations for the 1-week exposure group were 118.9 (± 1.2), 40.9 (± 2.0), and 0.0 (± 0.0) ppm for target exposure concentrations of 120, 40, and 0 ppm, respectively. Minimum to maximum daily mean ranges for the three exposure groups were 117.6 to 120.3 ppm, 37.8 to 43.4 ppm, and 0.0 to 0.0 ppm.

The grand means (± standard deviations) of analytical styrene concentrations for the 4-week exposure group were 119.1 (± 2.5), 39.6 (± 1.6), and 0.0 (± 0.0) ppm for target exposure concentrations of 120, 40, and 0 ppm, respectively. Minimum to maximum daily mean ranges for the three exposure groups were 109.9 to 122.1 ppm, 36.2 to 43.4 ppm, and 0.0 to 0.0 ppm.

The daily mean values during exposures for analytical chamber concentration, temperature, relative humidity, air flow rate, static pressure, FMI pump flow rate, and generator temperature for each exposure day are shown in Tables 5a, 5b, and 5c (target exposure styrene concentrations of 120, 40, and 0 ppm, respectively).

Deviations from the Protocol or Standard Operating Procedures

Protocol - Exposure Chamber Deviations

Table 6 lists the exposure chamber deviations for Study Day 19 (15-Nov-2013). The chamber parameter, protocol requirement, deviation, duration of deviation, and the deviation's impact on the study are shown. The deviations were caused by a power interruption causing failure of the prime and redundant fans to supply air flow to the exposure chambers. During the period of time when the exposure fans were not operating properly (65 minutes), the delivery of styrene test material to the J-tube generators was stopped. Chamber styrene concentrations deviated greater than 10% below the target exposure concentrations for approximately 30 minutes of the 65-minute power failure. To make

up this difference, the exposures were extended for 30 minutes thus providing a 6 hour daily exposure to target styrene concentrations.

Standard Operating Procedures

There were no SOP deviations.

Table 1 Chemical and Physical Properties of Styrene [1]

Synonyms	Styrene Monomer
Appearance	Colorless to Yellow Liquid
Molecular formula	$C_6H_{12}O$
CAS No.	100-42-5
Molecular weight	104.15 g/mole
Boiling point	293 °F (145 °C)
Flash point	88 °F (31 °C)
Lower flammability limit	~1 vol %
Vapor pressure	6.67 hPa (5.00 mm Hg) at 20 °C
Received from:	Lyondell Chemical Company, Houston, TX
Batch No.	130807.6008

[1] Source: MSDS LyondellBasell Chemical Company

Table 2 MIRAN [1] Operating Conditions

Instrument	MIRAN	MIRAN	MIRAN
Serial No.	4159	4432	3064
Sampling Location	Chamber 297	Chamber 299	Chamber 305
Concentration	120 ppm	40 ppm	0 ppm
Pathlength:	20.25 meters	20.25 meters	20.25 meters
Wavelength:	3.32 microns	3.2 microns	3.28 microns
Slit:	1 mm	1 mm	1 mm
Coarse Zero:	X 10	X 10	X 10
Range:	1A	1A	1A
Meter Response:	1	1	1
ELOD[2]	2.37 ppm	2.11 ppm	2.03 ppm

[1] MIRAN is an infrared spectrophotometer manufactured by The Foxboro Co., Foxboro, MA

[2] Estimated Limit of Detection

Table 3a Styrene Vapor Distribution in 120 ppm Exposure Chamber

120 ppm Styrene — Chamber 297

Sample Location	Total Port Concentration (TP) (ppm)	Temporal or Within Port Concentration (WP) (ppm)
Home Port[1] 9		119.9
1	120.30	
2	120.80	
3	121.80	
Home Port 9		120.30
4	120.80	
5	120.30	
6	119.90	
Home Port 9		120.30
7	122.70	
8	119.40	
Home Port 9		119.40
Home Port 9 (mean)	119.98	
Mean	120.66	119.98
Std. Dev.	1.02	0.43
N	9	4
Total Port Variation (TPCV)[2] =		0.85 %
Within Port Variation (WPCV)[3] =		0.36 %
Between Port Variation (BPCV)[4] =		0.77 %

[1] Sample location 9 is referred to as the Home Port for the Distribution test. The Home Port is located in the center of the chamber and represents the average of 3 or more samples taken at this position during the Distribution test. Other positions are located near the corners of the chamber or exposure cage units.

[2] TPCV = Total Port Measurements Coefficient of Variation: (St Dev TP/Average TP) * 100

[3] WPCV = Within Port Measurements Coefficient of Variation: (St Dev WP/Average WP) * 100

[4] BPCV = Between Port Measurements Coefficient of Variation: $SqRt[(TPCV)^2 - (WPCV)^2]$

Table 3b Styrene Vapor Distribution in 40 ppm Exposure Chamber

40 ppm Styrene	Chamber 299	
Sample Location	Total Port Concentration (TP) (ppm)	Temporal or Within Port Concentration (WP) (ppm)
Home Port[1] 9		40.1
1	40.1	
2	38.5	
3	41.0	
Home Port 9		40.1
4	41.4	
5	40.1	
6	38.9	
Home Port 9		40.1
7	44.4	
8	39.7	
Home Port 9		40.1
Home Port 9 (mean)	40.1	
Mean	40.47	40.1
Std. Dev.	1.73	0.0
N	9	4
Total Port Variation (TPCV)[2] =		4.27 %
Within Port Variation (WPCV)[3] =		0.0 %
Between Port Variation (BPCV)[4] =		4.27 %

[1] Sample location 9 is referred to as the Home Port for the Distribution test. The Home Port is located in the center of the chamber and represents the average of 3 or more samples taken at this position during the Distribution test. Other positions are located near the corners of the chamber or exposure cage units.

[2] TPCV = Total Port Measurements Coefficient of Variation: (St Dev TP/Average TP) * 100

[3] WPCV = Within Port Measurements Coefficient of Variation: (St Dev WP/Average WP) * 100

[4] BPCV = Between Port Measurements Coefficient of Variation: SqRt[$(TPCV)^2 - (WPCV)^2$]

Table 4a Summary of Exposure Data for 1-Week Exposure Group

	Styrene (ppm)	120	40	0.0
ACC (ppm)	**Group mean**	**118.94**	**40.93**	**0.0**
	Std Dev	1.19	2.02	0.0
	Minimum daily mean[1]	117.59	37.77	0.0
	Maximum daily mean[1]	120.31	43.35	0.0
	No. of Data Points	5	5	5
Exposure Temperature (°F)	**Group mean**	**72.1**	**72.0**	**72.1**
	Std Dev	0.0	0.1	0.1
	Minimum daily mean [1]	72.0	71.9	72.0
	Maximum daily mean[1]	72.1	72.1	72.2
	No. of Data Points	5	5	5
Exposure Relative Humidity (%)	**Group mean**	**50.6**	**50.1**	**51.9**
	Std Dev	0.9	0.2	1.7
	Minimum daily mean [1]	49.6	50.0	50.2
	Maximum daily mean[1]	52.0	50.4	54.6
	No. of Data Points	5	5	5
Air Flow (L/min)	**Group mean**	**2000.1**	**2001.2**	**2000.1**
	Std Dev	0.9	1.0	0.1
	Minimum daily mean [1]	1999.2	1999.8	2000.0
	Maximum daily mean[1]	2001.3	2002.3	2000.2
	No. of Data Points	5	5	5
Exposure Static Pressure (in H_20)	**Group mean**	**-0.100**	**-0.099**	**0.101**
	Std Dev	0.001	0.001	0.001
	Minimum daily mean [1]	-0.101	-0.101	0.100
	Maximum daily mean[1]	-0.099	-0.098	0.102
	No. of Data Points	5	5	5
Exposure FMI Pump Flow Rate (mL/min)	**Group mean**	**1.07**	**0.31**	**nap**
	Std Dev	0.01	0.01	nap
	Minimum daily mean [1]	1.06	0.31	nap
	Maximum daily mean[1]	1.08	0.33	nap
	No. of Data Points	5	5	nap
Generator Temperature (°C)	**Group mean**	**104.1**	**106.8**	**nap**
	Std Dev	0.5	2.0	nap
	Minimum daily mean [1]	103.6	105.1	nap
	Maximum daily mean[1]	104.9	110.1	nap
	No. of Data Points	5	5	nap

ACC: Analytical Chamber Concentration
Std Dev: Standard Deviation (± value)
nap: Not applicable
[1] See Table 5a – 5c for the individual daily means for the exposure

Table 4b Summary of Exposure Data for 4-Week Exposure Group

	Styrene (ppm)	120	40	0.0
ACC (ppm)	**Group mean**	**119.13**	**39.57**	**0.0**
	Std Dev	2.48	1.57	0.0
	Minimum daily mean[1]	109.86	36.20	0.0
	Maximum daily mean[1]	122.06	43.35	0.0
	No. of Data Points	20	20	20
Exposure Temperature (°F)	**Group mean**	**71.8**	**72.1**	**72.0**
	Std Dev	0.7	0.2	0.1
	Minimum daily mean [1]	69.1	71.8	71.9
	Maximum daily mean[1]	72.1	72.9	72.2
	No. of Data Points	20	20	20
Exposure Relative Humidity (%)	**Group mean**	**48.8**	**50.0**	**50.4**
	Std Dev	4.1	0.3	1.3
	Minimum daily mean [1]	35.6	49.4	48.4
	Maximum daily mean[1]	52.0	50.5	54.6
	No. of Data Points	20	20	20
Air Flow (L/min)	**Group mean**	**1989.8**	**1990.4**	**1987.9**
	Std Dev	43.4	43.8	54.8
	Minimum daily mean [1]	1805.7	1804.5	1754.9
	Maximum daily mean[1]	2001.3	2003.8	2001.6
	No. of Data Points	20	20	20
Exposure Static Pressure (in H_20)	**Group mean**	**-0.099**	**-0.098**	**0.100**
	Std Dev	0.004	0.007	0.003
	Minimum daily mean [1]	-0.101	-0.103	0.086
	Maximum daily mean[1]	-0.082	-0.072	0.103
	No. of Data Points	20	20	20
Exposure FMI Pump Flow Rate (mL/min)	**Group mean**	**1.11**	**0.32**	**nap**
	Std Dev	0.03	0.01	nap
	Minimum daily mean [1]	1.06	0.31	nap
	Maximum daily mean[1]	1.14	0.33	nap
	No. of Data Points	20	20	nap
Generator Temperature (°C)	**Group mean**	**105.6**	**107.0**	**nap**
	Std Dev	1.3	1.6	nap
	Minimum daily mean [1]	103.6	104.0	nap
	Maximum daily mean[1]	107.4	110.1	nap
	No. of Data Points	20	20	nap

ACC: Analytical Chamber Concentration
Std Dev: Standard Deviation (± value)
nap: Not applicable
[1] See Table 5a – 5c for the individual daily means for the exposure

Table 5a Inhalation Data for 120 ppm Exposure Group

Date	Study Day	Temp (deg F)	Humidity (%)	Air Flow (L/min)	Static Pressure (in of H_2O)	FMI Pump Flow Rate (mL/min)	Generator Temperature (deg C)	Analytical Chamber Concentration (ppm)
28-Oct-2013	1	72.0	52.0	1999.2	-0.100	1.06	104.9	117.59
29-Oct-2013	2	72.1	50.3	2001.3	-0.101	1.07	103.7	119.87
30-Oct-2013	3	72.1	50.8	2000.4	-0.100	1.07	104.0	119.03
31-Oct-2013	4	72.1	49.6	1999.3	-0.100	1.07	103.6	120.31
01-Nov-2013	5	72.1	50.1	2000.4	-0.099	1.08	104.4	117.90
04-Nov-2013	8	72.1	39.1	2000.3	-0.100	1.09	107.2	117.53
05-Nov-2013	9	71.8	45.8	2000.5	-0.099	1.10	107.4	120.06
06-Nov-2013	10	72.0	52.0	1999.8	-0.099	1.09	105.2	118.91
07-Nov-2013	11	72.0	50.4	1999.5	-0.101	1.10	105.1	119.56
08-Nov-2013	12	71.9	35.6	2000.4	-0.099	1.11	106.9	118.13
11-Nov-2013	15	72.1	50.0	2001.1	-0.100	1.12	105.8	122.06
12-Nov-2013	16	69.1	49.8	2000.4	-0.098	1.12	106.2	119.20
13-Nov-2013	17	72.1	49.9	1996.5	-0.100	1.12	105.0	120.48
14-Nov-2013	18	71.8	49.9	1995.7	-0.101	1.12	106.9	119.36
15-Nov-2013	19	71.9	49.6	1805.7	-0.082	1.13	107.1	109.86
18-Nov-2013	22	71.7	50.4	1999.4	-0.101	1.13	103.6	120.65
19-Nov-2013	23	72.1	50.0	2001.3	-0.100	1.13	106.1	120.09
20-Nov-2013	24	72.0	50.2	1998.7	-0.100	1.13	106.3	120.82
21-Nov-2013	25	72.0	50.0	1997.0	-0.101	1.13	106.7	120.78
22-Nov-2013	26	71.8	50.6	2000.0	-0.100	1.14	106.5	120.33
1-Week Group (Study Day 1 to Study Day 5)								
	Mean	72.1	50.6	2000.1	-0.100	1.07	104.1	118.94
	St Dev	0.0	0.9	0.9	0.001	0.01	0.5	1.19
Minimum Daily Mean		72.0	49.6	1999.2	-0.101	1.06	103.6	117.59
Maximum Daily Mean		72.1	52.0	2001.3	-0.099	1.08	104.9	120.31
	Count	5	5	5	5	5	5	5
4-Week Group (Study Day 1 to Study Day 26)								
	Mean	71.8	48.8	1989.8	-0.099	1.11	105.6	119.13
	St Dev	0.7	4.1	43.4	0.004	0.03	1.3	2.48
Minimum Daily Mean		69.1	35.6	1805.7	-0.101	1.06	103.6	109.86
Maximum Daily Mean		72.1	52.0	2001.3	-0.082	1.14	107.4	122.06
	Count	20	20	20	20	20	20	20

Table 5b Inhalation Data for 40 ppm Exposure Group

Date	Study Day	Temp (deg F)	Humidity (%)	Air Flow (L/min)	Static Pressure (in of H_2O)	FMI Pump Flow Rate (mL/min)	Generator Temperature (deg C)	Analytical Chamber Concentration (ppm)
28-Oct-2013	1	71.9	50.4	1999.8	-0.099	0.33	110.1	41.21
29-Oct-2013	2	72.0	50.0	2002.1	-0.101	0.31	105.1	43.35
30-Oct-2013	3	72.0	50.0	2000.7	-0.098	0.31	106.3	40.78
31-Oct-2013	4	72.0	50.0	2001.3	-0.100	0.31	106.7	41.54
01-Nov-2013	5	72.1	50.0	2002.3	-0.098	0.31	105.7	37.77
04-Nov-2013	8	72.1	49.4	1997.2	-0.096	0.31	109.1	37.10
05-Nov-2013	9	72.3	50.0	1999.2	-0.098	0.32	108.5	39.81
06-Nov-2013	10	72.9	49.8	1999.9	-0.093	0.32	106.0	38.67
07-Nov-2013	11	71.9	50.2	2000.3	-0.101	0.32	106.4	39.32
08-Nov-2013	12	71.9	50.0	1998.3	-0.100	0.32	108.0	39.33
11-Nov-2013	15	72.0	49.8	2003.8	-0.102	0.32	107.0	38.67
12-Nov-2013	16	72.1	49.7	1999.2	-0.099	0.32	109.9	39.39
13-Nov-2013	17	72.1	49.9	1999.8	-0.100	0.32	106.4	40.36
14-Nov-2013	18	72.0	49.8	1997.9	-0.098	0.32	107.8	39.29
15-Nov-2013	19	71.9	50.5	1804.5	-0.072	0.32	107.9	36.20
18-Nov-2013	22	71.8	50.2	1999.6	-0.103	0.32	104.0	39.46
19-Nov-2013	23	72.1	49.7	2001.3	-0.100	0.32	106.3	39.02
20-Nov-2013	24	72.0	50.2	1999.1	-0.101	0.32	107.4	39.56
21-Nov-2013	25	71.9	50.4	1999.4	-0.102	0.32	106.7	40.15
22-Nov-2013	26	72.0	50.4	2001.3	-0.103	0.32	105.2	40.34
1-Week Group (Study Day 1 to Study Day 5)								
	Mean	72.0	50.1	2001.2	-0.099	0.31	106.8	40.93
	St Dev	0.1	0.2	1.0	0.001	0.01	2.0	2.02
Minimum Daily Mean		71.9	50.0	1999.8	-0.101	0.31	105.1	37.77
Maximum Daily Mean		72.1	50.4	2002.3	-0.098	0.33	110.1	43.35
	Count	5	5	5	5	5	5	5
4-Week Group (Study Day 1 to Study Day 26)								
	Mean	72.1	50.0	1990.4	-0.098	0.32	107.0	39.57
	St Dev	0.2	0.3	43.8	0.007	0.01	1.6	1.57
Minimum Daily Mean		71.8	49.4	1804.5	-0.103	0.31	104.0	36.20
Maximum Daily Mean		72.9	50.5	2003.8	-0.072	0.33	110.1	43.35
	Count	20	20	20	20	20	20	20

