Embryotoxicant-Specific Transcriptomic Responses in Rat Postimplantation Whole-Embryo Culture

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Rat postimplantation whole-embryo culture (WEC) is a promising alternative test for the assessment of developmental toxicity. Toxicogenomic-based approaches may improve the predictive ability of the WEC model by providing a means to identify compound-specific mechanistic responses associated with embryotoxicity in vivo. Furthermore, alterations in gene expression may serve as a sensitive, objective, and robust marker, which precedes the observation of classical developmental toxicity endpoints in time. In this study, in combination with morphological developmental assessments, we studied transcriptomic responses associated with four distinct teratogens (caffeine [CAF], methylmercury [MM], monobutyl phthalate, and methoxyacetic acid) after 4 h of exposure, well before apparent embryotoxicity in WEC. We evaluated gene expression changes associated with similar levels of induced morphological embryotoxicity for each teratogen (as determined by total morphological score), evaluating for functional enrichment and quantitative changes in response. Concentrations selected for each of the four teratogens used induced a number of common effects on embryonic development (neural tube closure and optic/otic system). Despite inducing common morphological effects, our analysis suggests limited overlap in terms of toxicogenomic response at the gene expression level and at the level of biological processes across all four test chemicals. Many unique responses associated with each chemical correlated with previously hypothesized modes of developmental toxicity. For example, alterations in developmental signaling and cholesterol metabolism were observed with MM and CAF, respectively. This initial study suggests that distinct chemical-induced toxicogenomic responses precede morphological effects in WEC and that these responses are relevant with mechanisms of toxicity previously observed in vivo.

Key Words: toxicogenomics; whole embryo culture; development; alternative; microarray; neurulation; rat; mercury; phthalate; glycol ether; caffeine.

As a result of the implementation of European Registration, Evaluation, and Authorization of Chemicals legislation, toxicological information is required for approximately 30,000 existing chemicals used by manufacturers and users. As proposed, 1–2 million animals will be needed to conduct appropriate developmental toxicity testing (van der Jagt et al., 2004), representing an enormous cost in terms of resources and animal life. Therefore, there is an urgent need to develop alternative methods to reduce animal usage and increase efficiency in predicting developmental toxicity. Although no one existing in vitro method has been identified to be a full replacement for existing classical in vivo approaches, a select few have been recognized as potential alternative tests for screening embryotoxic compounds (Genschow et al., 2002).

One of the most established in vitro methods for assessing embryotoxicity is the rat whole-embryo culture (WEC) model. In WEC, postimplantation rat embryos are cultured successfully from embryonic days 10–12 with growth and development morphologically parallel with the in vivo system (New et al., 1976). During this developmental period, the embryo progresses through neurulation and subsequent early formation of the heart, eyes, ears, limbs, and central nervous system. Assessing 20 chemicals in four independent laboratories, a validation study demonstrated the ability of the WEC model to discriminate strong embryotoxins from weak and nonembryotoxicants with low interlaboratory variability (Piersma et al., 2004).

The predictive ability of the WEC model to determine embryotoxicity may be improved using gene expression profiling (toxicogenomics) by providing a mechanistic anchor relevant to in vivo modes of action associated with toxicity. In our initial WEC toxicogenomic study, we demonstrated retinoic acid (RA) to induce early effects (4 h) on gene expression following embryotoxic exposures, including multiple genes previously identified to be associated with...
RA-induced embryotoxicity in vivo (Luijten et al., 2010). Altered gene expression profiles in WEC may be usable as a predictor of malformations developed in vivo and furthermore provide a mechanistic basis for potential embryotoxicity. In addition, gene expression profiles may allow the detection of those embryotoxicants that cannot be detected by morphological scoring because their effects occur exclusively outside the 48-h culture window of the WEC system. Therefore, the predictive capacity of WEC may improve if toxicogenomics is used as the readout of embryotoxicity in WEC.

In the present study, we followed our initial assessments by investigating gene expression response associated with each of four classical teratogens in the WEC model system. These four compounds, methylmercury (MM), caffeine (CAF), monobutyl phthalate (MBP), and methoxyacetic acid (MAA), are well documented in their effects as teratogens and represent distinct classes in terms of mechanistic effects, metabolism, and kinetics. We aimed to determine if these four teratogens at concentrations of equipotency induce distinct alterations in transcription in WEC. These initial evaluations suggest that these four chemical compounds induce differing gene signatures, which precede morphological aberrations. Furthermore, analysis of responses at the gene and functional level suggests that select transcriptional changes in WEC overlap with previously identified responses associated with developmental toxicity in vivo.

