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A Comparison of Gene Expression Responses in Rat Whole Embryo Culture and *In Vivo*: Time-Dependent Retinoic Acid-Induced Teratogenic Response

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The whole embryo culture (WEC) model serves as a potential alternative for classical *in vivo* developmental toxicity testing. In the WEC, cultured rat embryos are exposed during neurulation and early organogenesis and evaluated for morphological effects. Toxicogenomic-based approaches may improve the predictive ability of WEC by providing molecular-based markers associated with chemical exposure, which can be compared across multiple parameters (e.g., exposure duration, developmental time, experimental model). Additionally, comparisons between *in vitro* and *in vivo* models may identify objective relevant molecular responses linked with developmental toxicity endpoints *in vivo*. In this study, using a transcriptomic approach, we compared all-*trans* retinoic acid (RA)–exposed and nonexposed Wistar rat embryos derived using WEC (RA, 0.5 μg/ml) or *in vivo* (RA, 50 mg/kg, oral gavage) to identify overlapping and nonoverlapping effects of RA on RNA expression in parallel with morphological changes. Across six time points (gestational day 10 + 2–48 h), we observed strong similarities in RA response at the gene (directionality, significance) and functional (e.g., embryonic development, cell differentiation) level which associated with RA-induced adverse morphological effects, including growth reduction as well as alterations in neural tube, limb, branchial, and mandible development. We observed differences between models in the timing of RA-induced effects on genes related to embryonic development and RA metabolism. These observations on the gene expression level were associated with specific differential morphological outcomes. This study supports the use of WEC to examine compound-induced molecular responses relative to *in vivo* and, furthermore, assists in defining the applicability domain of the WEC in determining complementary windows of sensitivity for developmental toxicological investigations.

Key Words: **development; toxicogenomics; whole embryo culture; retinoic acid; alternative; neurulation; rat; time-response; comparative genomics.**

Due to global pressures, such as the implementation of Registration, Evaluation, and Authorization of Chemicals European legislation, there is a high demand to use millions of animals to test for developmental toxicity for thousands of novel and preexisting chemical compounds. Therefore, there is an imperative need to reduce animal usage by improving alternative models specifically for developmental toxicity prediction (van der Jagt *et al.*, 2004). Although not one alternative model may be able to fully predict the vast range of compounds that disrupt the entire complex span of development, multiple select assays may act as potential screens for developmentally toxic compounds. In the ECVAM (The European Center for the Validation of Alternative Methods) validation study, the three alternative assays investigated (mouse embryonic stem cell test, the limb bud micromass test, and rat postimplantation whole embryo culture [WEC]) were identified as potential replacements for classical developmental toxicity testing (Genschow *et al.*, 2002; Piersma, 2006). Extensive research is being put forth to develop and standardize these tests, while at the same time, determine the applicability domain and relevance for *in vivo* testing to achieve further regulatory acceptance (Chapin *et al.*, 2008; Marx-Stoelting *et al.*, 2009).

Using a standardized WEC model, rat embryos can be explanted on gestational day (GD) 10 and cultured *in vitro* for 48 h (Piersma *et al.*, 2004). During this developmental period, the embryo progresses through neurulation and organogenesis, including early formation of the brain, heart, ear, eyes, and limbs similarly to embryonic development *in vivo*. Using an elaborate scoring method (Anon, 1999), embryos can be assessed for multiple endpoints corresponding with embryonic developmental progression. As mentioned above, by using 20 model compounds in four independent labs, the ECVAM validation study suggested the WEC assay, in combination with the 3T3-cell assay, to have the capacity to distinguish strong embryotoxicants from weak and nonembryotoxicants (Piersma *et al.*, 2004). A recent validation study verified conclusions of this initial study using 13 of 20 ECVAM model compounds, however, suggested insufficient predictivity for an additional 48 test chemicals (Thomson *et al.*, 2011).

The predictive ability of the WEC to screen for developmental toxicity may be improved using molecular-based markers (Piersma, 2006). Gene expression profiling (transcriptomics) provides a platform to assess compound molecular effects on a global scale within the embryo, which associate with adverse morphological outcomes. Recent WEC studies suggest that developmental toxic compounds induce specific distinct gene expression signatures in association with common embryotoxic morphological endpoints (Robinson *et al.,* 2010) indicating the promise of transcriptomics approaches to detect chemical (or class)-specific changes and the addition of detailed molecular information complimentary to classic morphological assessments.