Table 5c Inhalation Data for 0.0 ppm Exposure Group

Date	Study Day	Temp (deg F)	Humidity (%)	Air Flow (L/min)	Static Pressure (in of H_2O)	Analytical Chamber Concentration (ppm)
28-Oct-2013	1	72.1	54.6	2000.0	0.100	0.0
29-Oct-2013	2	72.2	50.8	2000.0	0.100	0.0
30-Oct-2013	3	72.1	50.2	2000.1	0.101	0.0
31-Oct-2013	4	72.0	51.7	2000.2	0.100	0.0
01-Nov-2013	5	72.0	52.1	2000.1	0.102	0.0
04-Nov-2013	8	71.9	49.8	2000.0	0.099	0.0
05-Nov-2013	9	72.0	50.0	2001.6	0.099	0.0
06-Nov-2013	10	72.0	50.6	2000.0	0.100	0.0
07-Nov-2013	11	72.0	49.5	1999.9	0.101	0.0
08-Nov-2013	12	72.0	49.6	1999.9	0.103	0.0
11-Nov-2013	15	72.0	49.8	2000.2	0.101	0.0
12-Nov-2013	16	72.0	50.3	2000.0	0.101	0.0
13-Nov-2013	17	71.9	50.2	1999.7	0.100	0.0
14-Nov-2013	18	72.0	49.7	2000.3	0.099	0.0
15-Nov-2013	19	72.2	51.9	1754.9	0.086	0.0
18-Nov-2013	22	71.9	49.7	2000.0	0.100	0.0
19-Nov-2013	23	72.1	48.4	2000.1	0.101	0.0
20-Nov-2013	24	72.1	50.0	2000.0	0.101	0.0
21-Nov-2013	25	72.0	49.6	2000.3	0.098	0.0
22-Nov-2013	26	72.0	50.1	2000.0	0.101	0.0
1-Week Group (Study Day 1 to Study Day 5)						
	Mean	72.1	51.9	2000.1	0.101	0.0
	St Dev	0.1	1.7	0.1	0.001	0.0
Minimum Daily Mean		72.0	50.2	2000.0	0.100	0.0
Maximum Daily Mean		72.2	54.6	2000.2	0.102	0.0
	Count	5	5	5	5	5
4-Week Group (Study Day 1 to Study Day 26)						
	Mean	72.0	50.4	1987.9	0.100	0.0
	St Dev	0.1	1.3	54.8	0.003	0.0
Minimum Daily Mean		71.9	48.4	1754.9	0.086	0.0
Maximum Daily Mean		72.2	54.6	2001.6	0.103	0.0
	Count	20	20	20	20	20

Table 6a Exposure Chamber Deviations for 120 ppm Exposure Group

Date	Study Day	Parameter	Protocol Requirement	Deviation	Duration	Impact on Study
15-Nov-2013	19	Air Flow	Approximately 2000 L/min	731.8 L/min	65 minutes	No
15-Nov-2013	19	Static Pressure	Slightly negative value relative to room	0.012 – 0.009 inches of H_20	65 minutes	No
15-Nov-2013	19	Styrene Concentration	Continuous 6 hours exposure of target concentration	> 10% below the target concentration	30 minutes	No

Table 6b Exposure Chamber Deviations for 40 ppm Exposure Group

Date	Study Day	Parameter	Protocol Requirement	Deviation	Duration	Impact on Study
15-Nov-2013	19	Air Flow	Approximately 2000 L/min	398.3 – 404.4 L/min	65 minutes	No
15-Nov-2013	19	Static Pressure	Slightly negative value relative to room	0.042 – 0.012 inches of H_20	65 minutes	No
15-Nov-2013	19	Styrene Concentration	Continuous 6 hours exposure of target concentration	> 10% below the target concentration	30 minutes	No

Table 6c Exposure Chamber Deviations for 0.0 ppm Exposure Group

Date	Study Day	Parameter	Protocol Requirement	Deviation	Duration	Impact on Study
15-Nov-2013	19	Air Flow	Approximately 2000 L/min	405.0 L/min	65 minutes	No

Figure 1 Example of Control Chamber Exposure System Diagram

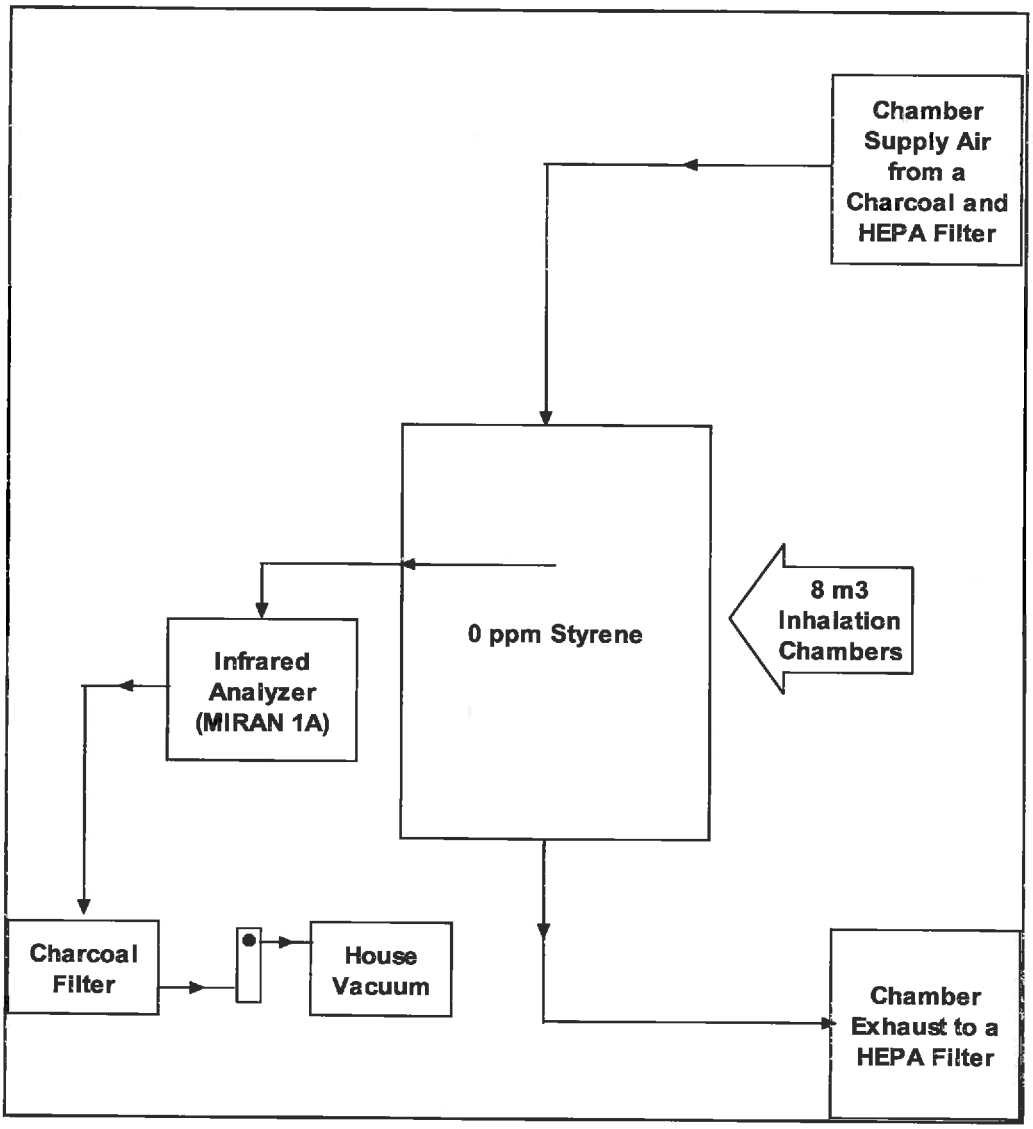
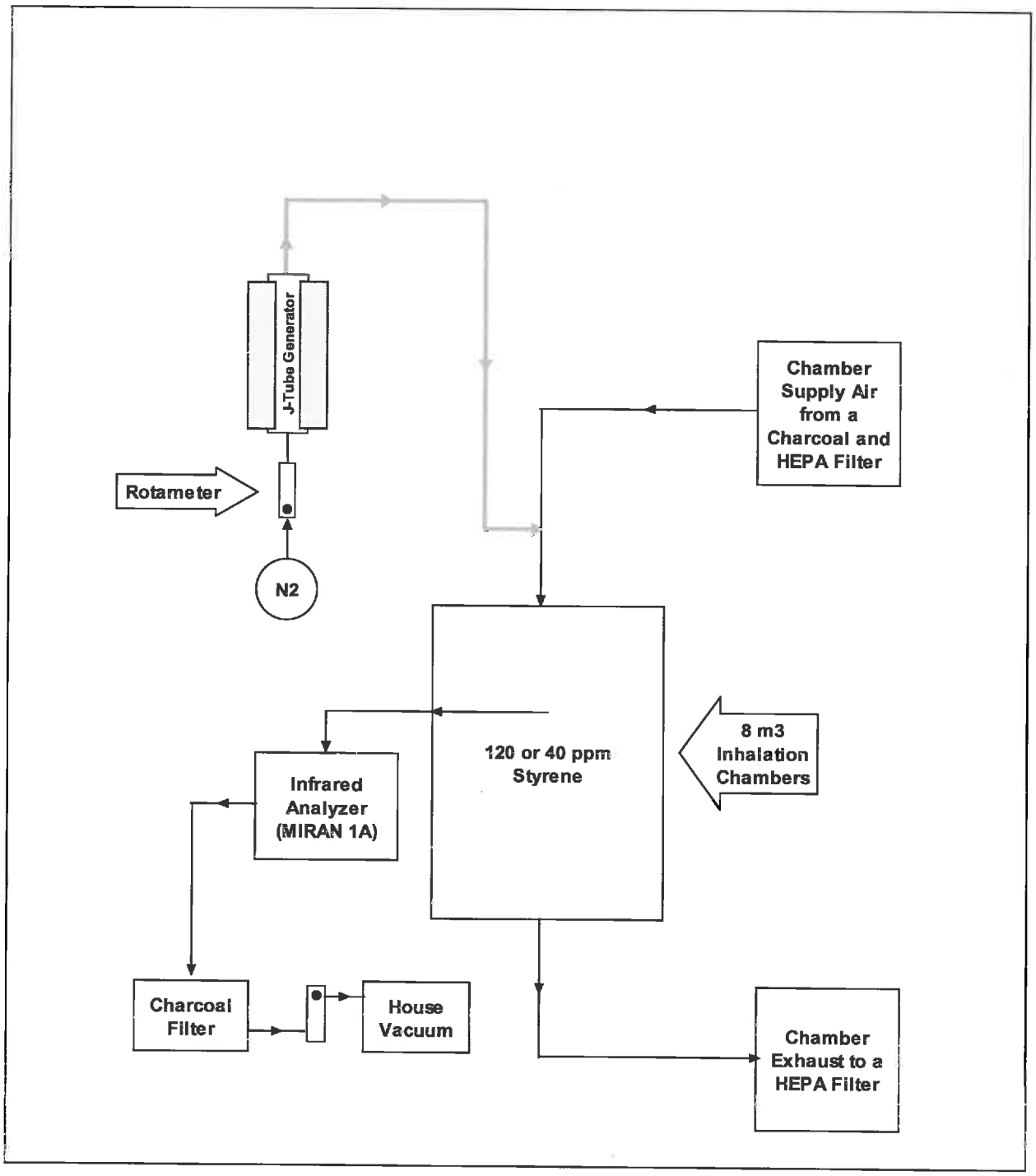


Figure 2 Example of Styrene Exposure System Diagram



Attachment 1 Certificate of Analysis

lyondellbasell

2502 Sheldon Road
Channelview, TX 77530

Temporary Certificate of Analysis for Styrene Monomer

Ship- To Address
The Hamner Institutes
6 Davis Drive
RTP, NC 27709

Lyondell Material 499243 Styrene Monomer **Date shipped** August 7, 2013

Styrene Monomer

Test Descriptions	Test Results	Sales Specifications Minimum	Maximum	Unit of Measure
Lot Number	130807.0008			
Styrene Monomer assay	99.98	99.95		weight %
Color (ASTM D-5386)	9		15	APHA Color
Ethylbenzene	84		100	ppm
Polymer	2		10	ppm
Aldehydes as Benzaldehyde	46		80	ppm
t-Butyl Catechol	13	10	15	ppm
Peroxides as H2O2	12		30	ppm
Ethylbenzene	84		100	ppm
a-Methyl Styrene	220		400	ppm
Benzene	<1		1	ppm
Water	133		220	ppm
Toluene	<1			ppm
Cumene	<1		250	ppm
2-MethylPentanol	36			ppm
o-Xylenes	<1			ppm
p/m Xylenes	4			ppm
Benzaldehyde	23			ppm
Alkyl Benzene	17			ppm
ACPBA	13			ppm
SO	0			ppm
Phenol	8			ppm
Total SM Impurities	485			ppm
Sum SM Olefins	104			
Total Vinylbenzene	3			
CIS-BETA-MS	8			
A-MS/B-MS	228			
Appearance	Pass			
Meets Manuf Spec	Conf			

Note: This is a temporary Certificate of Analysis based on sales specs.

This material was produced in an approved Quality Assurance Lab
Questions? Customer Service [illegible] 0232

Appendix 3

Histopathology and Cell Proliferation Report

44 pages

charles river

FINAL PATHOLOGY REPORT

Study Phase: Histopathology and Cell Proliferation

Test Site Phase Reference No. 20046536

Testing Facility Study No. DEDSTY131-1

4-Week Styrene Inhalation Lung Toxicity Study in Mice

SPONSOR:
Styrene Information and Research Center
910 17th Street NW, #500-B
Washington, DC 20006
USA

TESTING FACILITY:
The Hamner Institutes for Health Science
Post Office Box 12137
6 Davis Drive
Research Triangle Park, NC 27709
USA

TEST SITE:
Charles River Laboratories
Pathology Associates, Maryland
15 Worman's Mill Court, Suite I
Frederick, MD 21701
USA

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QUALITY ASSURANCE STATEMENT

Protocol: DEDSTY131-1

This phase has been audited by the Quality Assurance Unit in accordance with the applicable Good Laboratory Practice regulations. Reports were submitted in accordance with standard operating procedures as follows:

QA INSPECTION DATES

		Dates Findings Submitted to:			
Date(s) of Audit	Phase(s) Audited	Principal Investigator	Principal Investigator Management	Study Director	Study Director Management
08-Jan-2014 - 08-Jan-2014	Draft Phase Report - Pathology	08-Jan-2014	08-Jan-2014	13-Jan-2014	13-Jan-2014
07-Jan-2014 - 08-Jan-2014	Data Review - Histology	08-Jan-2014	08-Jan-2014	13-Jan-2014	13-Jan-2014
11-Feb-2014	Final Phase Report - Pathology	12-Feb-2014	12-Feb-2014	12-Feb-2014	12-Feb-2014

Process-based inspections relevant to this study were conducted according to a predetermined schedule. The outcome of each inspection was reported to Management and, where relevant for processes seen as part of a study, the Study Director.

Facilities relevant to this study are included in Charles River's annual facility inspection programme. The outcome of each inspection is reported to Management.

Carla L. Rein

Lind, Carla L
Quality Assurance Auditor

18-Feb-2014
Date

COMPLIANCE STATEMENT AND REPORT APPROVAL

The histopathology and cell proliferation phase of this study conducted in the USA was performed in accordance with the United States Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice), Japan (MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

This phase of the study was conducted in accordance with the procedures described herein. All deviations authorized/acknowledged by the Study Director are documented in the Study Records. The report represents an accurate and complete record of the results obtained for this study phase.

There were no deviations from the above regulations that affected the overall integrity of this study phase or the interpretation of the phase results and conclusions.