MATERIALS AND METHODS

Animal care. All animal studies were approved and conducted in accordance with the National Institute for Public Health and the Environment Animal Care facility and federal regulations. As described previously (Luijten et al., 2010), HsdCpb:WU (Harlan, The Netherlands) nulliparous female rats were housed with adult male rats. After a 3-h mating period (9–12 A.M.), copulatory plugs were identified. Following coitus (gestational day 0), females were housed in separate cages in climate-controlled rooms with a 12-h on/off light cycle. On a daily basis, animals were monitored for general health. Water (tap) and food were provided ad libitum.

Whole-embryo culture. As described previously (New, 1978; Pierson et al., 2004), on gestational day 10, rat embryos were extracted from the uterus with the yolk sac and ectoplacental cone left intact. Embryos with one to five somites were used at the onset of culture. Only embryos with initial counts of two to four somites were used for gene expression studies. Previous gene expression assessment studies from our laboratory suggest similar gene expression response between embryos of this stage in response to the classical teratogen, RA (Luijten et al., 2010). Embryos were cultured separately in 2 ml serum mixture (Biochrom, Berlin, Germany; #9890) consisting of 80% pregnant bovine serum, 10% rat serum, 1.57 mg/ml α-glucose, 75 μg/ml t-methionine, and supplemented 10% Hank’s solution (Gibco).

Exposure. Working concentrations for CAF (CAS 58-08-2; Sigma-Aldrich, Zwijndrecht, The Netherlands), MM chloride (CAS 115-09-3; Sigma-Aldrich), MBP (CAS 131-70-4; Frinton Laboratories, Vineland), and MAA (CAS no. 625-45-6; Sigma-Aldrich) were completed in the morning of each exposure culture day. Dilutions for CAF, MBP, and MAA were conducted using saline and dimethyl sulfoxide (DMSO; Merck) for MM. Prior to culture, chemical compounds were added to the serum mixture at a volume of 1 μl/ml at final concentrations of CAF (200mM), MM (6mM), MBP (1000mM), and MAA (250mM). Control (CON) (1 μl/ml saline) and DMSO (1 μl/ml) were also added for comparative control groups. Cultures were completed over a 5-week period. Appropriate controls (CON or DMSO) were performed concurrently with exposure groups within each separate culture.

RNA isolation. After 4 h exposure, embryos were quickly removed from the yolk sac and ectoplacental cone and scored for the amount of total somites and development of the neural tube. Immediately following morphological assessment, embryos were separately placed into 500 μl of RNA-Later Stabilization Solution (Ambion, Austin, TX) and stored at 4° C. In our previous study, we identified the 4-h time point to be an optimal window for assessing early effects for RA. Therefore, in this study, we chose 4 h as an initial assessment to compare and contrast gene expression signatures between these four distinct teratogens.

Following the manufacturer’s protocol, total RNA was isolated from single embryos using the RNeasy Micro Kit (Qiagen, Valencia, CA). RNA concentrations were determined using the Nanodrop ND-100 (Nanodrop Technologies Inc., Wilmington, DE), and RNA quality was assessed using the 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA). Total RNA samples with an RNA integrity number > 7 were used for further analysis.

Oligonucleotide microarrays. Affymetrix Rat Genome 230 2.0 Genechip Array (Affymetrix Inc., Santa Clara, CA) was used for gene expression profiling. In total, 60 arrays were used (n = 10 arrays for each exposure group). All technical aspects concerning microarray experimentation were performed at the University of Maastricht (Maastricht, The Netherlands). In brief, RNA targets were prepared using 150 ng of purified total RNA as a template for the Affymetrix GeneChip Expression 3′ Amplification Two-Cycle Target Labeling Kit (900494). Microarray hybridization was conducted according to the manufacturer’s instructions. Upon hybridization, arrays were washed and stained using the GeneChip Fluidics Station 450 and scanned by the GeneChip Scanner 3000 (Affymetrix Inc.).