In our recent complimentary transcriptomics study (Robinson *et al*., 2012), we compared rat embryo development over a 48-h period from GD 10 onward *in utero* versus *in vitro* (WEC). Gene expression patterns over time proved to be very similar, and moreover, could be matched extensively with an existing human database of gene expression in embryos undergoing neurulation and early embryogenesis (Fang *et al.,* 2010). In terms of teratogenic response, initial transcriptomic studies suggest compound-induced effects observed in WEC to be relevant to previously identified mechanisms of toxicity *in vivo* (Luijten *et al*., 2010; Robinson *et al.,* 2010; Zhou *et al*., 2011), suggesting molecular-based markers may provide a linkage between WEC and developmental toxicity assessments *in vivo*. Binary comparisons of previously published transcriptomic datasets examining teratogen-induced gene expression profiles in WEC and *in vivo*, further support similarities in response to developmental toxicants between models, moreover, reveal challenges in comparing studies not directly designed to make proper contrasts due to differing study designs (e.g., exposure, duration, timing, platform) (Robinson *et al.,* 2011). Direct comparisons between *in vivo* and *in vitro* models controlling for experimental variables (e.g., dose and exposure duration) may improve our understanding on how inherent model differences influence toxicity response across models.

Multiple studies have examined the normal critical role of retinoids during early embryonic development as well as the teratogenic response due to excess or deficiency (reviewed in Rosa *et al*., 1986). In the current study, we specifically investigated the effects of the model teratogen, all-*trans* retinoic acid (RA) in WEC and embryos *in vivo*. RA represents one of the most toxic retinoids metabolites in rat (Turton *et al*., 1992). Numerous studies conducted *in vivo* and *in vitro* have examined the dose-response relationship between RA and teratogenicity during early embryogenesis and associated transcriptional targets which underlie these outcomes (Cai *et al*., 2007; Danzer *et al*., 2005; Luijten *et al*., 2010; Tembe *et al*., 1996; Tzimas *et al*., 1995, 1996). Based on the extensive knowledge of RA, we selected doses of RA *in vivo* (Tembe *et al*., 1996) and in WEC (Luijten *et al*., 2010), which induced teratogenicity in both models, to compare toxicogenomic signatures in relation to induced adverse developmental outcomes. Specifically, we investigated the effect of RA on gene expression across several time points (six total) within this developmental window (GD 10 + 2–48 h), identifying commonalities and differences in RA-induced response in relation to embryonic morphological alterations.

MATERIALS AND METHODS

Animal care. As described in our previous WEC studies (Luijten *et al*., 2010; Robinson *et al.,* 2010, 2012), all animal studies were approved and conducted in accordance with the National Institute for Public Health and the Environment (RIVM) Animal Care facility and federal regulations. Wistar rats (HsdCpd:WU) (Harlan, the Netherlands) were housed at the RIVM in a climate-controlled room with a 12-h on/off light cycle (2:00 A.M.–14:00 P.M., dark). After acclimating for 2 weeks, virgin female rats were housed with adult male rats for a 3-h mating period (9:00 A.M.–12:00 P.M.). Pregnant dams showing evidence of a copulatory plug (declared GD 0) were housed in separate cages. Water and food were provided *ad libitum*. Rats were monitored daily for general health for the extent of the study.

Rat postimplantation whole embryo culture. Following previous published studies (Anon, 1999; Piersma *et al*., 2004; Robinson *et al*., 2010, 2012), on GD 10 pregnant dams were euthanized by intracardiac injection of T61 (Intervet, The Netherlands), and rat embryos were removed from the uterus with the yolk sac and the ectoplacental cone left intact. Euthanizations were performed between 9:00 A.M. and 12:00 P.M. Embryos with 1–5 somites were used at the onset of culture and equally distributed based on initial somite number for all experimental groups for both gene expression and morphological assessments. The preselection of this range of embryos for gene expression is based on our previous study (Luijten *et al*., 2010) suggesting similar expression response in select genes to RA in the setup of WEC as a screening tool. Embryos were cultured separately in a serum mixture (#S9001, consisting of 90% pregnant bovine serum and 10% rat serum; Biochrom, Berlin, Germany) diluted with 14% Hank's solution and supplemented with 1.57 mg/ml D-glucose and 75 μg/ml L-methionine (Gibco). The use of bovine serum mixture instead of 100% rat serum significantly reduces the usage of animals for this experiment. Furthermore, our previous work indicates that the use of a variation of this serum mixture (90% bovine and 10% rat) is suboptimal as compared with 100% rat serum but does not greatly influence development in control embryos (reduction of ~1 somite after 48 h) (Luijten *et al*., 2010). Gassing occurred five times during the 48 h of culture. After ~1 h and ~7 h of culture, the embryos were gassed for 30 s (5% O2, 5% CO2, and 75% N2). After ~23 h and ~30 h of culture, the embryos were gassed for 30 s (20% O2, 5% CO2, and 75% N2). Finally at ~47 h of culture, the embryos were gassed for 10 s (40% O2, 5% CO2, and 55% N2).