_____ Date: 21-Feb-2014

James T. Raymond, DVM, MS, DACVP
Principal Investigator

1. SUMMARY

Inhalation of 120 ppm styrene for 6 hours per day for 5 days by C57Bl/6 wild type (WT) mice resulted in an approximate 7-fold increase in BrdU labeling index of epithelial cells lining the lung terminal bronchioles compared with WT mice receiving 0 ppm styrene. WT mice receiving 40 or 120 ppm styrene for either 1-week or 4-weeks had microscopic lesions in the lungs consisting of decreased eosinophilia of bronchiolar epithelium mainly lining terminal bronchioles; degeneration, necrosis, exfoliation, and/or attenuation of individual bronchiolar epithelial cells (most likely Clara cells) mainly lining terminal bronchioles; and/or hyperplasia of the bronchiolar epithelium mainly lining the terminal bronchioles that sometimes extended into the distally connected alveolar duct. No CYP2F2(-/-) (knockout; KO) or CYP2F2(-/-)2F1, 2A13, 2B6-tg (transgenic; TG) mice had styrene-induced microscopic lesions after either 1- or 4-weeks of styrene inhalation at 40 or 120 ppm.

Overall, the microscopic findings in the WT mice at the 1- and 4-week timepoints did not appear to be dose dependent except for the degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium mainly lining terminal bronchioles at the 4-week timepoint, which appeared slightly more severe in WT mice receiving 120 ppm styrene compared with WT mice receiving 40 ppm styrene.

The mean severity scores for most microscopic lesions were greater in the WT mice from the 1-week timepoint compared with WT mice from the 4-week timepoint. However, the mean severity score for hyperplasia of bronchiolar epithelium mainly lining terminal bronchioles with extension into adjacent alveolar duct was greater in the WT mice from the 4-week timepoint compared with WT mice from the 1-week timepoint.

2. RESPONSIBLE PERSONNEL

Principal Investigator, Histopathology and Cell Proliferation	James T. Raymond, DVM, MS, DACVP Charles River Laboratories, Pathology Associates, Maryland
Test Site Management	Kevin S. McDorman, DVM, PhD, DACVP Charles River Laboratories, Pathology Associates, Maryland

3. INTRODUCTION

This report presents the histopathology findings and cell proliferation analysis in lung tissue from mice assigned to Study No. DEDSTY131-1.

The study was sponsored by Styrene Information and Research Center, Washington DC. Darol E. Dodd, PhD, served as the Study Director.

4. MATERIALS AND METHODS

Experimental procedures applicable to pathology investigations are summarized in Text Table 1. Deviations to the pathology procedures performed by Charles River Laboratories, Pathology Associates, Maryland, are listed in Appendix 1.

Text Table 1
Experimental Design

Group	Dose	Endpoints	Number and Strain	Animal Identification	Timepoint
Air only (control)	0 ppm	Genomics & Histopathology	8 WT 8 KO 8 TG	1-8 101-108 201-208	1-week
Air only (control)	0 ppm	Cell proliferation & Histopathology	6 WT	49-54	1-week
Styrene	40 ppm	Genomics & Histopathology	8 WT 8 KO 8 TG	9-16 109-116 209-216	1-week
Styrene	120 ppm	Genomics & Histopathology	8 WT 8 KO 8 TG	17-24 117-124 217-224	1-week
Styrene	120 ppm	Cell proliferation & Histopathology	6 WT	55-60	1-week
Air only (control)	0 ppm	Genomics & Histopathology	8 WT 8 KO 8 TG	25-32 125-132 225-232	4-week
Styrene	40 ppm	Genomics & Histopathology	8 WT 8 KO 8 TG	33-40 133-140 233-240	4-week
Styrene	120 ppm	Genomics & Histopathology	8 WT 8 KO 8 TG	41-48 141-148 241-248	4-week
WT = wild type; KO = knockout; TG = transgenic					

All surviving animals from the genomics and cell proliferation endpoints were submitted for necropsy one day after the last inhalation exposure (1-week or 4-week timepoint).

4.1. Histopathology

For the animals assigned for histopathology (but not cell proliferation), the left lobe of the lung was collected, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at approximately 5 µm, mounted on slides, and stained with hematoxylin and eosin by the Hamner Institutes for Health Sciences personnel. The slides were then shipped to Charles River Laboratories, Pathology Associates, Maryland for microscopic evaluation. Microscopic evaluation was conducted by the Principal Investigator, a board-certified veterinary pathologist.

Tissues were evaluated by light microscopy including identification of animal numbers and treatment groups. Severity of microscopic findings were scored as follows.

0=normal/not present

1=minimal

2=mild

3-moderate

4=marked

5=severe

Mean severity scores were calculated as total score for all animals evaluated divided by number of animals evaluated.

4.2. Cell Proliferation

For the animals assigned to the cell proliferation and histopathology endpoint, the whole lung and a small piece of the proximal duodenum were collected and fixed in 10% neutral buffered formalin by the Hamner Institutes for Health Sciences personnel. A section of the left lobe and the duodenum were embedded in paraffin, and the resulting blocks were shipped to Charles River Laboratories, Pathology Associates, Maryland. A limited number of the duodenum blocks were used only as a positive control for staining consistency across all staining runs, and cell proliferation analysis and microscopic evaluation was not conducted for this tissue. Each lung block was sectioned at approximately 5 µm to produce slides for cell proliferation analysis and histopathology evaluation.

The slides designated for histopathology evaluation were stained with hematoxylin and eosin by Charles River Laboratories, Pathology Associates, Maryland personnel for microscopic evaluation. Microscopic evaluation was conducted by the Principal Investigator, a board-certified veterinary pathologist. Tissues were evaluated by light microscopy.

The slides designated for cell proliferation analysis were immunohistochemically (IHC) stained with a commercially available marker to detect BrdU. One slide was stained with a rat anti-BrdU antibody (detection reagent), while another slide was stained with rat IgG2a (negative control antibody). The third slide was used as an assay control, wherein the detection reagent or negative control antibody was omitted from the assay. For the lung cell proliferation analysis, approximately 300-650 stained and unstained epithelial cells lining the terminal bronchioles (5-12 per animal) were counted per animal. A terminal bronchiole was identified as a small diameter conducting airway, lined by simple cubodal respiratory epithelium, with its distal end connected to an alveolar duct. The cumulative BrdU labeling index was calculated for each animal by dividing the total number of BrdU positive cells by the total number of counted epithelial cells (stained and unstained).

4.3. Computerized Systems

Critical computerized systems used in the study by the Test Site are listed in Text Table 2.

Text Table 2
Computerized Systems

System Name	Version No.	Description of Data Collected and/or Analyzed
Microsoft Office Excel	2007	Histopathology and Cell Proliferation

4.4. Disposition of Study Materials

All study-specific raw data, pathology materials, documentation, and Final Report generated from this study phase will be sent to the following address for archiving:

Nigel Edgerton, BS
The Hamner Institutes for Health Sciences
6 Davis Drive
Research Triangle Park, NC 27709
Tel: 919.558.1321
E-mail: nedgerton@thehamner.org

Study materials will be retained for a period of 6 months following issue of the Final Report.

5. RESULTS AND DISCUSSIONS

5.1. Mortality

There were no unscheduled deaths during the course of this study. Prior to the start of the study (during housing acclimation period), one KO mouse (#148) was found dead with no obvious cause of death. Additional KO animals were not available to replace this animal on the study.

5.2. Cell Proliferation

The BrdU labeling index is presented by animal in Table 1.

For the control group (0 ppm styrene for 5 days), the BrdU labeling index was 5.11% (± 1.35%) with a range of 2.84%-6.42%. For the treatment group (120 ppm styrene for 5 days), the BrdU labeling index was 34.21% (± 9.81%) with a range of 21.74%-49.15%.

Microscopic findings in the lung for the animals assigned to the cell proliferation endpoint are presented in Table 2.

No histopathologic findings were noted in any control group animal. In all animals treated with 120 ppm styrene for 5 days, there was minimal degeneration, necrosis, exfoliation, and/or attenuation of individual bronchiolar epithelial cells (most likely Clara cells) mainly lining terminal bronchioles. In addition, there was minimal hyperplasia of the bronchiolar epithelium mainly lining the terminal bronchioles in four animals (56, 58, 59, and 60), which in two animals (56 and 60) extended into the distally connected alveolar duct. One animal (60) also had

minimal, nonsuppurative peribronchiolitis, while another animal (57) also had minimal, histiocytic alveolitis.

5.3. Histopathology

Microscopic findings are presented in Table 3 (WT mice at 1-Week Timepoint), Table 4 (KO mice at 1-Week Timepoint), Table 5 (TG mice at 1-Week Timepoint), Table 6 (WT mice at 4-Week Timepoint), Table 7 (KO mice at 4-Week Timepoint), and Table 8 (TG mice at 4-Week Timepoint). Additionally, microscopic findings are summarized in Text Table 3 (1-Week Timepoint) and Text Table 4 (4-Week Timepoint).

Text Table 3
Summary (Mean Severity Scores) of Microscopic Findings (1-Week Timepoint)

	Mean Severity								
Strain	**WT**			**KO**			**TG**		
Group	Control	Styrene	Styrene	Control	Styrene	Styrene	Control	Styrene	Styrene
Dose	0 ppm	40 ppm	120 ppm	0 ppm	40 ppm	120 ppm	0 ppm	40 ppm	120 ppm
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0.00	1.38	1.13	0.00	0.00	0.00	0.00	0.00	0.00
Epithelium, bronchiolar (mainly terminal),degeneration/necrosis/ exfoliation/attenuation	0.00	2.13	1.38	0.00	0.00	0.00	0.00	0.00	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0.00	0.75	0.75	0.00	0.00	0.00	0.00	0.00	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0.00	0.38	0.38	0.00	0.00	0.00	0.00	0.00	0.00
Perivasculitis, subacute	0.00	0.50	0.63	0.00	0.00	0.00	0.00	0.00	0.00
Alveolitis, histiocytic	0.00	0.50	0.38	0.00	0.00	0.00	0.00	0.00	0.00

Text Table 4
Summary (Mean Severity Scores) of Microscopic Findings (4-Week Timepoint)

	Mean Severity								
Strain	**WT**			**KO**			**TG**		
Group	Control	Styrene	Styrene	Control	Styrene	Styrene	Control	Styrene	Styrene
Dose	0 ppm	40 ppm	120 ppm	0 ppm	40 ppm	120 ppm	0 ppm	40 ppm	120 ppm
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0.00	0.13	0.13	0.00	0.00	0.00	0.00	0.00	0.00
Epithelium, bronchiolar (mainly terminal),degeneration/necrosis/ exfoliation/attenuation	0.00	0.50	1.13	0.00	0.00	0.00	0.00	0.00	0.00

	Mean Severity								
Strain	**WT**			**KO**			**TG**		
Group	Control	Styrene	Styrene	Control	Styrene	Styrene	Control	Styrene	Styrene
Dose	0 ppm	40 ppm	120 ppm	0 ppm	40 ppm	120 ppm	0 ppm	40 ppm	120 ppm
Lung									
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0.00	0.50	0.13	0.00	0.00	0.00	0.00	0.00	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0.00	0.75	0.75	0.00	0.00	0.00	0.00	0.00	0.00
Perivasculitis, subacute	0.00	0.13	0.13	0.13	0.00	0.14	0.00	0.00	0.00
Alveolitis, histiocytic	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

No microscopic lesions were noted in any control (0 ppm styrene) WT mice after the 1-week timepoint. After 1 week of styrene inhalation at 40 or 120 ppm, there was minimal to mild decreased eosinophilia of the bronchiolar epithelium mainly lining terminal bronchioles in WT mice. In addition, 1 week of styrene inhalation at 40 or 120 ppm by WT mice resulted in minimal to moderate degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium mainly lining terminal bronchioles that was sometimes associated with minimal to mild hyperplasia of the bronchiolar epithelium with or without extension into the adjacent alveolar duct. Minimal subacute perivasculitis and minimal to moderate histiocytic alveolitis were noted in few WT mice after 1 week of styrene inhalation at 40 or 120 ppm. Overall, the microscopic changes were not dose dependent and in some cases were more severe, based upon the mean severity scores for the lesions, in WT mice receiving 40 ppm styrene compared with those WT mice receiving 120 ppm styrene. No KO or TG mice had microscopic lesions after 1 week of styrene inhalation at 0, 40, or 120 ppm except for one KO mouse (117) that had mild interstitial mixed cell infiltrates, which was considered an incidental finding.

No microscopic lesions were noted in any control (0 ppm styrene) WT mice after the 4-week timepoint. After 4 weeks of styrene inhalation at 40 or 120 ppm, there was minimal decreased eosinophilia of the bronchiolar epithelium mainly lining terminal bronchioles in few WT mice. In addition, 4 weeks of styrene inhalation at 40 or 120 ppm by WT mice was associated with minimal (mild in one animal) degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium mainly lining terminal bronchioles that was also sometimes associated with minimal to mild hyperplasia of the bronchiolar epithelium with or without extension into the adjacent alveolar duct. Minimal subacute perivasculitis and minimal to mild nonsuppurative peribronchiolitis were noted in few WT mice after 4 weeks of styrene inhalation at 40 or 120 ppm. Overall, the microscopic changes were not dose dependent based upon the mean severity scores for the lesions except for the degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium mainly lining terminal bronchioles, which appeared slightly more severe based upon mean severity score in WT mice receiving 120 ppm styrene compared with those WT mice receiving 40 ppm styrene. No KO or TG mice had microscopic lesions after 4 weeks of styrene inhalation at 0, 40, or 120 ppm except for minimal acute hemorrhage in one KO

mouse (133) and minimal subacute perivasculitis noted in two KO mice receiving either 0 or 120 ppm styrene, which were all judged as incidental findings.

The mean severity scores for the decreased eosinophilia of bronchiolar epithelium mainly lining terminal bronchioles; degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium mainly lining terminal bronchioles; and hyperplasia of bronchiolar epithelium mainly lining terminal bronchioles without extension into adjacent alveolar duct were greater in the WT mice from the 1-week timepoint compared with WT mice from the 4-week timepoint. However, the mean severity score for hyperplasia of bronchiolar epithelium mainly lining terminal bronchioles with extension into adjacent alveolar duct was greater in the WT mice from the 4-week timepoint compared with WT mice from the 1-week timepoint. The latter was most likely due to the longer chronicity of the hyperplasia after 4 weeks compared with 1 week, resulting in further extension into the adjacent alveolar ducts.

6. CONCLUSIONS

Inhalation of 0, 40, or 120 ppm styrene for 6 hours per day for 5 days for either 1 week or 4 weeks by mice resulted in no deaths. At the end of the treatment periods, no gross findings were noted in any animal. Microscopic findings were noted in the lung of all styrene-treated WT mice and consisted of decreased eosinophilia of bronchiolar epithelium mainly lining terminal bronchioles; degeneration, necrosis, exfoliation, and/or attenuation of individual bronchiolar epithelial cells (most likely Clara cells) mainly lining terminal bronchioles; and/or hyperplasia of the bronchiolar epithelium mainly lining the terminal bronchioles that sometimes extended into the distally connected alveolar duct. These lesions are consistent with those previously described in mice exposed to styrene for 1 or more weeks.[1, 2] These findings also correlate with previously published results in that inhalation of ≥20 ppm styrene is toxic to Clara cells in lung terminal bronchioles of mice, and this cytotoxicity is due to ring-oxidized metabolites produced in situ by CYP2F2 metabolism.[1, 3-5] Furthermore, the cytotoxicity results in subsequent regenerative hyperplasia.[6] No KO or TG mice had styrene-induced microscopic lesions after 1 or 4 weeks of styrene inhalation at 40 or 120 ppm. This correlates with the reported literature that KO mice lacking CYP2F2 or TG mice containing human CYP2F1 in place of mouse CYP2F2 do not produce cytotoxicity from styrene exposure.[1]

For the control group (0 ppm styrene for 5 days), the BrdU labeling index was 5.11% (± 1.35%), while the BrdU labeling index for the treatment group (120 ppm styrene for 5 days) was 34.21% (± 9.81%). The approximate 7-fold increase in BrdU labeling index in WT mice treated with 120 ppm styrene is due to cytotoxicity to Clara cells followed by regeneration.[1, 6, 7]

Overall, the microscopic changes noted in the WT mice were not dose dependent and in the 1-week timepoint, actually appeared slightly more severe in WT mice receiving 40 ppm styrene compared with those WT mice receiving 120 ppm styrene. In the WT mice receiving styrene for 4 weeks, the microscopic changes were not dose dependent except for the degeneration, necrosis, exfoliation, and/or attenuation of bronchiolar epithelium mainly lining terminal bronchioles which appeared slightly more severe in WT mice receiving 120 ppm styrene compared with WT mice receiving 40 ppm styrene.