Microarray analysis—data processing. Scanned microarray images were inspected visually, and raw values were examined for average background, scale factors, percentage of present calls, 3′/5′ ratios of glyceraldehyde 3-phosphate dehydrogenase, and 3′/5′ ratios of Bactin. Affymetrix CEL files were normalized using the Robust Multichip Average algorithm (Irizarry et al., 2003) using a custom CDF developed by de Leeuw et al. (2008) (http://mad-db.science.uva.nl/~wdeleeuw/HybridAnnot/version6.html). Of the hybrid probe sets included within the custom annotation, 11,427 probe sets defined by the Brainarray custom CDF version 11 (http://brainarray.mbi.umi-ch.edu/Brainarray/Database/CustomCDF) (Dai et al., 2005) and 2374 additional probe sets defined by Affymetrix chip annotation 26 were used in further analyses. In total, we assessed 13,801 probe sets. Affymetrix internal controls or probes that did not correspond to an Entrez Gene ID were not used in further analyses.

Microarray analysis—identification of significantly altered genes. Normalized data were log transformed and imported into BRBArrayTools (National Cancer Institute) (Simon et al., 2007). We employed an all-effects model (ANOVA, F-test) to determine the significance of impact on genes by any one of the six exposure groups using the random variance model of all available probes (Wright and Simon, 2003). p Values were corrected for multiple testing by calculating the false discovery rate (FDR) according to Benjamini and Hochberg (1995). Significant effects were determined using a p value cutoff of p < 0.0001 (FDR < 1%). Post hoc analyses (Student’s t-test) were conducted to determine the distribution of response in terms of significance. Significant genes were identified with a cutoff of p < 0.05, controlled modestly by a FDR < 50%.

Because of extreme differences in distribution and the complexity of using differing sizes of gene lists for later comparison, a ranking approach was implemented to make comparisons regarding common gene expression responses and assessing function changes in gene expression (gene ontology [GO] analysis). The top 1000 genes were determined using t-tests between each exposure group and their respective control (CON or DMSO). This approach
translated into different criteria in terms of \( p \) value cutoff for each one of the compounds, \( p \) value cutoffs for each compound were approximately the following: CAF (\( p < 0.05 \)), MM (\( p < 0.05 \)), MBP (\( p < 0.01 \)), and MAA (\( p < 0.0001 \)). Enrichment and quantification of significantly enriched GO biological processes were conducted using GenMAPP (Doniger et al., 2003) and GO-Quant (Yu et al., 2005), respectively. Enriched GO biological processes were determined to contain > 3 genes, \( Z > 2 \), and a \( p < 0.05 \). The absolute average fold change in all genes identified to be within the top 1000 of one of the four teratogenic compounds (CAF, MM, MBP, and MAA) for each enriched GO term was determined and color coded by intensity of change. Similar results were observed with assessments conducted using the top 500 (not shown).

**Morphological validation.** In parallel with RNA studies, morphological assessments were conducted to validate comparable embryotoxic concentrations of CAF, MM, MBP, and MAA in comparison with vehicle controls (CON or DMSO). Embryos initially (0 h) with 1–5 somites were cultured for 48 h and evaluated for their total morphological score (TMS) to determine potential effects of each compound on yolk sac and embryonic development (\( n \geq 10 \) for each exposure group). Developed previously by Brown and Fabro (1981), the TMS system was used to determine effects of chemical compounds on developmental morphological hallmarks in the WEC. Over 20 endpoints were assessed including evaluation of yolk sac, heart, neural tube, ear, limb, mandibulars, and somite development. Average scores were determined for specific parameters to identify common effects on embryonic development across exposure groups. \( t \)-Tests (one sided, \( p < 0.05 \)) were employed to determine significant effects between exposure and respective control (CON or DMSO). Images of embryos were executed using an Olympus SZX9 and Olympus DP Software. Examples of embryos at 4 and 48 h were taken at \( \times 32 \) and \( \times 20 \) magnification, respectively.