Retinoic acid exposure—WEC. All-*trans*-RA (CAS No. 302-79-4) (MP Biomedical, Irvine, CA) at a concentration of 0.5 μg/ml or vehicle control (1 μl/ml dimethyl sulfoxide, DMSO) were added to 2 ml of culture medium prior to culturing (8:00–9:00). The concentration of RA selected for WEC in this study was based on interpolation of a previous study conducted in our lab investigating RA effects on gene expression and morphology (Luijten *et al*., 2010). Based on this analysis, we expected 0.5 μg/ml to induce teratogenicity, no signs of embryonic mortality and an estimated 25–30% decline in total morphology score (TMS) after 48-h exposure. New solutions were prepared in the morning of each culture day. A previous study from our lab has indicated that the concentration of DMSO used does not significantly alter morphology or gene expression (Robinson *et al.,* 2010). Cultures were completed over a 7-week period.

Embryo isolation for gene expression analysis in WEC. Following 2, 4, 6, 12, 24, and 48 h of vehicle or RA exposure, embryos were isolated and scored for total somite number and development of the neural tube. The yolk sac and ectoplacental cone were completely removed. Cultured embryos collected at each time point for RNA analysis were originally extracted from at least three different pregnant dams.

In vivo *rat gestational exposure studies to retinoic acid.* During the same general time period as WEC experiments, *in vivo* exposure studies were conducted within the RIVM Animal Care facility. RA was administered via oral gavage (50 mg/kg body weight [BW]) on GD 10 (at 10:00 or 11:00, only one dam was exposed per time point per day). RA was administered at 50 mg/kg BW due to a previous study (Tembe *et al.*, 1996) suggesting this dose induces teratogenicity, including specific malformations (e.g., neural tube defects, craniofacial abnormalities) in Wistar rats. Additionally, the dose selected from this study did not induce significant embryonic death (post hoc chi-squared test, $p > 0.05$) nor signs of maternal toxicity. RA solutions were prepared the day of administration in corn oil. Total volumes of vehicles did not exceed 2 ml/kg BW. In a similar fashion, controls were also dosed with corn oil via oral gavage.

***Embryo isolation for gene expression analysis* in vivo.** At 2, 4, 6, 12, 24, and 48 h after oral dosing (50 mg/kg BW), the dams were euthanized via intracardiac injection of T61 (Intervet) and embryos were extracted from the uterus. All extraembryonic membranes were removed. Embryos were scored for total number of somites and development of the neural tube. Embryos collected for each time point were extracted from at least three different pregnant dams.

Embryonic RNA isolation. As previously described (Robinson *et al.*, 2012), in brief, following extraction of embryos derived from WEC or *in vivo*, separate whole embryos were immediately placed into RNAlater RNA Stabilization Solution (Ambion, Austin, TX) and placed at 4°C for 1 week and then ≤ -20°C until further processing. To begin RNA isolation, after thawing on ice, each embryo was homogenized by passing through a 26G needle and 1 ml syringe 10 times. RNA was isolated using the RNeasy Micro Plus RNA Isolation Kit following the manufacturer's protocol for animal tissues (Qiagen). RNA was eluted with 13 µl nuclease-free water and stored at −80°C. RNA quantity and quality were measured via the Nanodrop (Nanodrop Technologies Inc., Wilmington, DE) and also the 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA samples with an absorbance value between 1.9 and 2.2 (260/280 nm) and RNA integrity number > 7 were used for further assessments. Embryos with RNA quantity within 2 SDs of each exposure group were chosen for further analyses due to possible loss of embryonic tissue during homogenization or collection.