The mean severity scores for most microscopic lesions were greater in the WT mice from the 1-week timepoint compared with WT mice from the 4-week timepoint. However, the mean severity score for hyperplasia of bronchiolar epithelium mainly lining terminal bronchioles with extension into adjacent alveolar duct was greater in the WT mice from the 4-week timepoint compared with WT mice from the 1-week timepoint. The latter was most likely due to the longer chronicity of the hyperplasia after 4 weeks compared with 1 week, resulting in further extension into the adjacent alveolar ducts.

7. REFERENCES

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(2) Cruzan G, Cushman JR, Andrews LS et al. Chronic toxicity/oncogenicity study of styrene in CD-1 mice by inhalation exposure for 104 weeks. *J Appl Toxicol* 2001 May;21(3):185-98.

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Table 1
BrdU Cell Counts

Table 1. BrdU Cell Counts

Animal No.		Terminal Bronchioles I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	Total Cells Counted	BrdU Labeling Index	Mean Labeling Index	Standard Deviation
49	No. of Positive Epithelial Cells	1	2	1	2	0	3	2	2	0	2	4	2	21	6.42%	5.11%	1.35%
	No. of Negative Epithelial Cells	7	23	35	22	19	21	17	23	29	42	35	33	306			
50	No. of Positive Epithelial Cells	1	3	2	1	5	1	0	4	5				22	5.53%		
	No. of Negative Epithelial Cells	28	74	44	42	57	25	14	37	55				376			
51	No. of Positive Epithelial Cells	4	0	0	3	1	0	0	6	1	3			18	2.84%		
	No. of Negative Epithelial Cells	26	60	91	86	30	12	77	94	49	91			616			
52	No. of Positive Epithelial Cells	0	0	1	4	1	1	5	3	7				22	4.26%		
	No. of Negative Epithelial Cells	101	28	63	94	23	16	38	57	75				495			
53	No. of Positive Epithelial Cells	2	3	6	4	4								19	6.23%		
	No. of Negative Epithelial Cells	8	24	65	136	53								286			
54	No. of Positive Epithelial Cells	3	4	3	2	2	6	2	9					31	5.36%		
	No. of Negative Epithelial Cells	120	43	26	70	44	68	26	150					547			
55	No. of Positive Epithelial Cells	4	1	7	5	4	6	5	8	7	13	2	13	75	21.74%	34.21%	9.81%
	No. of Negative Epithelial Cells	28	15	23	14	25	12	18	15	24	16	32	48	270			
56	No. of Positive Epithelial Cells	16	6	18	36	34	17	17	8					152	33.04%		
	No. of Negative Epithelial Cells	40	39	34	20	42	64	40	29					308			
57	No. of Positive Epithelial Cells	3	6	8	63	44	13	11	10					158	29.10%		
	No. of Negative Epithelial Cells	19	48	44	104	47	47	45	31					385			
58	No. of Positive Epithelial Cells	5	42	8	8	27	5	4	5	13				117	30.31%		
	No. of Negative Epithelial Cells	20	72	22	21	52	6	20	19	37				269			
59	No. of Positive Epithelial Cells	28	46	34	9	8	31	28	19					203	41.94%		
	No. of Negative Epithelial Cells	59	52	34	10	16	32	55	23					281			
60	No. of Positive Epithelial Cells	38	12	23	18	15	53	48	24					231	49.15%		
	No. of Negative Epithelial Cells	48	25	31	30	12	33	32	28					239			

Table 2
Microscopic Findings for WT Mice Assigned to Cell Proliferation Group

Table 2. Microscopic Findings for WT Mice Assigned to Cell Proliferation Group												
Animal Number	**49**	**50**	**51**	**52**	**53**	**54**	**55**	**56**	**57**	**58**	**59**	**60**
Group	Control	Control	Control	Control	Control	Control	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene
Dose	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm
Strain	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT	WT
Tissue/Diagnosis												
Lung												
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0	0	0	0
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	1	1	1	1	1	1
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0	1	1	0
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	1	0	0	0	1
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	0	0	0	0	1
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	1	0	0	0

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5 = severe

Table 3
Microscopic Findings for WT Mice Assigned to 1-Week Genomics Group

Table 3. Microscopic Findings for WT Mice Assigned to 1-Week Genomics Group

Animal Number	1	2	3	4	5	6	7	8	Mean
Group	Control	Control	Control	Control	Control	Control	Control	Control	
Dose	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	
Strain	WT	WT	WT	WT	WT	WT	WT	WT	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5 = severe, M = missing

Table 3. Microscopic Findings for WT Mice Assigned to 1-Week Genomics Group

Animal Number	9	10	11	12	13	14	15	16	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	
Strain	WT	WT	WT	WT	WT	WT	WT	WT	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	1	2	2	1	1	1	1	2	1.38
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	2	2	2	2	2	3	1	3	2.13
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	1	0	1	1	1	1	1	0.75
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	1	0	2	0	0	0	0	0	0.38
Perivasculitis, subacute	0	1	1	0	1	0	0	1	0.50
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	1	1	0	0	0	1	1	0.50

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5 = severe, M = missing

Table 3. Microscopic Findings for WT Mice Assigned to 1-Week Genomics Group

Animal Number	17 (1)	17 (11)	18	19	20	21	22	23	24	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	
Strain	WT	WT	WT	WT	WT	WT	WT	WT	WT	
Tissue/Diagnosis										
Lung										
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	M	1	2	1	1	1	1	1	1	1.13
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	M	1	2	0	1	1	2	2	2	1.38
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	M	1	0	1	1	1	1	0	1	0.75
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	M	0	2	0	0	0	0	1	0	0.38
Perivasculitis, subacute	0	0	1	1	0	1	1	1	0	0.63
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	3	0	0	0.38

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5 = severe, M = missing

Table 4
Microscopic Findings for KO Mice Assigned to 1-Week Genomics Group

Table 4. Microscopic Findings for KO Mice Assigned to 1-Week Genomics Group

Animal Number	101	102	103	104	105	106	107	108	Mean
Group	Control	Control	Control	Control	Control	Control	Control	Control	
Dose	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	
Strain	KO	KO	KO	KO	KO	KO	KO	KO	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5 = severe

Table 4. Microscopic Findings for KO Mice Assigned to 1-Week Genomics Group

Animal Number	109	110	111	112	113	114	115	116	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	
Strain	KO	KO	KO	KO	KO	KO	KO	KO	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5 = severe

Table 4. Microscopic Findings for KO Mice Assigned to 1-Week Genomics Group

Animal Number	117	118	119	120	121	122	123	124	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	
Strain	KO	KO	KO	KO	KO	KO	KO	KO	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	2	0	0	0	0	0	0	0	0.25
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5 = severe

Table 5
Microscopic Findings for TG Mice Assigned to 1-Week Genomics Group

Table 5. Microscopic Findings for TG Mice Assigned to 1-Week Genomics Group

Animal Number	201	202	203	204	205	206	207	208	Mean
Group	Control	Control	Control	Control	Control	Control	Control	Control	
Dose	0 ppm	0 ppm	205	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	
Strain	TG	TG	TG	TG	TG	TG	TG	TG	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe

Table 5. Microscopic Findings for TG Mice Assigned to 1-Week Genomics Group

Animal Number	209	210	211	212	213	214	215	216	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	
Strain	TG	TG	TG	TG	TG	TG	TG	TG	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe

Table 5. Microscopic Findings for TG Mice Assigned to 1-Week Genomics Group

Animal Number	217	218	219	220	221	222	223	224	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	
Strain	TG	TG	TG	TG	TG	TG	TG	TG	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe

Table 6
Microscopic Findings for WT Mice Assigned to 4-Week Genomics Group

Table 6. Microscopic Findings for WT Mice Assigned to 4-Week Genomics Group

Animal Number	25	26	27	28	29	30	31	32	
Group	Control	Control	Control	Control	Control	Control	Control	Control	
Dose	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	
Strain	WT	WT	WT	WT	WT	WT	WT	WT	Mean
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	0	0.00
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe

Table 6. Microscopic Findings for WT Mice Assigned to 4-Week Genomics Group

Animal Number	33	34	35	36	37	38	39	40	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	
Strain	WT	WT	WT	WT	WT	WT	WT	WT	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	1	0.13
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	1	1	0	1	1	0.50
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	1	1	1	0	0	0	1	0.50
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	2	0	0	0	1	2	1	0	0.75
Perivasculitis, subacute	0	0	0	0	0	0	1	0	0.13
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	0	0.00
Inflammation, peribronchiolar, nonsuppurative	0	0	0	2	0	1	1	1	0.63
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe

Table 6. Microscopic Findings for WT Mice Assigned to 4-Week Genomics Group

Animal Number	41	42	43	44	45	46	47	48	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	
Strain	WT	WT	WT	WT	WT	WT	WT	WT	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	1	0	0	0	0	0	0	0.13
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	1	1	1	2	1	1	1	1	1.13
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	1	0	0	0	0	0	0.13
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	1	1	0	1	1	0	1	1	0.75
Perivasculitis, subacute	0	0	0	0	0	1	0	0	0.13
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	0	0.00
Inflammation, peribronchiolar, nonsuppurative	0	1	0	2	0	0	1	0	0.50
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe

Table 7
Microscopic Findings for KO Mice Assigned to 4-Week Genomics Group

Table 7. Microscopic Findings for KO Mice Assigned to 4-Week Genomics Group

Animal Number	125	126	127	128	129	130	131	132	Mean
Group	Control	Control	Control	Control	Control	Control	Control	Control	
Dose	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	
Strain	KO	KO	KO	KO	KO	KO	KO	KO	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	1	0	0.13
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	0	0.00
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe, M=missing

Table 7. Microscopic Findings for KO Mice Assigned to 4-Week Genomics Group

Animal Number	133	134	135	136	137	138	139	140	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	
Strain	KO	KO	KO	KO	KO	KO	KO	KO	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	1	0	0	0	0	0	0	0	0.13
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe, M=missing

Table 7. Microscopic Findings for KO Mice Assigned to 4-Week Genomics Group

Animal Number	141	142	143	144	145	146	147	148	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	
Strain	KO	KO	KO	KO	KO	KO	KO	KO	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	M	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	M	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	M	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	M	0.00
Perivasculitis, subacute	0	0	0	1	0	0	0	M	0.14
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	M	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	M	0.00
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	M	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	M	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe, M=missing

Table 8
Microscopic Findings for TG Mice Assigned to 4-Week Genomics Group

Table 8. Microscopic Findings for TG Mice Assigned to 4-Week Genomics Group

Animal Number	225	226	227	228	229	230	231	232	Mean
Group	Control	Control	Control	Control	Control	Control	Control	Control	
Dose	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	0 ppm	
Strain	TG	TG	TG	TG	TG	TG	TG	TG	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	0	0.00
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe, M=missing

Table 8. Microscopic Findings for TG Mice Assigned to 4-Week Genomics Group

Animal Number	233	234	235	236	237	238	239	240	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	40 ppm	
Strain	TG	TG	TG	TG	TG	TG	TG	TG	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	0	0.00
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe, M=missing

Table 8. Microscopic Findings for TG Mice Assigned to 4-Week Genomics Group

Animal Number	241	242	243	244	245	246	247	248	Mean
Group	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	Styrene	
Dose	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	120 ppm	
Strain	TG	TG	TG	TG	TG	TG	TG	TG	
Tissue/Diagnosis									
Lung									
Epithelium, bronchiolar (mainly terminal), decreased eosinophilia	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), degeneration/necrosis/exfoliation/attenuation	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia without extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Epithelium, bronchiolar (mainly terminal), hyperplasia with extension into alveolar duct	0	0	0	0	0	0	0	0	0.00
Perivasculitis, subacute	0	0	0	0	0	0	0	0	0.00
Infiltrates, interstitial, mixed cell	0	0	0	0	0	0	0	0	0.00
Hemorrhage, acute, multifocal	0	0	0	0	0	0	0	0	0.00
Inflammation, peribronchiolar, nonsuppurative	0	0	0	0	0	0	0	0	0.00
Alveolitis, histiocytic	0	0	0	0	0	0	0	0	0.00

Key: 0=normal/not present; 1=minimal; 2=mild; 3=moderate; 4=marked, 5=severe, M=missing

Appendix 1
Deviations

DEVIATIONS

All deviations that occurred during this study phase have been acknowledged by the Study Director, assessed for impact, and documented in the study records. All protocol deviations and those SOP deviations regarded as significant are listed below. None of the deviations were considered to have impacted the overall integrity of the study or the interpretation of the study results and conclusions.

- The Lung Cell Proliferation Evaluation section of the protocol states that 350-400 stained and unstained epithelial cells lining the terminal bronchioles would be counted per animal. However, approximately 300-650 cells were actually counted per animal. This deviation did not impact the study as less than 350 cells were counted only in the instance where not enough airways were present in the section to meet the required number. The counting of greater than 400 cells was an inadvertent.

Appendix 4

Genomics Analyses Summary Report

17 pages

Styrene Inhalation Study
Genomics Analyses Summary

Final Report
Protocol Number: DEDSTY131-1

Authors:
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February, 2014

Reviewed and Approved by:

Michael B. Black, PhD

Michael B. Black 02/18/2014

Signature/Date

Melvin E. Andersen, PhD, DABT

Melvin E. Anderson 2/20/2014.

Signature/Date

Summary:

A total of 90 male C57BL/6 mice were exposed to two inhalation doses of styrene (40ppm and 120ppm) for 1 week and 4 weeks. For each treatment a total of 5 biological replicates were used, from 3 strains of mice; wild type (WT), CYP2F2(-/-) knock-out (KO) and CYP2F2(-/-),2F1,SA13,2B6 transgenic (TG), with strain specific controls included (0ppm).

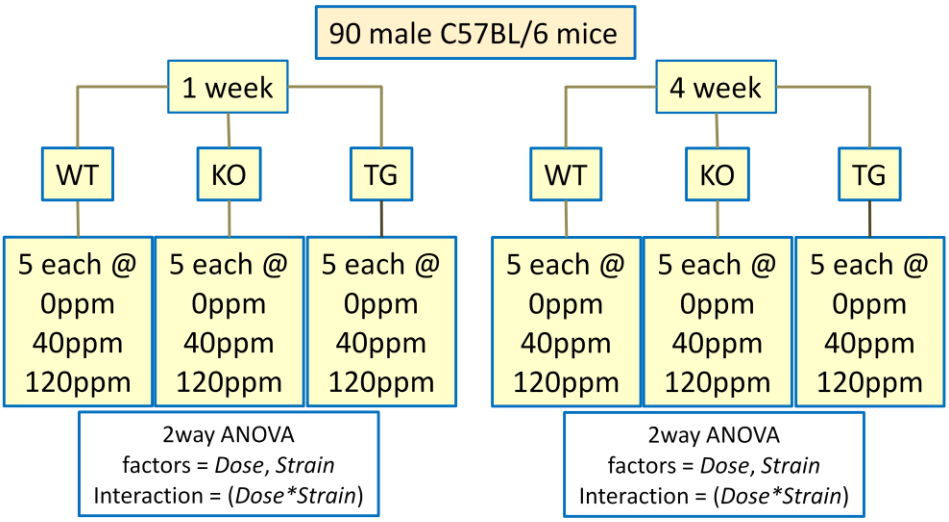


Figure 1. Genomics analysis design

Animals were harvested and RNA extracted according to the study's protocol (Hamner protocol DEDSTY131-1) and samples were run on a single 96-peg Affymetrix HT_MG-430_PM[1] GeneTitan array system. A PCA analysis of the normalized array data indicated a distinct separation of the data by the two time points. Subsequently, each time point was analyzed independently by conventional 2way ANOVA with orthogonal linear contrasts for pair-wise comparisons (fig.1). Differential expression was determined by the simultaneous application of a statistical threshold (a False Discovery Rate < 0.05) and a fold change threshold using linear fold change greater than +1.5 OR less than -1.5. Fold change was computed as the ratio of the geometric mean $\log_2$ expression for each strain and dose specific treatment, over the geometric mean $\log_2$ expression for each strain specific vehicle control. Linear fold change is used throughout this report however, and is computed as the simple anti-log of the $\log_2$ ratio for ratio's greater than 1.0 and as (-1/(anti-log of the $\log_2$ ratio)) for ratio's less than 1.0.