**RESULTS**

**Embryotoxic Compounds Induce Morphological Alterations in WEC in a Time-Dependent Manner**

In embryos used for microarray analysis, we assessed the impact of embryotoxic compounds on development progression by evaluating somitogenesis and neural tube morphology at 4 h (Fig. 1). Cultures of whole embryos across all treatment groups initially averaged ~3 somites (\( S(0 \text{h}) \)) and developed ~2 additional somites over the 4-h period of culture. No differences in initial (\( S(0 \text{h}) \)), final somite (\( S(4 \text{h}) \)), or differences in somite count (\( S_D \)) were observed between treatment groups (\( p > 0.05 \)). No differences in neural tube progression or heart development were observed between toxicant-exposed or control embryos at 4 h (\( p > 0.05 \)) at this time (not shown). In parallel with these studies, embryos were evaluated for morphological alterations following exposure for 48 h. In contrast to 4-h assessments, at 48 h, significant differences were observed between embryotoxic compounds and their respective control (CON or DMSO) for multiple developmental endpoints. As observed in Figure 2A, selected concentrations of CAF, MM, MBP, and MAA induced equivalent and significant reduction in TMS scores at 48 h (\( p < 0.05 \)). Common compound-specific significant effects were observed on somitogenesis (\( S_P \)), yolk sac development, forebrain neural tube closure, and ear and eye development (otic and optic system). Other significant effects observed with at least two of the four compounds included mid/hindbrain neural tube, branchial, mandibular, heart, and limb development. No effects were observed on caudal neural tube morphogenesis across all four compounds in comparison to their respective concurrent control. No significant differences were observed in DMSO-exposed embryos compared with CON (\( p > 0.05 \)). Examples of embryotoxic-induced abnormalities of the neural tube, eye, branchial bars, and forelimb are displayed in Figure 2B.

**Differential Significance of Gene Expression Response across Embryotoxic Compounds in WEC**

Using principal components analysis, we evaluated single arrays for similarities in gene expression across the 1733 genes

![FIG. 1.](https://academic.oup.com/toxsci/article/118/2/675/1655132/1826751655132)
identified to be significantly altered with exposure ($B_{dose}$, $p < 0.0001$; FDR < 1%) (Fig. 3A). Embryos exposed to MAA (purple) showed consistent separation from all other treatment groups. Considerable overlap was observed between MBP, CAF, MM, and control groups (CON and DMSO). As observed in Figure 3B, post hoc analyses suggested MAA to uniquely alter the majority (96%) of genes identified to be significantly affected across all groups, suggesting MAA to be much more responsive than the remaining compounds tested at the 4-h time point. MBP significantly affected expression of  

![Diagram](https://example.com/diagram.png)
24% of the 1733 genes. CAF and MM both showed small changes in terms of the amount of genes altered (12 and 10%, respectively), whereas DMSO showed minimal changes compared with CON (<1%). Hierarchical clustering plots assessing quantitative changes further confirmed greater responsiveness in the MAA exposure group compared with MBP, CAF, MM, and DMSO (not shown). Because of apparent differences in the magnitude of response and significance, we employed a ranking approach to compare and examine functional changes occurring across exposure groups.

Embryotoxic Compounds Induce Differential Qualitative and Quantitative Enrichment of GO Biological Processes in WEC

Within the top 1000 genes altered by each compound in comparison with their respective control (p value), we observed a modest overlap across all groups (Fig. 4A). Most comparisons showed approximately a 10% overlap in altered genes between exposure groups, with CAF and MBP showing the highest similarity (16.6%). To identify possible overlap in effects on previously identified as well as potentially unknown

![Diagram](https://academic.oup.com/toxsci/article/118/2/675/1655132)