Microarray hybridization. RNA hybridization and microarray experimentation were performed by ServiceXS B.V (Leiden, the Netherlands). RNA targets were prepared using 100 ng of purified total RNA as a template for the Affymetrix 3′IVT Express Labeling Kit (901229). Fragmentation reactions were performed using 7.5 µg complementary RNA (cRNA) for each sample. At a concentration of 0.0375 ng/µl fragmented/denatured cRNA, hybridization was conducted using Affymetrix HT RG-230 PM Array Plate (Affymetrix Inc., Santa Clara, CA) according to the manufacturer's instructions. The Affymetrix HWS Kit (901530) was used for hybridization, washing, and staining of chips. In total, 204 arrays were used ($n = 8$ for each of the 24 experimental groups + 12 technical replicates).

Microarray analysis-data processing. Quality of microarray images was inspected visually and raw values were examined for average background scale factors, 3′/5′ ratios of glyceraldehydes 3-phosphate dehydrogenase and 3′/5′ ratios of Bactin, NUSE, and RLE signal quality metrics. Affymetrix CEL files were normalized using the Robust Multichip Average algorithm (Irizarry *et al.*, 2003) using the Brainarray custom CDF version 13 (http://brainarray.mbni.medumich.edu/Brainarray/Database/CustomCDF) (Dai *et al.*, 2005). Affymetrix internal controls ID were not used in further analyses, leaving a total of 11,521 probe sets for Entrez Gene ID mapped genes. Transcriptomic data were deposited in NCBI GEO under accession number GSE33195.

Identification of genes altered by RA. Normalized data were log transformed and imported into BRBarraytools (National Cancer Institute, Simon *et al.*, 2007). Due to high similarity between technical replicates, arrays of technical replicates were averaged. Across the 192 arrays, representing 192 separate embryos, we employed three statistical models (ANOVA) to identify genes altered by RA exposure independent of both models (MODEL 1), only *in vivo* (MODEL 2), and only in WEC (MODEL 3).

$$\text{MODEL 1}: \text{Log}_2[\text{Exp}_n]\,\text{WEC\_INVIVO} = B_{\text{RA}}X_1 + B_{\text{TIME}}X_2 + B_{MODELSYSTEM}X_3$$

$$\text{MODEL 2}: \text{Log}_2[\text{Exp}_n]\text{INVIVO} = B_{\text{RA}}X_1 + B_{\text{TIME}}X_2$$

$$\text{MODEL 3}: \text{Log}_2[\text{Exp}_n]\text{WEC} = B_{\text{RA}}X_1 + B_{\text{TIME}}X_2$$

An *F*-test was applied for each term, and corresponding *p* values were calculated to determine the significance of effects due to RA. To correct for multiple testing, we calculated the false discovery rate (FDR) according to Benjamini and Hochberg (1995).

Significant genes were identified to be altered by RA with an FDR of $\leq 1\%$ and an absolute fold change (FC) (RA/Con) ≥ 1.2 at any of the six time points. FC values were determined by calculating the arithmetic mean of each exposure group and determining ratios between exposed and control at each time point. Within genes identified to be significantly altered by RA across time using MODEL 1–3 (as discussed above), a secondary post hoc test (two-sided *t*-test, $p \leq 0.05$) was applied between RA and control exposed embryos within each time group to examine the number of genes altered by RA at each time point in WEC and embryos *in vivo*.

Functional analysis of differentially expressed genes. Genes identified to be significantly altered by RA in at least one of the two model systems were assessed for enrichment of gene ontology (GO) terms (specifically, biological processes) (GenMAPP, Doniger *et al.*, 2003). Significantly enriched terms were identified to contain at least five genes, *Z*-score ≥ 2 and *p* value ≤ 0.05. GO biological processes were grouped based on GO classification (Carbon *et al.*, 2009; geneontology.org) to determine themes. To quantitatively describe changes on a biological process level, we employed GO-Quant (Yu *et al.*, 2006) to calculate the absolute average FC within genes significantly altered by RA within each enriched GO biological process across all six time points.

RA-induced developmental gene expression alterations. Genes related to the GO biological process term "Developmental Process" and related subcategories were identified using DAVID (Dennis *et al.*, 2003). Hierarchical clustering of average FC values (log 2 scale) was completed using average linkage and Euclidean Distance (TIGR MEV, Saeed *et al.*, 2003).

RA metabolism gene expression alterations. To examine genes involved in the metabolism of RA, genes significantly altered by RA which were associated with GO associations "Retinoic Acid Metabolism" or "Retinol Metabolism" were identified via DAVID (Dennis *et al.*, 2003) and examined for time-dependent RA effects on gene expression in WEC and *in vivo*.