There was almost no significantly differentially expressed genes in either the knock-out nor the transgenic mice at any time point for any dose. A single significant gene was detected for transgenic mice at 120ppm exposure and 1 week (Npas2), and a single gene at 40ppm at 4 weeks (Dcaf4).

Significant differential gene expression was observed in wild type mice at 1 week with 378 differentially expressed genes in the 40ppm exposure groups (relative to the wild type specific vehicle controls), and 516 differentially expressed genes in the 120ppm treatment group. Of these, 287 genes were significantly differentially expressed at both doses. However, by 4 weeks, there were fewer differentially expressed genes detected: 19 at 40ppm and 69 at 120ppm with 17 of these significant at

[1] The HT_MG-430_PM array contains 45,141 total probes. Of these, 38,011 include non-promiscuous annotated probes representing 20,678 unique genes by Entrez Gene ID. A promiscuous probe is a probe that has the potential to bind to multiple target sequences and thus does not unambiguously indicate the presence of a single transcript.

both doses. A total of ten genes were significantly differentially expressed at all doses and both time points.

Gene ontology enrichment of the 1 week wild type DE genes showed a strong dominance of cell cycle regulatory pathways. The greatest significant enrichment was observed with the 287 genes that were differentially expressed at both 40ppm and 120ppm at 1 week. At 4 weeks, significant enrichment was only observed for a single pathway due to the limited numbers of DE genes detected, and this pathway was also significantly enriched at 1 week.

Genomic Analysis

Affymetrix CEL files were normalized in Partek Genome Suite 6.6 (Partek Inc.) using a standard Robust Multi-array Average (RMA[2]) algorithm (RMA background correction, quantile normalization and Median Polish probe summary). A standard principle components analysis was applied to all 90 samples (fig. 2). This indicated a clear distinction in the data clouds between the two time points. Subsequently, each time point (45 samples) was analyzed independently by 2-way ANOVA using *Dose* and *Strain* as ANOVA factors, and with an interaction factor of these two (*Dose*Strain*).

Principle components plots of the two separate time points indicated a distinction in wild type samples at 1 week relative to the other two strains (fig. 3). A 2 standard deviation ellipse drawn about the WT samples indicate a data group orthogonal to the other two strains. This relationship disappeared by 4 weeks, and the wild type data coalesced with the knock-out and the transgenic samples into a single undifferentiated data cloud (fig. 4).

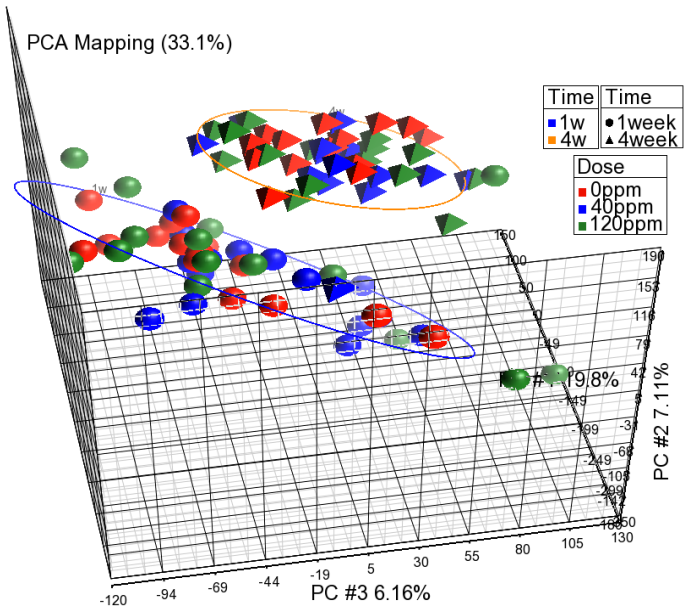


Figure 2. PCA plot of normalized array intensity across all probes, for all doses, strains and times showing primary data clustering by time. Time-based ellipses are 2 standard deviations. Symbol colors represent dose.

[2] Irizarry, R. A., et al. (2003). "Exploration, normalization, and summaries of high density oligonucleotide array probe level data." Biostatistics **4**(2): 249-264.

Complete ANOVA results (table 1) show the extent of the response as indicated by the PCA plots. By far the majority of significantly differentially expressed genes were detected amongst the wild type samples at 1 week. Both doses showed hundreds of significant probes as determined by the simultaneous application of a statistical threshold (FDR <0.05) and a fold change threshold where a gene was considered significant if the linear fold change was either greater than positive 1.5 fold or less than negative 1.5 fold. The FDR correction used was a standard Benjamini & Hochberg (1995)[3] step-up False Discovery Rate corrected p-value.

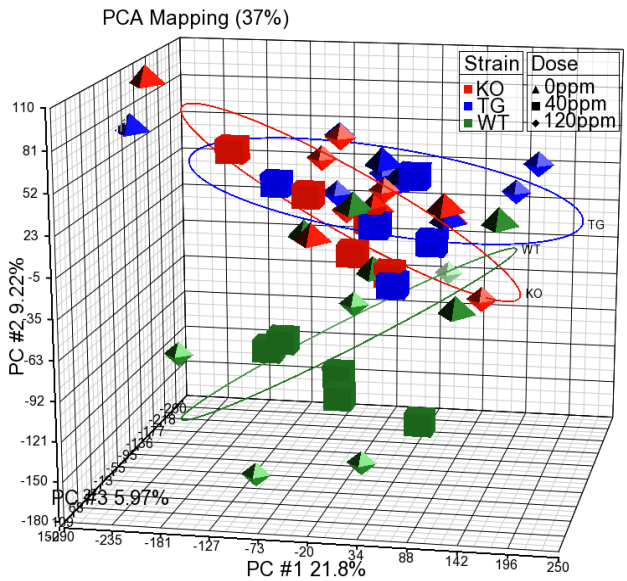


Figure 3. PCA plot of normalized array intensity for the 45 1 week samples only, across all probes, for all doses and strains. The wild type data cloud (green) appears nearly orthogonal to the other two strains (KO = red and TG = blue)

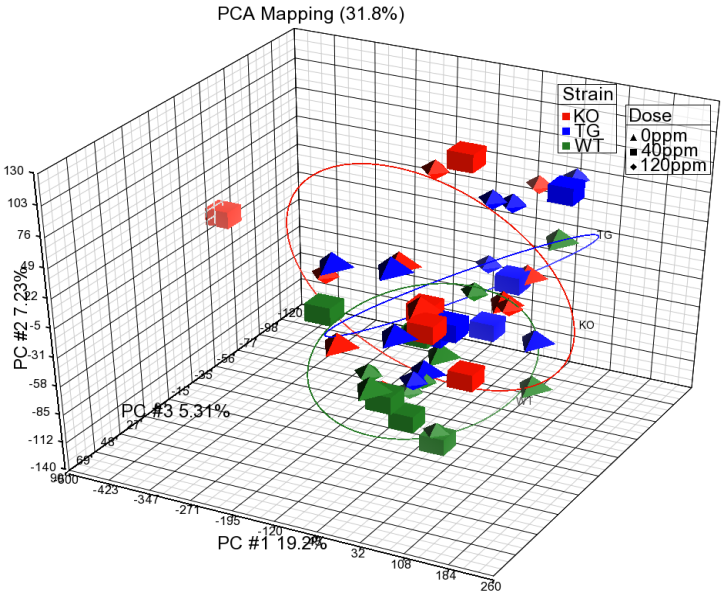


Figure 4. PCA plot of normalized array intensity for the 45 4 week samples only, across all probes, for all doses and strains. The wild type data cloud (green) now has coalesced with the other two strains to form a single undifferentiated data cloud.

[3] Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate: A practical and powerful approach to multiple testing." Journal of the Royal Statistical Society, Series B (Methodological) **57**(1): 289-300.

Table 1: Significantly differentially expressed probes from independent ANOVA analyses at each time point

1 week samples (N=45)	# of probes								
Significant by Dose (FDR<0.05)	141								
Significant by Strain (FDR<0.05)	3841								
Significant by interaction (Dose*Strain) (FDR<0.05)	248								
Linear Contrast results (Dose*Strain)	***KO vs WT***	***TG vs WT***	***TG vs KO***	***KO 40ppm***	***KO 120ppm***	***TG 40ppm***	***TG 120ppm***	***WT 40ppm***	***WT 120ppm***
Significant by FDR <0.05	2856	4283	95	0	0	0	1	926	1719
Significant by Fold Change >±1.5*	395	428	79	122	108	91	229	890	1336
Significant by FDR <0.05 & FC >±1.5*	331	379	41	0	0	0	1	**512**	**727**
4 week samples (N=45)	# of probes								
Significant by Dose (FDR<0.05)	156								
Significant by Strain (FDR<0.05)	1395								
Significant by interaction (Dose*Strain) (FDR<0.05)	11								
Linear Contrast results (Dose*Strain)	***KO vs WT***	***TG vs WT***	***TG vs KO***	***KO 40ppm***	***KO 120ppm***	***TG 40ppm***	***TG 120ppm***	***WT 40ppm***	***WT 120ppm***
Significant by FDR <0.05	628	1282	234	0	0	1	0	30	151
Significant by Fold Change >±1.5*	121	135	74	108	96	246	134	165	233
Significant by FDR <0.05 & FC >±1.5*	86	92	41	0	0	1	0	23	88

*A probe was considered significant if the linear fold change was greater than +1.5 OR less than -1.5

When the 1 week wild type probe level data shown in table 1 was broken out by unique annotated genes, the results indicated 378 genes significantly differentially expressed at 40ppm and 516 genes at 120ppm (fig. 5). These values represent the number of unique genes once un-annotated, promiscuous and redundant probes were excluded from the lists.

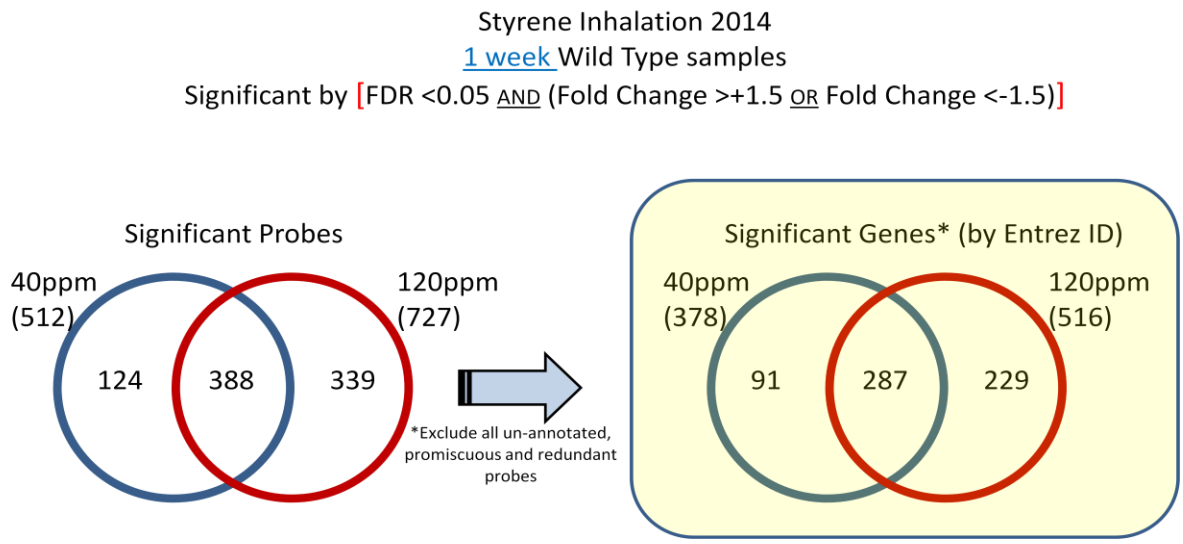


Figure 5. Differentially expressed genes for 1 week wild type samples.

In contrast, there were only 19 significant genes at 4 week 40ppm and only 69 at 4 week 120ppm and only 10 of these were shared with the 1 week samples (fig. 6 and 7). The complete list of significant genes for wild type samples at both doses and both time points is provided in the supplemental excel spreadsheet *WT_significant_genes.xlsx*.

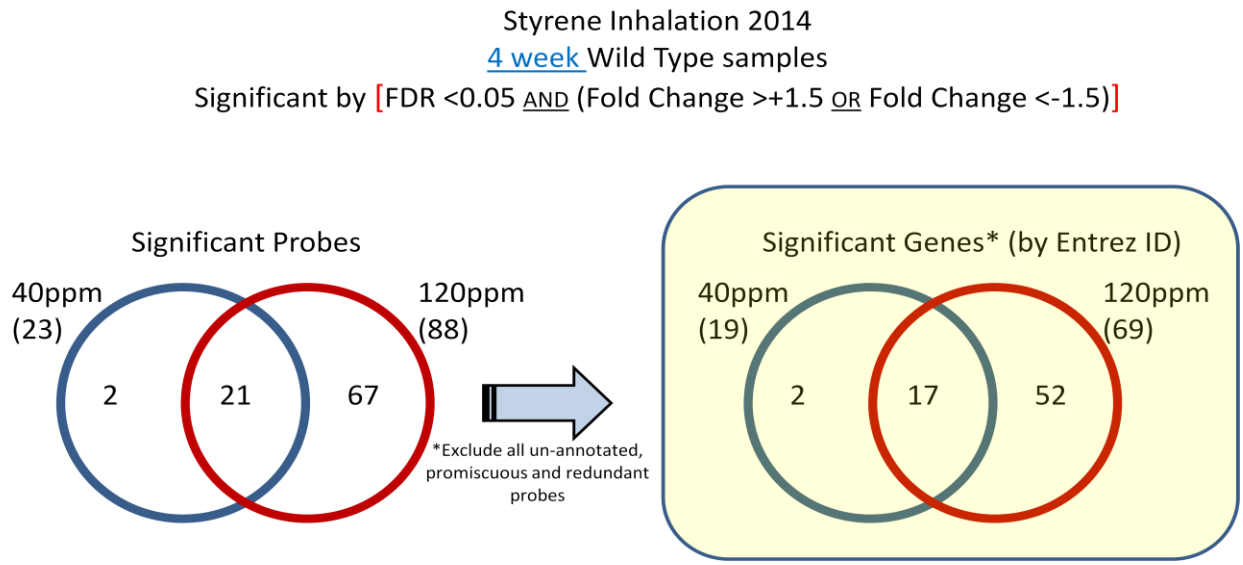


Figure 6. Differentially expressed genes for 4 week wild type samples.

Styrene Inhalation 2014
1 week & 4 week Wild Type samples
Genes Significant by [FDR <0.05 AND (Fold Change >+1.5 OR Fold Change <-1.5)]

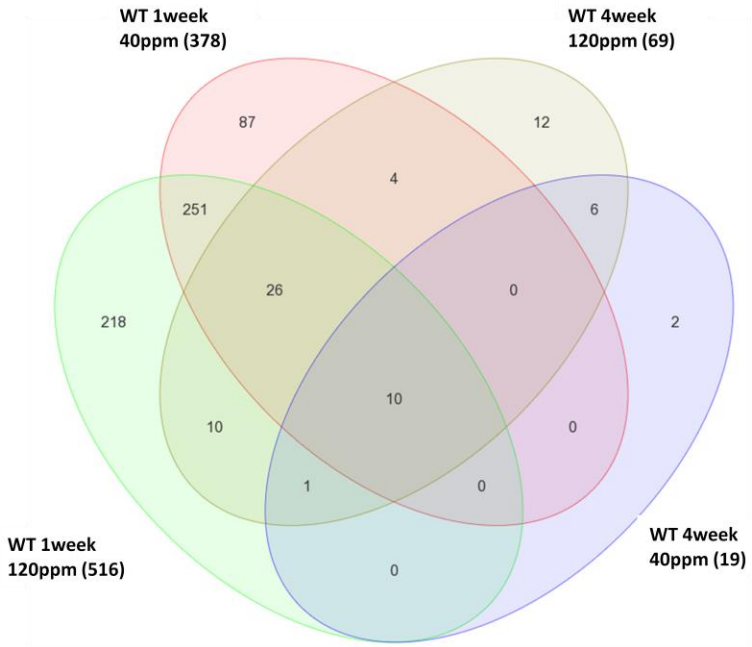


Figure 7. Differentially expressed genes for Wild Type strain across all doses and time points.

Gene ontology enrichment for the wild type significant genes was performed against the MetaCore curated ontology database (Thomson and Reuters Inc., (http://thomsonreuters.com/metacore) as well as the public consortium Gene Ontology's (http://www.geneontology.org) Biological Processes sub-division. An ontology was considered to be enriched if statistically significant (FDR <0.05) and it had at least 5 of the styrene data query genes found within the ontology. Enrichment for the wild type significant genes from 1 week indicated that most of the significant enrichment obtained occurred with the 287 genes that were common to both the 40ppm and the 120ppm doses at week 1 (table 2).