**FIG. 4.** Enrichment and quantitative analysis of GO biological processes altered by embryotoxic compounds in WEC. (A) The overlap of genes between exposure groups within the top 1000 ranked genes for each chemical compound. (B) Supervised hierarchical clustering of enriched GO biological processes associated with the top 1000 ranked genes altered by each chemical compound. The degree of enrichment (−log(p) > 2) is indicated by increased color. GO terms were considered to be significantly enriched with the criteria of p < 0.05, Z > 2, and >3 genes altered (degree of enrichment >2.6). The amount of altered genes related to each specific GO biological process is labeled for each of the four chemicals. GO terms enriched by more than one compound are indicated ($). (C) Quantitative differences in the degree of change within genes associated with enriched GO biological processes between exposure groups using GO-Quant. The absolute average fold change of all genes within each enriched GO biological process identified to be disrupted (top 1000) by any of the four compounds is indicated using a color gradient. The total number of genes identified to be altered by any of the four chemicals is listed to the right of each GO term. Colored bars reflect similar general GO categories to show potential overlap in general effects.
mechanisms of developmental toxicity at the functional level, we investigated for enrichment of gene expression–linked GO biological processes. Corresponding with the top 1000 genes to be significantly altered by each compound, we identified distinctive enrichment of GO biological processes across embryotoxic compounds, CAF, MM, MBP, and MAA (Fig. 4B). With CAF, we observed unique significant enrichment of GO terms, lipid biosynthesis, immune effector process, tube morphogenesis, and intracellular receptor–mediated signaling. MM disrupted categories of genes related to development (organ, nervous system, heart, and somitogenesis), cell development (cell maturation and negative regulation of cell differentiation), cell adhesion, RNA transport, and cell cycle arrest. Enrichment of genes related to organelle (mitochondrial and actomyosin) organization, cell cycle, response to endogenous stimulus, response to DNA damage, protein folding, and bone morphogenetic protein signaling were observed with MBP. MAA altered genes associated with biopolymer modification, phosphate metabolic process, water soluble vitamin metabolic process, glucose transport, glutathione metabolic process, and regulation of DNA metabolic process. Limited overlap in enrichment in GO terms was observed across chemical compounds. Common enrichment was observed for sterol and cholesterol metabolism (MM and CAF), RNA processing (CAF and MBP), and negative regulation of enzyme, transferase, and protein kinase activity (MM and MBP). Interestingly, even in commonly enriched categories, <20% of the genes overlapped within these categories between the two compounds (not shown). Quantitative analyses of all genes identified to be altered by at least one of the four compounds (top 1000) within each enriched GO biological process were evaluated by calculating the absolute average fold change (Fig. 4C). In general, enriched categories related to each exposure group displayed greater changes compared with nonenriched categories within the same column. Comparing quantitative changes across exposure groups revealed potential common changes in the degree of response within genes related to a GO term, irrespective of enrichment. These observations were most apparent with MAA, which impacted most groups of genes, reflective of the robustness of gene change observed with earlier analyses. For example, within GO-enriched terms identified to be significant for CAF (e.g., lipid biosynthetic process), MM (e.g., organ development), or MBP (e.g., cell cycle process), MAA showed higher response in genes related to these GO terms than any of the other three compounds, despite not showing enrichment of these terms within the top 1000 MAA-responsive genes.

**Embryotoxic Compounds Induce Limited Overlapping Response at the Gene Level in WEC**

To further investigate potential overlap in response at the gene level across each of the four compounds, we identified the top 200 genes identified to be the most commonly altered on average (average rank) across all four compounds (Fig. 5). By conducting k-means clustering analysis, in total, five clusters, or in other words, five relationships, were observed in terms of gene expression regulation across the four compounds within this subset. Clusters showing similar direction of regulation across all four embryotoxic compounds were identified to fall within cluster I (upregulated) and cluster V (downregulated). In general, only 35% of 200-gene subset showed common directionality across all four compounds. Furthermore, within these clusters, not one gene was identified to be commonly regulated within the top 1000 of each of the four compounds, suggesting limited overlap in response. Only 13 genes (highlighted) were significant in 3/4 exposure groups, which showed common directionality.

**Embryotoxic Compounds Induce Relative Gene Expression Responses Observed In Vivo**

At the gene level, embryotoxicant-induced responses in WEC corresponded with previously proposed mechanisms of toxicity identified in other toxicological models (Table 1). Here, we show how proposed mechanisms of toxicity for CAF (fat metabolism inhibition and alterations in cholesterol levels), MM (developmental signaling, cell differentiation, cell proliferation, and cell adhesion), MBP (cell cycle alterations and DNA damage), and MAA (protein kinase pathways and oxidative stress) are observed in WEC on a gene expression–based level. For example, previous human- and cell-based studies suggest CAF to reduce lipid and cholesterol metabolism. In this study, CAF disrupted expression of genes involved in lipid biosynthetic process and cholesterol metabolism. Genes related with lipid biosynthetic process (nine total, Dhcr24, Fdft1, Hmgcs1, Hsd17b7, Idi1, Insig1, Ohrs1, Sd1, and Scd1) and cholesterol metabolism (six total, Dhcr24, Hmgcs1, Hsd17b7, Idi1, Insig1, and Sfle) were all identified to be downregulated with CAF (top 1000, 20% increase or decrease in expression).