Morphological scoring. In comparisons with our RNA expression studies, morphological assessments were completed in concurrently cultured embryos to determine and compare morphological effects induced by RA in WEC and *in vivo*. Embryos ($n \geq 12$ embryos derived from ≥8 dams) with initially one to five somites (0 h) were evaluated after 48 h in culture, and embryos extracted *in vivo* ($n \geq 9$ embryos from ≥3 dams) on GD 10 + 48 h were evaluated for morphological effects. Adapted from previous studies (Brown and Fabro, 1981; van Maele-Fabry *et al.*, 1990, 1992), the TMS quantitative scoring system (Anon, 1999) was used to determine RA effects in WEC and *in vivo* on developmental morphological hallmarks. Endpoints evaluated included yolk sac, cranial and caudal neural tube, heart, ear, eye, forelimb and hindlimb, facial, and somite development. To determine if there was a difference in morphological development between RA-exposed embryos and time-matched controls, the full battery of morphological endpoints (summarized as a TMS) were compared using a Student's *t*-test (two-sided), treating each embryo as an independent sample. Although not presented, in general, accounting for litter effects (maternal origin) in WEC or *in vivo* in our statistical analyses did not influence our conclusions. In embryos specifically used for microarray analysis, Mann-Whitney *U* tests (one-sided) were applied to determine significant differences for binary outcomes (cranial and caudal neural tube closure) between

RA-exposed and control embryos. Significant differential RA effects between WEC and *in vivo* were determined using ANOVA to assess significant interactions (model system × exposure).

RESULTS

Comparison of RA-Induced Morphological Effects Between In Vivo *and WEC*

As shown in Figure 1A, RA significantly impacted embryonic morphology in WEC and *in vivo*. Developmental effects included delayed formation of the neural tube in the cranial (x) and caudal (z) regions of the neural tube as well as alterations in branchial bar morphology (w, y). RA induced a significant 25 and 19% decline in TMS in WEC and *in vivo* ($p \leq 0.005$, respectively) (Fig. 1B). Common RA effects were observed on somite (S_{48h}) and crown rump length at 48 h in both models; however, these effects were greater *in vivo* as compared with WEC (ANOVA, $p \leq 0.05$). Additionally, in both models ($p \leq 0.05$), we observed significant alterations in development of the forebrain and caudal regions of the neural tube, branchial bars, mandible, maxillary, forelimb, and hindlimb were observed in both models. Uniquely, RA significantly impacted midbrain and hindbrain, ear (otic), and eye (optic) development in the WEC and not *in vivo* ($p \leq 0.05$). RA did not alter heart development in either model ($p > 0.05$). As shown in Figures 1C–E, we observed differences in the