MetaCore enriched pathway maps were almost entirely cell cycle or related processes. Of these, only the *Neurophysiological process_Circadian rhythm* pathway map was also statistically significant at 4 weeks for both 40ppm and 120ppm, but with only 3 query genes (4 week, 40ppm) or 4 query genes (4 week, 120ppm) found in the ontology. None of the other pathways enriched with the 267 common genes at 1 week were also significantly enriched at 4 weeks. This is not unexpected given the very few genes differentially expressed at 4 weeks and available for enrichment analysis. The 1 week GO Biological Process category enrichment indicated cell and mitotic cycle process as those primarily enriched, as well as several DNA repair and associated processes (Table 3). Pathways for cholesterol synthesis were enriched at 40 ppm at 1 week using either Metacore or GO ontologies.

Included as a short appendix are selected MetaCore pathway map images for enriched pathway maps from table 2.

Table 2. MetaCore pathway enrichment for 1 week WT differentially expressed genes (see fig.4). Red indicates a significantly enriched pathway, with FDR <0.05 and with a minimum of 5 elements from the WT query data found in the ontology

MetaCore version 6.16 build 63671		***40ppm unique genes***		***Common genes***		***120ppm unique genes***	
MetaCore Pathway Maps	Total entries in ontology	FDR	elements from query data	FDR	elements from query data	FDR	elements from query data
Cell cycle_The metaphase checkpoint	36	1.00000		1.745E-14	13	0.33082	2
Cell cycle_Role of APC in cell cycle regulation	32	1.00000		4.546E-12	11	0.08467	3
Cell cycle_Chromosome condensation in prometaphase	21	1.00000		6.619E-11	9	0.00492	4
Cell cycle_Spindle assembly and chromosome separation	33	1.00000		1.909E-07	8	0.08467	3
DNA damage_ATM / ATR regulation of G2 / M checkpoint	26	1.00000		7.009E-07	7	0.28337	2
Cell cycle_Transition and termination of DNA replication	28	1.00000		1.031E-06	7	0.46482	1
Cell cycle_Start of DNA replication in early S phase	32	1.00000		0.00006	6	0.08467	3
Cell cycle_Role of Nek in cell cycle regulation	32	1.00000		0.00101	5	1.00000	
Neurophysiological process_Circadian rhythm	47	1.00000		0.00447	5	1.00000	
dCTP/dUTP metabolism	75	1.00000		0.00465	6	0.05177	5
Cell cycle_Role of SCF complex in cell cycle regulation	29	1.00000		0.00620	4	0.00162	5
dATP/dITP metabolism	95	1.00000		0.01441	6	0.18446	4
ATP/ITP metabolism	124	1.00000		0.04974	6	0.68592	1
SCAP/SREBP Transcriptional Control of Cholesterol and FA Biosynthesis	46	0.00001	6	0.48881	2	1.00000	
Cholesterol Biosynthesis	88	0.00002	7	0.59005	2	1.00000	

Table 3. GO Biological Processes for 1 week WT differentially expressed genes (see fig.4). Red indicates a significantly enriched pathway, with FDR <0.05 and with a minimum of 5 elements from the WT query data found in the ontology

The Gene Ontology		***40ppm unique genes***		***Common genes***		***120ppm unique genes***	
GO Biological Processes	Total entries in ontology	FDR	elements from query data	FDR	elements from query data	FDR	elements from query data
cell cycle	1493	1.00000		1.227E-43	76	8.571E-10	33
mitotic cell cycle	852	1.00000		2.595E-40	61	8.935E-09	25
cell division	515	1.00000		3.975E-37	55	3.730E-07	21
mitosis	353	1.00000		2.256E-35	49	3.629E-07	19
DNA replication	255	1.00000		5.258E-17	27	0.00098	11
chromosome segregation	176	1.00000		2.372E-14	18	0.14994	3
DNA strand elongation involved in DNA replication	39	1.00000		3.965E-12	12	0.04907	3
G1/S transition of mitotic cell cycle	236	0.62554	1	7.604E-12	23	0.00626	10
meiosis	211	1.00000		3.612E-11	17	1.00000	
regulation of transcription involved in G1/S phase of mitotic cell cycle	29	1.00000		2.469E-09	9	1.00000	
regulation of cell cycle	1025	1.00000		8.400E-08	16	0.05691	6
response to DNA damage stimulus	770	1.00000		1.559E-07	26	0.02140	13
DNA replication initiation	28	1.00000		2.171E-07	8	0.00800	4
DNA repair	482	1.00000		3.368E-07	24	0.00713	14
regulation of attachment of spindle microtubules to kinetochore	10	1.00000		7.391E-07	5	1.00000	

Table 3, continued

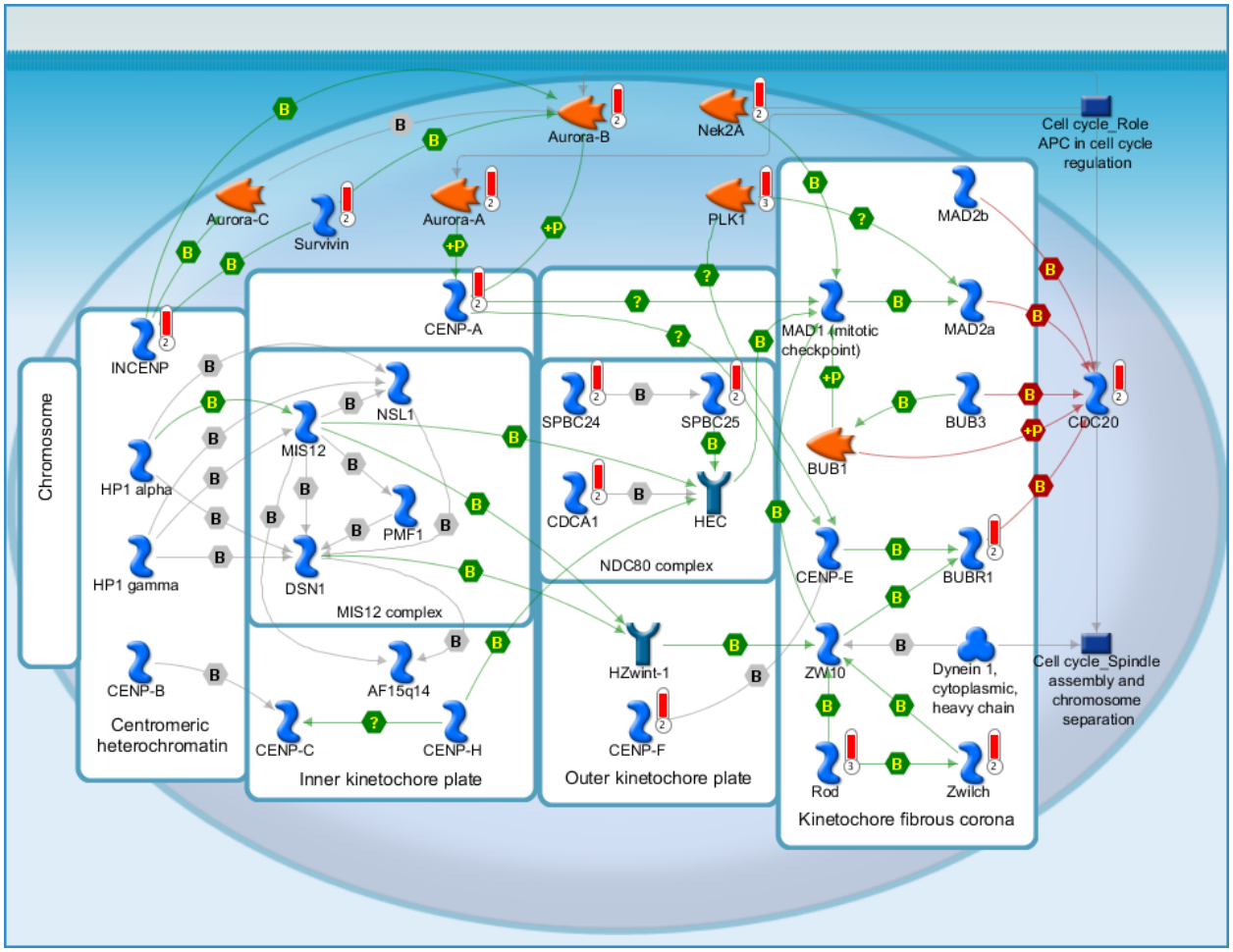
The Gene Ontology		*40ppm unique genes*		*Common genes*		*120ppm unique genes*	
GO Biological Processes	Total entries in ontology	FDR	elements from query data	FDR	elements from query data	FDR	elements from query data
positive regulation of cytokinesis	24	1.00000		1.594E-06	7	1.00000	
G2/M transition of mitotic cell cycle	169	1.00000		2.865E-06	14	0.02180	7
cytokinesis	124	1.00000		4.500E-06	11	0.08945	4
regulation of cyclin-dependent protein serine/threonine kinase activity	115	1.00000		4.500E-06	10	1.00000	
organ regeneration	131	0.44026	1	9.829E-06	12	0.06516	5
double-strand break repair	126	1.00000		0.00005	9	1.00000	
nucleosome assembly	130	1.00000		0.00006	11	1.00000	
double-strand break repair via homologous recombination	60	1.00000		0.00007	8	1.00000	
phosphatidylinositol-mediated signaling	178	1.00000		0.00010	12	1.00000	
rhythmic process	354	1.00000		0.00010	7	0.16613	2
DNA recombination	233	1.00000		0.00018	10	1.00000	
positive regulation of mitotic cell cycle	49	1.00000		0.00028	6	1.00000	
mitotic sister chromatid segregation	61	1.00000		0.00030	5	0.01941	3
spindle organization	106	1.00000		0.00030	5	0.01941	3

Table 3, continued

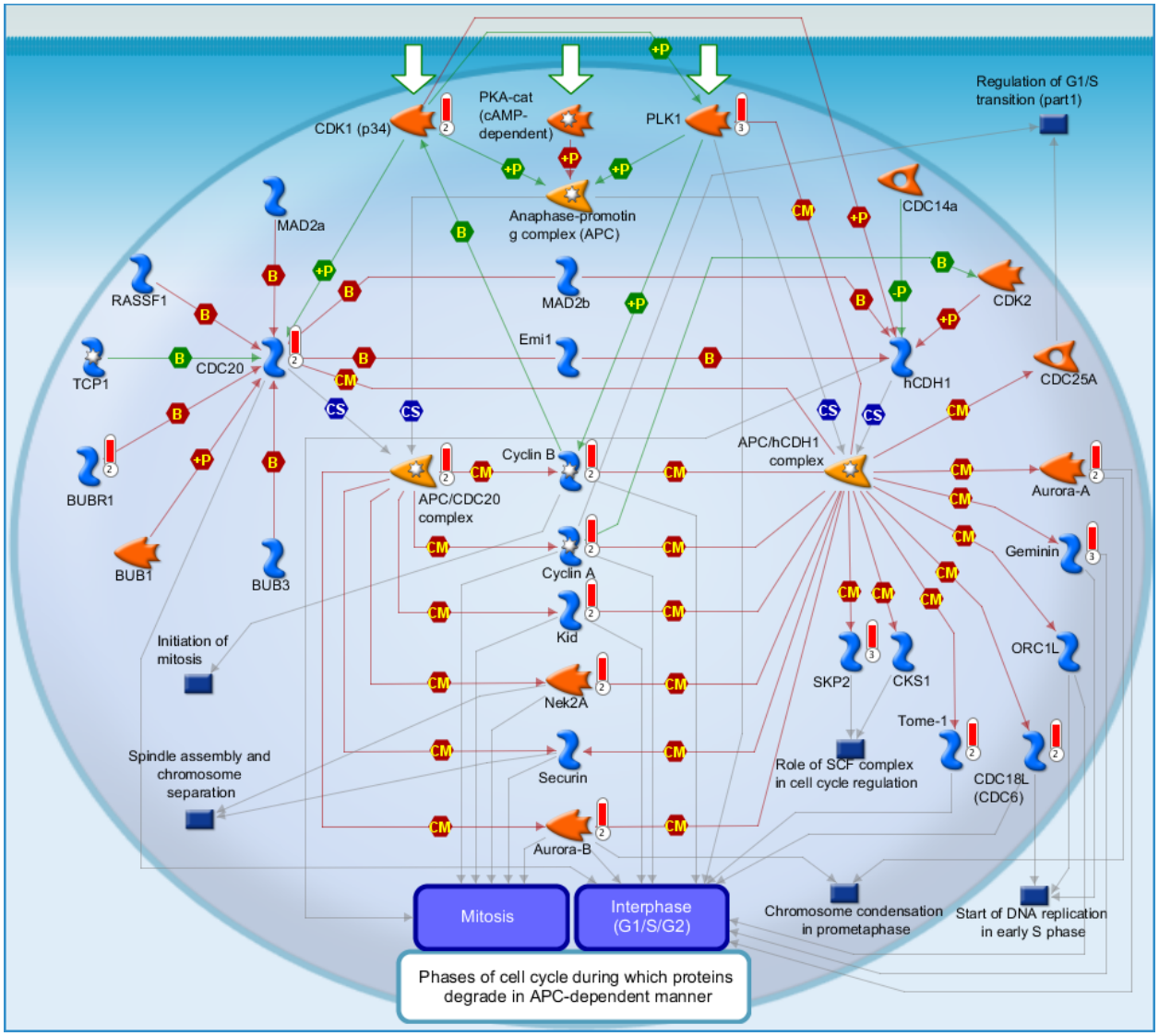
The Gene Ontology		*40ppm unique genes*		*Common genes*		*120ppm unique genes*	
GO Biological Processes	Total entries in ontology	FDR	elements from query data	FDR	elements from query data	FDR	elements from query data
response to drug	745	0.18650	5	0.00031	24	0.16129	11
lipid metabolic process	1387	3.366E-06	15	0.00398	20	1.00000	
telomere maintenance via semi-conservative replication	27	1.00000		0.00780	4	0.00095	5
telomere maintenance	79	1.00000		0.07713	4	0.00095	7
response to estradiol stimulus	210	0.10005	3	0.08652	7	0.00095	11
sterol biosynthetic process	51	8.674E-07	6	0.17474	2	1.00000	
cholesterol biosynthetic process	44	2.016E-07	7	0.22496	2	1.00000	
steroid metabolic process	340	3.366E-06	9	0.27956	4	1.00000	
positive regulation of Ras GTPase activity	211	1.00000		1.00000		0.00003	7
steroid biosynthetic process	149	0.00010	6	1.00000		1.00000	
cholesterol metabolic process	152	1.463E-07	10	1.00000		1.00000	

Appendix: Select MetaCore Pathway from table 2 with 1 week WT data genes present in the ontology indicated (red bar symbols beside ontology elements)