**DISCUSSION**

The WEC model represents a well-established and promising alternative test to assess developmental toxicity. The predictive ability of the WEC model to determine developmental toxicity may be improved by evaluating gene expression in combination with classical measures of toxicity. Our initial study (Luijten et al., 2010), using RA, suggested that the identification of gene expression alterations in WEC to be feasible and relevant to previously identified mechanisms of RA-induced developmental toxicity. In this study, we support and expand these initial assessments by determining if a group of four diverse embryotoxicants tested at equipotent concentrations induce toxicogenomic signatures in WEC in association with developmental toxicity. Our results suggest that all four embryotoxicants induced distinct toxicogenomic signatures in terms of specificity and functionality. Furthermore, specific toxicogenomic changes observed correlate with previous...
hypothesized mechanisms of action. These results suggest that a toxicogenomic approach provides a more detailed description of compound effects than morphological observations alone.

Classic Teratogens Impact Embryonic Development in the WEC Model Similar to In Vivo

We observed all four teratogens (CAF, MM, MBP, and MAA) to cause developmental toxicity in WEC. Selected concentrations of CAF, MM, MBP, and MAA induced equivalent levels of embryotoxicity as determined by TMS evaluation at 48 h (Fig. 2). Associated with a reduction of TMS by ~20%, an array of teratogenic effects were observed (e.g., neural tube closure, eye, and ear abnormalities) including many documented in vivo using animal models with CAF, MM, MBP, and MAA during similar exposure windows (Christian and Brent, 2001; Ema et al., 1995; Su and Okita, 1976; Terry et al., 1994).

Teratogens Induce Distinct Gene Expression Alterations in WEC: Overlap in Response

Assessments of equal proportions of genes (top 1000) altered by each compound indicated distinct responses between compounds (Fig. 4). In general, with disregard to directionality of response, only ~10% of genes overlapped between exposure groups with the highest overlap of significantly altered genes between CAF and MBP (16.6%). Despite some overlap in comparing exposure group to exposure group, not one gene was commonly identified across all four compounds within the top 1000, and additionally, only 13 genes were represented that showed common directionality and were within the top 1000 for three out of the four compounds (Fig. 5). These results suggest a limited overlap in gene expression response between these four test chemicals. Functional analysis of these genes strengthened these observations, suggesting dissimilarity in response at even the functional level. Not one GO biological
process term was commonly enriched across all four compounds or, for that matter, three out of the four exposure groups (Fig. 4B). These observations stress that the most prominent responses induced by these teratogens at this early time point (4 h) reflect differently altered genes at the functional and gene level across the unique teratogens tested in this study. Although these compounds induced equivalent levels of toxicity, including specific developmental toxic effects morphologically at 48 h (Fig. 2), gene expression signatures at 4 h did not reflect these similarities in subsequent altered morphology. Furthermore, gene expression alterations preceded morphological alterations as no morphological differences were observed between exposure groups at 4 h in contrast to 48-h morphological assessments (Figs. 1 and 2).

### Table 1

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Proposed mechanism</th>
<th>GO biological process enrichment</th>
<th>Genes significantly altered (top 1000, 20% increase or decrease)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAF</td>
<td>Reduced lipid biosynthesis</td>
<td>Lipid biosynthetic process</td>
<td>Dhcr24, Fdft1, Hmgcs1, Hsd17b7, Idi1, Insig1, Oespl, Scl6, Sc4mol</td>
</tr>
<tr>
<td></td>
<td>Reduced cholesterol</td>
<td>Cholesterol metabolism</td>
<td>Fdft1, Hmgcs1, Hsd17b7, Idi1, Insig1, Scle</td>
</tr>
<tr>
<td></td>
<td>Delayed neurulation</td>
<td>Tube morphogenesis</td>
<td>Bbs, Cobl, Gpc3, Wnt4</td>
</tr>
<tr>
<td>MM</td>
<td>Developmental signaling</td>
<td>Nervous system development</td>
<td>Abr, Agr, Calmb1, Cel51, Chd7, Cxcl12, Cyp26a1, Dll1, Dll3, Dnch2, Dpy4, Edg8, Etv4, Fabp7, Fez1, Fgf18, Fgf8, Hes1, Hes3, Igfr, Irs2, Ket, Kit5c, Lrp4, Nes, Nkx6-1, Nkx6-2, Notch1, Robo2, Smarca4, Robo3</td>
</tr>
</tbody>
</table>