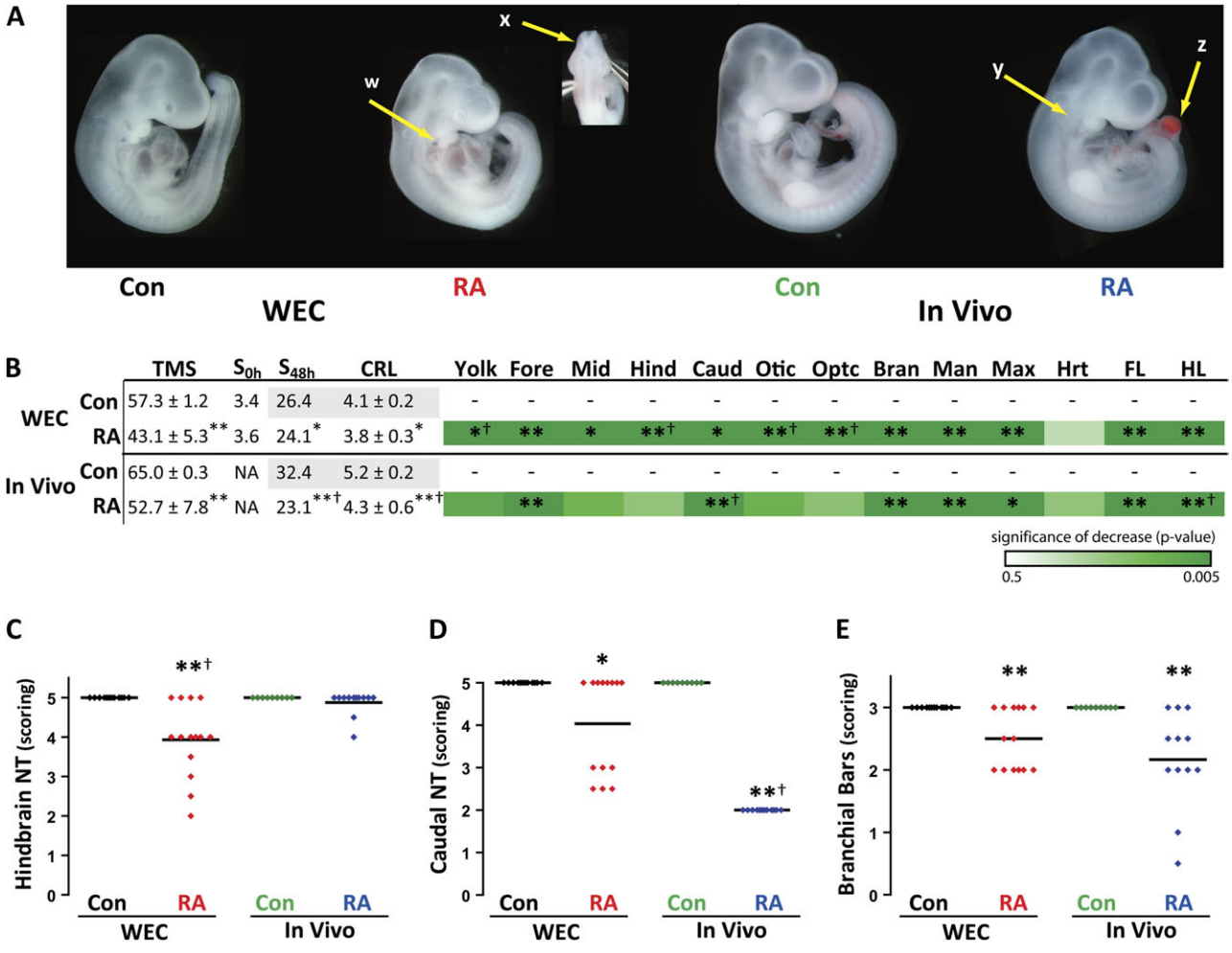

FIG. 1. Total morphological scoring of RA-exposed embryos *in vivo* and *in vitro*. Representative examples of control- and RA-exposed embryos in WEC and *in vivo* (A). Arrows (x, w, y, z) highlight specific developmental aberrations of the branchial bars and neural tube in these examples of RA-exposed embryos. Summary of developmental parameters evaluated at 48 h postexposure to RA in WEC and *in vivo* (B). Abbreviations include: total morphological score (TMS), somites at 0 h (S_{0h}), somites at 48 h (S_{48h}), crown rump length (CRL), yolk sac (Yolk), forebrain (Fore), midbrain (Mid), hindbrain (Hind), caudal (Caud), otic (Otic), optic (optc), branchial (Bran), mandible (Man), maxillary (Max), heart (Hrt), forelimb (FL), and hindlimb (HL). Distribution of morphological scoring of embryos for hindbrain neural tube region (C), caudal neural tube region (D), and branchial bars (E). Bars represent scoring average. Asterisks (*) represent significant effects between RA-exposed and control embryos in either model system (*$p \leq 0.05$; **$p \leq 0.005$, *t*-test). Crosses (†) represent significantly differentially affected by RA between models ($p \leq 0.05$, ANOVA). Shaded areas represent control data, which was previously reported in our complimentary study assessing differences between WEC and embryo *in vivo* under control conditions (Robinson *et al.*, 2012).