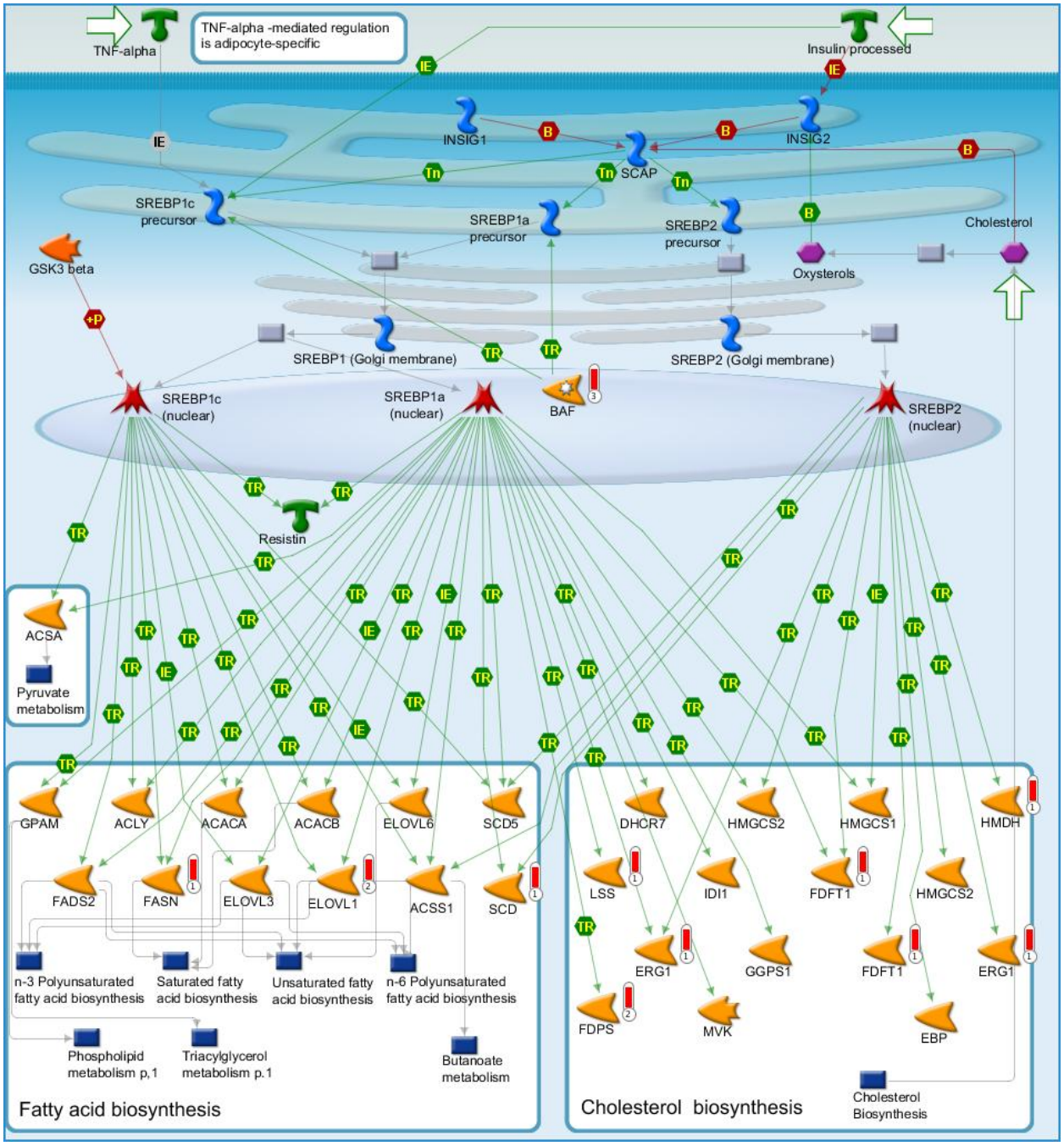
MetaCore's ***Cell cycle_The metaphase checkpoint*** pathway map (red poles beside elements represent a query element found in the ontology). This was the top ranked enriched pathway for the 1 week data enrichment with 13 query elements found in the ontology



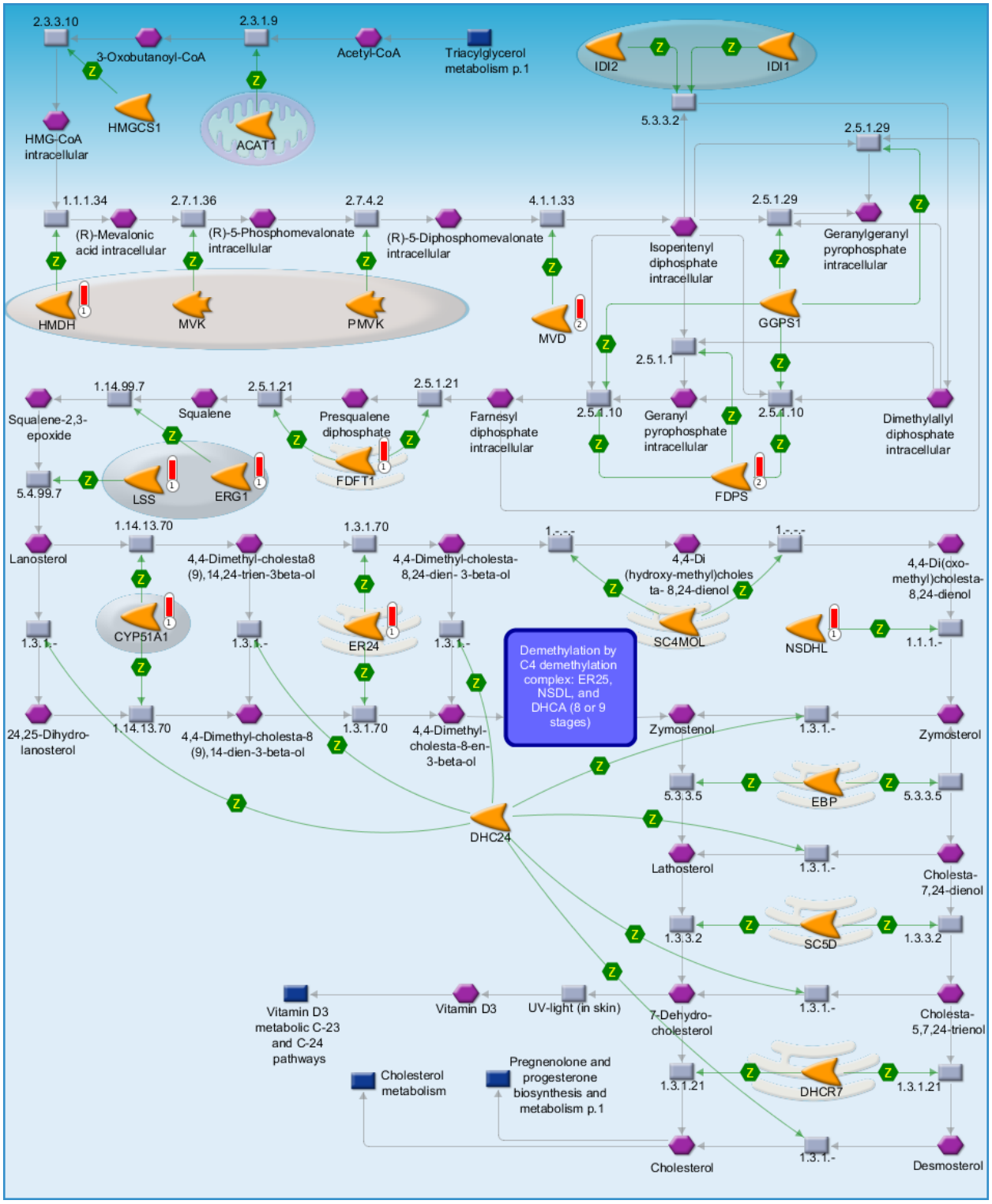
MetaCore's ***Cell cycle_Role of APC in cell cycle regulation*** pathway map (red poles beside elements represent a query element found in the ontology). The second ranked enriched pathway at 1 week with 11 query elements found in the ontology.



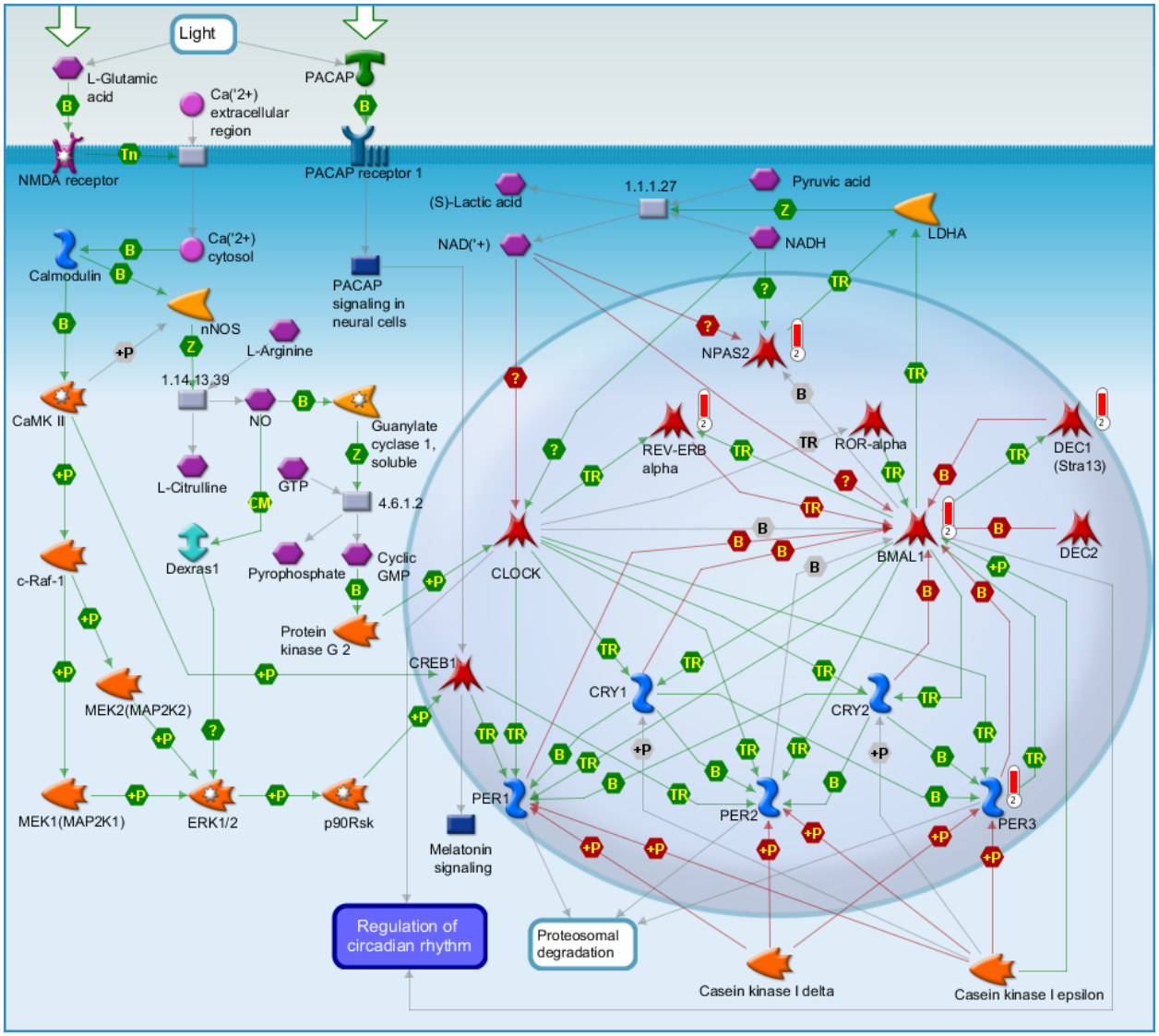
MetaCore's ***SCAP/SREBP Transcriptional Control of Cholesterol and FA Biosynthesis*** pathway map
(red poles beside elements represent a query element found in the ontology



MetaCore's ***Cholesterol Biosynthesis*** pathway map (red poles beside elements represent a query element found in the ontology



MetaCore's ***Neurophysiological process_Circadian rhythm*** pathway map (red poles beside elements represent a query element found in the ontology). This pathway was statistically significantly enriched at both 1 week and 4 week, although less than 5 query elements were found in the ontology at 4 weeks due to the limited number of significant genes for enrichment



Appendix 5a

Mean Body Weight and Mean Body Weight Gain Tables

10 pages

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Wild Type Mice #1-48

Bodyweight (Grams)

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
-12	Mean	18.975	19.038	19.238
	SD	0.907	0.792	0.812
	N	16	16	16
-6	Mean	21.531	21.488	21.513
	SD	0.599	0.566	0.623
	N	16	16	16
1	Mean	22.306	22.469	22.088
	SD	0.955	0.634	1.021
	N	16	16	16
6	Mean	21.665	21.160	20.933
	SD	0.642	0.968	1.417
	N	8	8	8
8	Mean	23.400	23.275	23.325
	SD	1.643	0.636	1.026
	N	8	8	8
15	Mean	24.263	23.775	23.713
	SD	1.693	0.911	1.131
	N	8	8	8

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Wild Type Mice #1-48

Bodyweight (Grams)

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
22	Mean	25.050	24.863	24.413
	SD	1.717	1.025	1.287
	N	8	8	8
27	Mean	24.193	24.398	23.955
	SD	1.830	1.258	1.148
	N	8	8	8

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Knock Out Mice #101-148

Bodyweight (Grams)

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
-12	Mean	19.488	19.113	20.500
	SD	4.279	4.464	3.845
	N	16	16	16
-6	Mean	22.681	22.050	22.669
	SD	2.273	2.493	2.432
	N	16	16	16
1	Mean	23.825	23.000	23.647
	SD	2.122	2.184	1.609
	N	16	16	15
6	Mean	23.285	22.236	22.956
	SD	1.329	1.670	1.547
	N	8	8	8
8	Mean	24.300	23.263	24.371
	SD	2.534	2.602	1.614
	N	8	8	7
15	Mean	25.088	23.825	25.271
	SD	2.339	2.371	1.291
	N	8	8	7

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Knock Out Mice #101-148

Bodyweight (Grams)

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
22	Mean	26.475	24.788	25.543
	SD	2.244	2.009	1.237
	N	8	8	7
27	Mean	25.471	24.115	24.689
	SD	1.897	2.196	1.188
	N	7	8	7

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Transgenic Mice #201-248

Bodyweight (Grams)

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
-12	Mean	21.319	21.106	21.331
	SD	1.332	1.461	1.042
	N	16	16	16
-6	Mean	23.169	23.131	23.181
	SD	1.145	1.128	1.267
	N	16	16	16
1	Mean	24.425	24.006	23.700
	SD	1.055	1.098	1.194
	N	16	16	16
6	Mean	22.823	22.863	27.053
	SD	1.336	1.097	11.580
	N	8	8	8
8	Mean	25.263	24.963	24.988
	SD	0.899	1.010	1.630
	N	8	8	8
15	Mean	25.800	25.513	25.363
	SD	1.041	1.279	1.945
	N	8	8	8

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Transgenic Mice #201-248

Bodyweight (Grams)

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
22	Mean	26.688	26.688	25.850
	SD	1.256	1.099	2.125
	N	8	8	8
27	Mean	25.498	25.505	25.539
	SD	1.488	1.019	2.110
	N	8	8	8

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

Bodyweights - Intergroup Comparison of Bodyweight Gains

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Wild Type Mice #1-48

Body Weight Gain (Grams)

Week numbers relative to Start Date

Group	Sex	Base Weight Week 1	From: To:	-2 -1	-1 1	1 2	2 3	3 4	Abs Gain 1 4	% Gain 1 4
0 ppm	m	22.306	Mean	2.556	0.775	1.275	0.863	0.788	2.925	13.14
		0.955	S.D.	0.826	0.775	0.757	0.226	0.323	0.761	3.09
40pp	m	22.469	Mean	2.450	0.981	0.625	0.500	1.088*(120)	2.213	9.81
		0.634	S.D.	0.720	0.418	0.652	0.535	0.402	0.813	3.72
120	m	22.088	Mean	2.275	0.575	1.063	0.388	0.700	2.150	9.70
		1.021	S.D.	0.542	0.670	0.453	0.572	0.273	0.793	3.71

Abs Gain = absolute bodyweight gain between base period and end of the analysis period
% Gain = percentage bodyweight gain between base period and end of the analysis period

Group 0 ppm - Control Group 40pp - 40 ppm Styrene Group 120 - 120 ppm Styrene

Bodyweights - Intergroup Comparison of Bodyweight Gains

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Knock Out Mice #101-148

Body Weight Gain (Grams)

Group	Sex	Base Weight Week 1	From: To:	Week numbers relative to Start Date -2 -1	-1 1	1 2	Abs Gain 1 4	% Gain 1 4
0 ppm	m	23.825	Mean	3.188	1.150*(120)	1.150	3.325	14.87
		2.122	S.D.	3.934	0.682	1.013	1.178	6.60
40pp	m	23.000	Mean	2.938	0.950	0.713	2.238	10.31
		2.184	S.D.	2.292	0.987	0.508	0.756	4.38
120	m	23.647	Mean	2.169	0.507*	0.614	1.786	7.87
		1.609	S.D.	1.582	0.740	0.628	1.181	5.93

Abs Gain = absolute bodyweight gain between base period and end of the analysis period
% Gain = percentage bodyweight gain between base period and end of the analysis period

Group 0 ppm - Control Group 40pp - 40 ppm Styrene Group 120 - 120 ppm Styrene

Bodyweights - Intergroup Comparison of Bodyweight Gains

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Knock Out Mice #101-148

Body Weight Gain (Grams)

Group	Sex	Base Weight Week 1	From: To:	Week numbers relative to Start Date 2 3	3 4	Abs Gain 1 4	% Gain 1 4
0 ppm	m	23.825	Mean	0.788	1.388*(40pp),**(120)	3.325	14.87
		2.122	S.D.	0.405	0.196	1.178	6.60
40pp	m	23.000	Mean	0.563	0.963*,**(120)	2.238	10.31
		2.184	S.D.	0.267	0.526	0.756	4.38
120	m	23.647	Mean	0.900	0.271**	1.786	7.87
		1.609	S.D.	0.416	0.415	1.181	5.93