**Note.** Gene expression alterations in the WEC were compared with previously proposed mechanisms of action specific to CAF, MM, MBP, or MAA to identify common changes occurring across toxicological models. Enriched GO biological processes that correspond with proposed mechanisms of toxicity were determined using GenMAPP ($p < 0.05, Z > 2, \# \text{ changed} > 3$). Genes significantly altered (top 1000, 20% increase or decrease) by each compound are displayed.

These observations may also support the use of gene expression to detect defects that develop outside the 48-h window of culture and emerge later in embryofetogenesis (e.g., cleft palate). In general, these observations suggest that the compound specificity of gene expression response in WEC provides a more detailed picture of toxicity than scoring of morphological changes alone.

### Teratogens Induce Distinct Gene Expression Alterations in WEC: Quantitative Changes in Response

Although our observations within the top 1000 genes of each teratogen suggest a lack of overlap for the most prominent responses associated with each teratogen, they do not fully account for possible overlapping quantitative changes in
response independent of significance. Despite showing only ~10% overlap within the top 1000 genes with other teratogens, MAA clearly impacted all categories of genes impacted by any of the four teratogens (Fig. 4C). At the gene level, transcriptional responses in MAA-exposed embryos were more robust in terms of fold change and significance compared with the other exposure groups in this study (Fig. 3). Because of our analysis containing only one time point (4 h) and one dose, we are limited in our conclusions regarding qualitative and quantitative comparisons across the four teratogens used in this study. However, this initial snapshot in gene expression response in the WEC does implicate the potential complexity of assessing common and unique transcriptional changes between teratogens because of differences in kinetic and dynamic response. Although our study, in general, does suggest that the derivation of a single gene set for prediction of embryotoxicity does not seem to be feasible in this system, further analyses examining dose- and time-dependent response should be completed to examine these relationships. The current study focused on earlier gene expression changes in time (4 h). Later responses may correspond with common disruption of developmental pathways, which regulate specific developmental processes critical for neural tube or eye development or, perhaps, common pathways representative of irreversible damage (apoptosis). As an example, previous studies indicate teratogens such as cadmium, cyclophosphamide, 2-methoxyethanol, and CAF all induce increased apoptosis within the neural tube in relation with disturbances in neural tube formation (Ambroso et al., 1998; Fernandez et al., 2003; Marret et al., 1997; Terry et al., 1996; Xiao et al., 2007). In parallel with toxicokinetic and morphological assessments, the combination of dose and time toxicogenomic data will be critical in determining the value of toxicogenomics in predicting developmental toxicity in alternative models like the WEC model.

Responses in WEC Correspond with Previously Identified Mechanisms of Toxicity In Vivo

Several mechanisms underlying embryotoxicity have been proposed for the teratogens used in this study. Using a systems-based approach, we identified gene expression alterations in WEC, many of which relate with previously proposed mechanisms of toxicity in vivo. Although toxicogenomic observations are primarily descriptive in nature, our results support future detailed mechanistic studies, which may explore specific mechanistic links with chemical-induced teratogenesis.