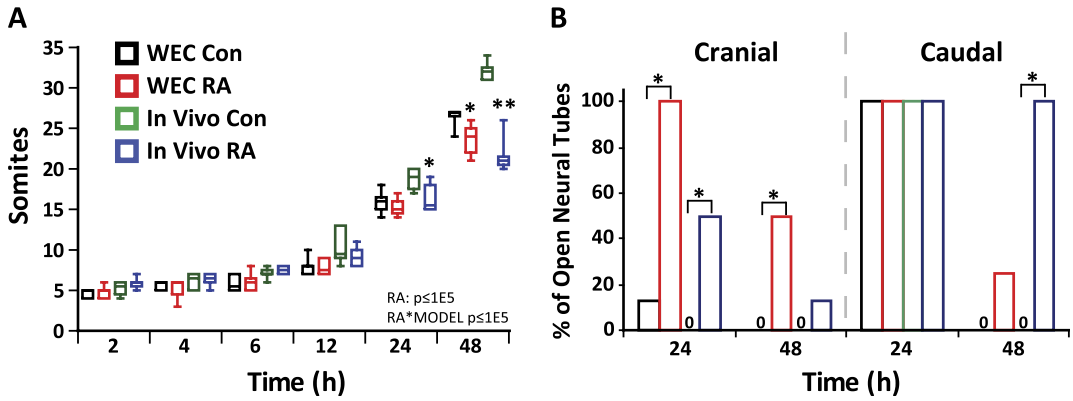

FIG. 2. Development of RA-exposed and nonexposed embryos over time. Somite development in RA-exposed and nonexposed embryos used for microarray analysis in WEC or *in vivo* (A). Significance values (ANOVA) are indicated for RA and RA* model effects. Dark lines indicate median somite number or (total RNA) within each group ($n = 8$ within each group). Cranial and caudal neural tube effects in RA-exposed and nonexposed embryos in WEC or *in vivo* (B). Values represent percentages of embryos with open neural tube in cranial and caudal regions at 24 and 48 h. Asterisks (*) represent significant effects between RA-exposed and control embryos in each of the respective controls (*$p \leq 0.05$; **$p \leq 0.005$, *t*-test). Control somite data were previously reported in our complimentary study assessing differences between WEC and embryo *in vivo* under control conditions (Robinson *et al.*, 2012).

distribution of affected versus nonaffected RA-exposed single embryos between WEC and *in vivo* for specific morphological endpoints. For example, 10/14 RA-exposed embryos in WEC showed adverse effects (scoring < 5) on midbrain morphology, whereas *in vivo*, only 2/12 showed adverse RA effects (Fig. 1C). In contrast, caudal neural tube RA effects were observed in 6/14 in the WEC and 12/12 embryos *in vivo* (< 5), respectively (Fig. 1D). RA effects on branchial bar morphology were more similar between WEC (8/14 abnormal, < 3) and *in vivo* (9/12, < 3) (Fig. 1E).

In embryos used for microarray analysis, we observed significant inhibition of somite development by RA in WEC and *in vivo* across the six evaluated time points (ANOVA, $p \leq$ 1E-5) (Fig. 2A). In particular, we observed significant effects on somite development at 24 h (*in vivo* only) and 48 h (WEC and *in vivo*) (*t*-test, $p \leq 0.05$). As observed in embryos only used for embryonic morphological assessments (Fig. 1B), at 48 h, greater effects on somite development were observed between RA-exposed embryos *in vivo* versus WEC (ANOVA, $p \leq 0.05$). No significant differences were observed in total RNA isolated in RA-exposed and nonexposed embryos across the six time points, either *in vivo* or *in vitro* ($p > 0.05$) (data not shown).

Additionally, in embryos specifically used for microarray analysis, in WEC and *in vivo*, and similar to full morphological assessments (see above), RA inhibited closure in both the cranial and caudal regions of the neural tube (Fig. 2B). Significant increases in embryos with open neural tubes in the cranial region were observed both *in vivo* (50%↑) and WEC (87%↑) at 24 h and in WEC at 48 h (50%↑) ($p \leq 0.05$). Significant increases in caudal neural tube alterations were observed *in vivo* at 48 h ($p \leq 0.05$). In general, greater cranial neural tube effects were observed in WEC and greater caudal effects were observed *in vivo* across both time points.

RA-Induced Gene Expression Alterations in WEC and In Vivo

Using three ANOVA models, which identified genes which were significantly altered by RA in both model systems or uniquely in the WEC or *in vivo*, in total, 845 genes were identified to be significantly altered by RA (FDR $\leq$ 0.01, absolute FC $\geq$ 1.2) (Fig. 3A). Post hoc analysis of RA effects of all 845 genes at each time point revealed a peak response *in vivo* at 6 h (Fig. 3B). In WEC, less specificity in peak gene response was observed, with 4 and 24 h being the most responsive time points. RA induced upregulated and downregulated genes at all six time points. By conducting hierarchical clustering of the average FC response of all 845 significantly altered genes, we identified two clusters (I and II) of genes based on directionality of regulation of expression across time (Fig. 3C and Supplemental Fig. 1). In general, cluster I consisted of genes downregulated by RA and cluster II consisted of genes upregulated by RA in both model systems. In many cases, temporal differences in RA-induced regulation were apparent between the two models.