Abs Gain = absolute bodyweight gain between base period and end of the analysis period
% Gain = percentage bodyweight gain between base period and end of the analysis period

Group 0 ppm - Control Group 40pp - 40 ppm Styrene Group 120 - 120 ppm Styrene

Bodyweights - Intergroup Comparison of Bodyweight Gains

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Transgenic Mice #201-248

Body Weight Gain (Grams)

Week numbers relative to Start Date

Group	Sex	Base Weight Week 1	From: To:	-2 -1	-1 1	1 2	2 3	3 4	Abs Gain 1 4	% Gain 1 4
0 ppm	m	24.425	Mean	1.850	1.256**(120)	0.738	0.538	0.888	2.163	8.82
		1.055	S.D.	0.746	0.631	0.524	0.272	0.364	0.852	3.48
40pp	m	24.006	Mean	2.025	0.875*	0.950	0.550	1.175**(120)	2.675	11.23
		1.098	S.D.	1.591	0.603	0.568	0.417	0.403	0.709	3.27
120	m	23.700	Mean	1.850	0.519**	1.113	0.375	0.488	1.975	8.16
		1.194	S.D.	0.625	0.550	0.376	0.520	0.398	0.855	3.00

Abs Gain = absolute bodyweight gain between base period and end of the analysis period
% Gain = percentage bodyweight gain between base period and end of the analysis period

Group 0 ppm - Control Group 40pp - 40 ppm Styrene Group 120 - 120 ppm Styrene

Appendix 5b

Mean Food Consumption Relative to Body Weight Tables

3 pages

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Wild Type Mice #1-48

Mean Food Consum rel to BW

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
1 → 6 aa[1]	Mean	171.55	119.35 dd[2]	109.64 dd[2]
	SD	15.64	27.88	46.22
	N	8	8	8
1 → 8	Mean	159.79	149.98	156.72
	SD	26.35	19.78	14.21
	N	8	8	8
8 → 15	Mean	186.75	199.42	193.30
	SD	26.94	21.54	10.37
	N	8	8	8
15 → 22	Mean	147.97	158.40	162.98
	SD	21.17	10.76	7.30
	N	8	8	8
22 → 27 aa[1]	Mean	164.46	164.02	182.36 dd[2]
	SD	13.32	9.83	7.35
	N	8	8	8

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

[1][Group Factor Anova & Dunnett's Test: aa - Statistical Test: Analysis of Variance $p < 0.01$]
[2][dd - Statistical Test: Dunnett 2 Sided $p < 0.01$]

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Knock Out Mice #101-148

Mean Food Consum rel to BW

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
1 → 6	Mean	165.75	165.02	140.55
	SD	22.34	19.61	23.58
	N	8	8	8
1 → 8	Mean	163.72	156.07	170.40
	SD	58.55	18.75	12.47
	N	8	8	7
8 → 15	Mean	201.24	185.62	188.83
	SD	132.87	11.10	16.08
	N	8	8	7
15 → 22	Mean	138.05	145.67	156.71
	SD	40.64	9.83	13.95
	N	8	8	7
22 → 27 aa[1]	Mean	145.29	167.47 d[2]	176.50 dd[3]
	SD	14.20	14.05	16.08
	N	7	8	7

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

[1][Group Factor Anova & Dunnett's Test: aa - Statistical Test: Analysis of Variance $p < 0.01$]
[2][d - Statistical Test: Dunnett 2 Sided $p < 0.05$]
[3][dd - Statistical Test: Dunnett 2 Sided $p < 0.01$]

Generalised Results - Group Summary by Time - Fixed Parameter

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice
Transgenic Mice #201-248

Mean Food Consum rel to BW

Sex: Male Day(s) Relative to Start Date		Control	40 ppm Styrene	120 ppm Styrene
1 → 6	Mean	158.41	176.56	143.58
	SD	46.90	21.24	23.84
	N	8	8	8
1 → 8	Mean	150.18	159.84	184.48
	SD	42.17	21.97	38.03
	N	8	8	8
8 → 15	Mean	183.25	181.94	183.16
	SD	32.21	11.66	17.17
	N	8	8	8
15 → 22	Mean	131.23	145.06	146.34
	SD	18.33	10.24	10.13
	N	8	8	8
22 → 27	Mean	154.29	164.53	174.91
	SD	22.61	10.22	11.70
	N	8	8	8

Statistical Test: Anova & Dunnett's Test Transformation: Identity (No Transformation)

Appendix 5c

Clinical Observations

6 pages

Clinical Observations - Clinical Signs by Animal

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	-12	-6	1	6	8	15	22
0 ppm	m	1	No Abnormalities Detected	X	X	X	X	.	.	.
		2	No Abnormalities Detected	X	X	X	X	.	.	.
		3	No Abnormalities Detected	X	X	X	X	.	.	.
		4	No Abnormalities Detected	X	X	X	X	.	.	.
		5	No Abnormalities Detected	X	X	X	X	.	.	.
		6	No Abnormalities Detected	X	X	X	X	.	.	.
		7	No Abnormalities Detected	X	X	X	X	.	.	.
		8	No Abnormalities Detected	X	X	X	X	.	.	.
		25	No Abnormalities Detected	X	X	X	.	X	X	X
		26	No Abnormalities Detected	X	X	X	.	X	X	X
		27	No Abnormalities Detected	X	X	X	.	X	X	X
		28	No Abnormalities Detected	X	X	X	.	X	X	X
		29	No Abnormalities Detected	X	X	X	.	X	X	X
		30	No Abnormalities Detected	X	X	X	.	X	X	X
		31	No Abnormalities Detected	X	X	X	.	X	X	X
		32	No Abnormalities Detected	X	X	X	.	X	X	X
		101	No Abnormalities Detected	X	X	X	X	.	.	.
		102	No Abnormalities Detected	X	X	X	X	.	.	.
		103	No Abnormalities Detected	X	X	X	X	.	.	.
		104	No Abnormalities Detected	X	X	X	X	.	.	.
		105	No Abnormalities Detected	X	X	X	X	.	.	.
		106	No Abnormalities Detected	X	X	X	X	.	.	.
		107	No Abnormalities Detected	X	X	X	X	.	.	.
		108	No Abnormalities Detected	X	X	X	X	.	.	.
		125	No Abnormalities Detected	X	X	X	.	X	X	X
		126	No Abnormalities Detected	X	X	X	.	X	X	X
		127	No Abnormalities Detected	X	X	X	.	X	X	X
		128	No Abnormalities Detected	X	X	X	.	X	X	X
		129	No Abnormalities Detected	X	X	X	.	X	X	X
		130	No Abnormalities Detected	X	X	X	.	X	X	X
		131	No Abnormalities Detected	X	X	X	.	X	X	X

Severity Codes: X = Present

Group 0 ppm - Control
Group 0 CP - Control Cell Proliferat
Group 40pp - 40 ppm Styrene
Group 120CP - 120 ppm Styrene Cell
Group 120 - 120 ppm Styrene

Clinical Observations - Clinical Signs by Animal

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	12	6	1	6	8	15	22
0 ppm	m	132	No Abnormalities Detected	X	X	X	.	X	X	X
		201	No Abnormalities Detected	X	X	X	X	.	.	.
		202	No Abnormalities Detected	X	X	X	X	.	.	.
		203	No Abnormalities Detected	X	X	X	X	.	.	.
		204	No Abnormalities Detected	X	X	X	X	.	.	.
		205	No Abnormalities Detected	X	X	X	X	.	.	.
		206	No Abnormalities Detected	X	X	X	X	.	.	.
		207	No Abnormalities Detected	X	X	X	X	.	.	.
		208	No Abnormalities Detected	X	X	X	X	.	.	.
		225	No Abnormalities Detected	X	X	X	.	X	X	X
		226	No Abnormalities Detected	X	X	X	.	X	X	X
		227	No Abnormalities Detected	X	X	X	.	X	X	X
		228	No Abnormalities Detected	X	X	X	.	X	X	X
		229	No Abnormalities Detected	X	X	X	.	X	X	X
		230	No Abnormalities Detected	X	X	X	.	X	X	X
		231	No Abnormalities Detected	X	X	X	.	X	X	X
		232	No Abnormalities Detected	X	X	X	.	X	X	X

Severity Codes: X = Present

Group 0 ppm - Control
Group 0 CP - Control Cell Proliferat
Group 40pp - 40 ppm Styrene
Group 120CP - 120 ppm Styrene Cell
Group 120 - 120 ppm Styrene

Clinical Observations - Clinical Signs by Animal

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	-12	-6	1	6	8	15	22
40pp	m	9	No Abnormalities Detected	X	X	X	X	.	.	.
		10	No Abnormalities Detected	X	X	X	X	.	.	.
		11	No Abnormalities Detected	X	X	X	X	.	.	.
		12	No Abnormalities Detected	X	X	X	X	.	.	.
		13	No Abnormalities Detected	X	X	X	X	.	.	.
		14	No Abnormalities Detected	X	X	X	X	.	.	.
		15	No Abnormalities Detected	X	X	X	X	.	.	.
		16	No Abnormalities Detected	X	X	X	X	.	.	.
		33	No Abnormalities Detected	X	X	X	.	X	X	X
		34	No Abnormalities Detected	X	X	X	.	X	X	X
		35	No Abnormalities Detected	X	X	X	.	X	X	X
		36	No Abnormalities Detected	X	X	X	.	X	X	X
		37	No Abnormalities Detected	X	X	X	.	X	X	X
		38	No Abnormalities Detected	X	X	X	.	X	X	X
		39	No Abnormalities Detected	X	X	X	.	X	X	X
		40	No Abnormalities Detected	X	X	X	.	X	X	X
		109	No Abnormalities Detected	X	X	X	X	.	.	.
		110	No Abnormalities Detected	X	X	X	X	.	.	.
		111	No Abnormalities Detected	X	X	X	X	.	.	.
		112	No Abnormalities Detected	X	X	X	X	.	.	.
		113	No Abnormalities Detected	X	X	X	X	.	.	.
		114	No Abnormalities Detected	X	X	X	X	.	.	.
		115	No Abnormalities Detected	X	X	X	X	.	.	.
		116	No Abnormalities Detected	X	X	X	X	.	.	.
		133	No Abnormalities Detected	X	X	X	.	X	X	X
		134	No Abnormalities Detected	X	X	X	.	X	X	X
		135	No Abnormalities Detected	X	X	X	.	X	X	X
		136	No Abnormalities Detected	X	X	X	.	X	X	X
		137	No Abnormalities Detected	X	X	X	.	X	X	X
		138	No Abnormalities Detected	X	X	X	.	X	X	X
		139	No Abnormalities Detected	X	X	X	.	X	X	X

Severity Codes: X = Present

Group 0 ppm - Control
Group 0 CP - Control Cell Proliferat
Group 40pp - 40 ppm Styrene
Group 120CP - 120 ppm Styrene Cell
Group 120 - 120 ppm Styrene

Clinical Observations - Clinical Signs by Animal

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	-12	-6	1	6	8	15	22
40pp	m	140	No Abnormalities Detected	X	X	X	.	X	X	X
		209	No Abnormalities Detected	X	X	X	X	.	.	.
		210	No Abnormalities Detected	X	X	X	X	.	.	.
		211	No Abnormalities Detected	X	X	X	X	.	.	.
		212	No Abnormalities Detected	X	X	X	X	.	.	.
		213	No Abnormalities Detected	X	X	X	X	.	.	.
		214	No Abnormalities Detected	X	X	X	X	.	.	.
		215	No Abnormalities Detected	X	X	X	X	.	.	.
		216	No Abnormalities Detected	X	X	X	X	.	.	.
		233	No Abnormalities Detected	X	X	X	.	X	X	X
		234	No Abnormalities Detected	X	X	X	.	X	X	X
		235	No Abnormalities Detected	X	X	X	.	X	X	X
		236	No Abnormalities Detected	X	X	X	.	X	X	X
		237	No Abnormalities Detected	X	X	X	.	X	X	X
		238	No Abnormalities Detected	X	X	X	.	X	X	X
		239	No Abnormalities Detected	X	X	X	.	X	X	X
		240	No Abnormalities Detected	X	X	X	.	X	X	X

Severity Codes: X = Present

Group 0 ppm - Control
Group 0 CP - Control Cell Proliferat
Group 40pp - 40 ppm Styrene
Group 120CP - 120 ppm Styrene Cell
Group 120 - 120 ppm Styrene

Clinical Observations - Clinical Signs by Animal

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	-12	-6	1	6	8	15	22
120	m	17	No Abnormalities Detected	X	X	X	X	.	.	.
		18	No Abnormalities Detected	X	X	X	X	.	.	.
		19	No Abnormalities Detected	X	X	X	X	.	.	.
		20	No Abnormalities Detected	X	X	X	X	.	.	.
		21	No Abnormalities Detected	X	X	X	X	.	.	.
		22	No Abnormalities Detected	X	X	X	X	.	.	.
		23	No Abnormalities Detected	X	X	X	X	.	.	.
		24	No Abnormalities Detected	X	X	X	X	.	.	.
		41	No Abnormalities Detected	X	X	X	.	X	X	X
		42	No Abnormalities Detected	X	X	X	.	X	X	X
		43	No Abnormalities Detected	X	X	X	.	X	X	X
		44	No Abnormalities Detected	X	X	X	.	X	X	X
		45	No Abnormalities Detected	X	X	X	.	X	X	X
		46	No Abnormalities Detected	X	X	X	.	X	X	X
		47	No Abnormalities Detected	X	X	X	.	X	X	X
		48	No Abnormalities Detected	X	X	X	.	X	X	X
		117	No Abnormalities Detected	X	X	X	X	.	.	.
		118	No Abnormalities Detected	X	X	X	X	.	.	.
		119	No Abnormalities Detected	X	X	X	X	.	.	.
		120	No Abnormalities Detected	X	X	X	X	.	.	.
		121	No Abnormalities Detected	X	X	X	X	.	.	.
		122	No Abnormalities Detected	X	X	X	X	.	.	.
		123	No Abnormalities Detected	X	X	X	X	.	.	.
		124	No Abnormalities Detected	X	X	X	X	.	.	.
		141	No Abnormalities Detected	X	X	X	.	X	X	X
		142	No Abnormalities Detected	X	X	X	.	X	X	X
		143	No Abnormalities Detected	X	X	X	.	X	X	X
		144	No Abnormalities Detected	X	X	X	.	X	X	X
		145	No Abnormalities Detected	X	X	X	.	X	X	X
		146	No Abnormalities Detected	X	X	X	.	X	X	X
		147	No Abnormalities Detected	X	X	X	.	X	X	X

Severity Codes: X = Present

Group 0 ppm - Control
Group 0 CP - Control Cell Proliferat
Group 40pp - 40 ppm Styrene
Group 120CP - 120 ppm Styrene Cell
Group 120 - 120 ppm Styrene

Clinical Observations - Clinical Signs by Animal

DED-STY131-1 - 4-Week Styrene Inhalation Lung Toxicity Study in Mice

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	-12	-6	1	6	8	15	22
120	m	148	No Abnormalities Detected	X	X	.	.	.	.	.
		217	No Abnormalities Detected	X	X	X	X	.	.	.
		218	No Abnormalities Detected	X	X	X	X	.	.	.
		219	No Abnormalities Detected	X	X	X	X	.	.	.
		220	No Abnormalities Detected	X	X	X	X	.	.	.
		221	No Abnormalities Detected	X	X	X	X	.	.	.
		222	No Abnormalities Detected	X	X	X	X	.	.	.
		223	No Abnormalities Detected	X	X	X	X	.	.	.
		224	No Abnormalities Detected	X	X	X	X	.	.	.
		241	No Abnormalities Detected	X	X	X	.	X	X	X
		242	No Abnormalities Detected	X	X	X	.	X	X	X
		243	No Abnormalities Detected	X	X	X	.	X	X	X
		244	No Abnormalities Detected	X	X	X	.	X	X	X
		245	No Abnormalities Detected	X	X	X	.	X	X	X
		246	No Abnormalities Detected	X	X	X	.	X	X	X
		247	No Abnormalities Detected	X	X	X	.	X	X	X
		248	No Abnormalities Detected	X	X	X	.	X	X	X

Severity Codes: X = Present

Group 0 ppm - Control
Group 0 CP - Control Cell Proliferat
Group 40pp - 40 ppm Styrene
Group 120CP - 120 ppm Styrene Cell
Group 120 - 120 ppm Styrene