Several hypothesized molecular effects for CAF have been suggested, including the disruption of calcium levels through interactions with ryanodine receptors (Tsai and Barish, 1995), alterations in cell division (Marret et al., 1997; Schlegel and Pardee, 1986), and decreased lipid (Nakabayashi et al., 2008) and cholesterol synthesis (Yazdani et al., 1990). Concerning the latter, as observed in Figure 5, we observed enrichment of cholesterol metabolism genes within the top 1000 genes identified to be impacted by CAF. All genes related to cholesterol metabolism altered by CAF were identified to be downregulated (Table 1, Fig. 4B). These results correspond with gestational exposure studies suggesting decreased cholesterol synthesis in fetal mouse brain following exposure to CAF during key windows in brain development (Yazdani et al., 1990). Although cholesterol is known to be important for cellular membrane architecture, it is also critical for the molecular signaling molecule Shh (Beachy et al., 1997), which is necessary for early embryogenesis. Furthermore, human and animal studies suggest that elimination of key cholesterol metabolism–related genes, including two observed to be downregulated in this study (Dhcr24 and Fdft1), are linked with a variety of developmental effects, including craniofacial abnormalities, neural tube and limb malformations, and viability (FitzPatrick et al., 1998; Mirza et al., 2006; Ohashi et al., 2003; Salen et al., 1996; Tozawa et al., 1999). This study highlights potential interactions with CAF, cholesterol metabolism, and induced teratogenesis relevant for mechanisms of developmental toxicity in vivo.

Also in this study, we observed MM to specifically disrupt expression of genes related to morphological and cellular development (Table 1, Fig. 4B). These changes in gene expression are supported by a wealth of evidence in animal-based models indicating MM to disrupt the balance of proliferation and differentiation in developing organisms by inducing changes in cell cycle regulatory molecules (e.g., p53 and Cdkn1a) (Faustman et al., 2002) and altering expression of key developmental signaling molecules (Bland and Rand, 2006; Robinson et al., 2010a,b; Tamm et al., 2008; Theunissen et al., 2010). Recent toxicogenomic in vivo studies assessing MM impacts on gene expression in mouse embryos undergoing neurulation suggest similar impacts within GO-based biological processes as the current study, including common enrichment of morphological (nervous system development) and cellular development (cell differentiation, cell cycle, and cell adhesion), GO-based categories, as well as specific targets, such as the cadherin receptor, Celsr1 (Robinson et al., 2010a,b). MM-induced transcriptional changes in WEC correspond with previous in vivo–based studies, including embryonic toxicogenomic evaluations.

Phthalates as a class of chemicals have been studied because of their selective endocrine disruptive effects as well as their potential as developmental toxicants (Ema et al., 1995). Limited mechanistic information exists concerning specifically MBP, but phthalates as a class disrupt multiple pathways. In whole-animal and cell-based models, phthalates alter cellular proliferation and induce DNA damage (Rusyn et al., 2006). Here, in this study, we observed alterations related to the GO categories, cell cycle, and response to DNA damage stimulus (Table 1, Fig. 4B). MBP-induced alteration included upregulation of well-known targets of induced cell cycle arrest and DNA damage response (Brcal, Gadd45a, Trp53, and Xrcc1) (Taylor and Stark, 2001), supporting previous
hypothesized mechanism of toxicity of the phthalate class of compounds.

Glycol ethers represent a class of chemicals known to induce structural defects including neural tube defects (Clarke et al., 1992; Horton et al., 1985; Nagano et al., 1981; Sleet et al., 1996) because of their ability to impact multiple pathways, including oxidative stress, kinase (Bagchi et al., 2009; Jindo et al., 2001) and androgen receptor/estrogen receptor signaling (Bagchi et al., 2009; Henley et al., 2009) proliferation (van Dartel et al., 2010), and cell death pathways (Rao and Shaha, 2002). Here, in this study, we also identified MAA to elicit a robust response across multiple pathways at 4 h (Fig. 4C). In particular, genes involved in glutathione metabolism were identified to be a primary response because of MAA exposure, including upregulation of key genes (Ggt5, Gstt1, and Mgst1) critical for restoring glutathione stores and their involvement in stress response (Heisterkamp et al., 2008) (Table 1, Fig. 4B).

Using the WEC model, we observe MAA-induced responses, which support previous hypothesized pathways of MAA toxicity.

For the past 20 years, the WEC model has been recognized as an alternative model to evaluate embryotoxicity. Toxicogenomic evaluations may improve the predictive ability of the WEC model by adding a robust mechanistic anchor to morphological alterations comparable across in vitro and in vivo systems. Here, in this study, we support the usage of toxicogenomics in WEC, showing distinct and relevant alterations in gene expression, which precede embryotoxicity. In addition, because of the complexity of proposed dynamic and possibly kinetic differences between chemicals, we highlight the need for dose- and time-response data in making cross-toxicogenomic comparisons in the WEC system.

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