Quantitative Functional Analysis of RA-Induced Gene Expression Alterations

Within the 845 genes significantly altered by RA, we identified 154 GO biological processes to be significantly enriched (Fig. 4). Over 90% of all enriched GO terms were categorized within six themes: development (central nervous system, embryonic, circulatory, ear, and organ), differentiation (cell and neuron), lipid/hormone metabolism, transcription, sugar metabolism, and biological regulation. Quantitative analysis of genes within enriched GO biological processes suggested time-dependent regulation of all functional groups by RA in both WEC and *in vivo*. For example, in

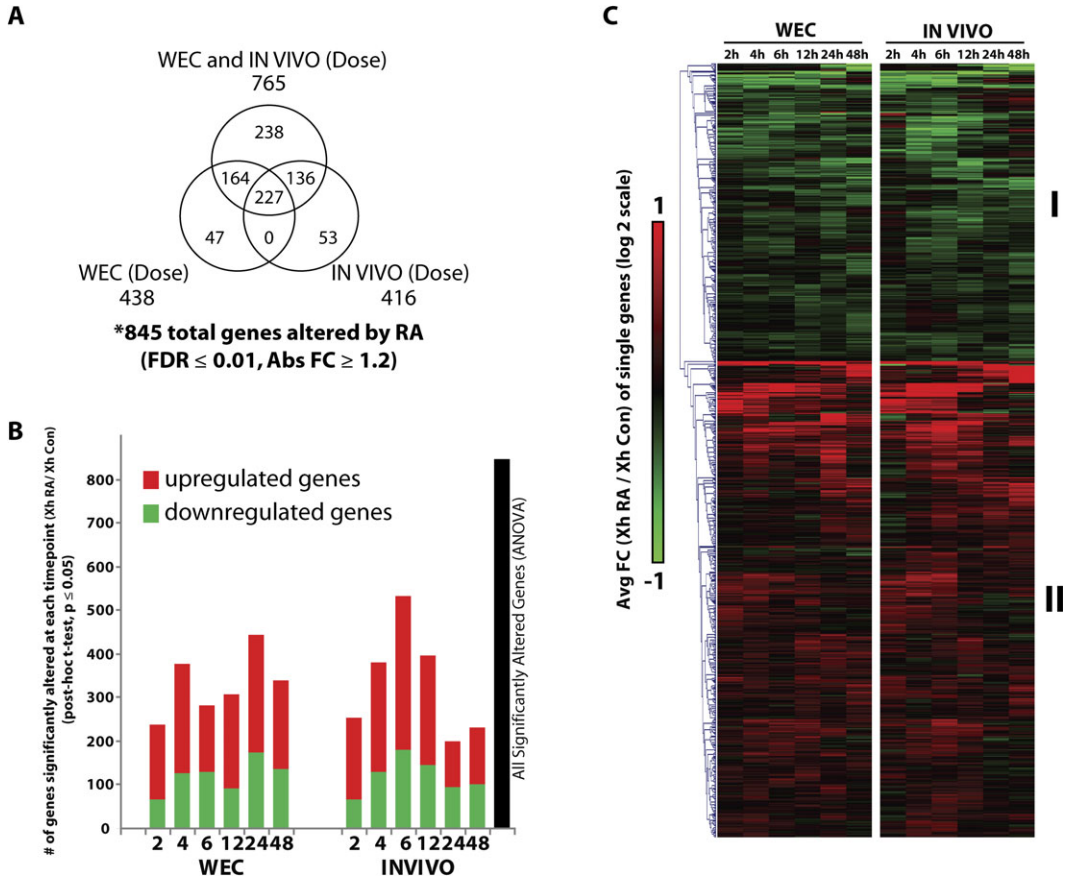

FIG. 3. Gene expression alterations induced by RA in WEC and *in vivo*. Number of differentially expressed genes identified to be altered by RA in only WEC, in only embryos *in vivo*, and in both model systems represented by Venn Diagram (FDR $\leq$ 0.01, absolute FC $\geq$ 1.2) (A). In total, 845 genes were identified to be significantly altered by RA in WEC and/or *in vivo* model systems. Time-dependent regulation of RA-induced gene expression using post hoc analysis (*t*-test, $p \leq$ 0.05) (B). Hierarchical clustering of average degree of RA impact on genes (845 genes) significantly altered by RA in WEC and/or *in vivo*. Color intensities depict average FC of average normalized intensities between RA and control at each time point (log 2 scale) (C).

general, in both models, genes related to ear (shaded yellow) and neural crest cell development (NCD) (shaded green) were observed to be impacted on average at a higher degree $\leq$ 6 h as compared with $\geq$ 12 h. In contrast, genes linked with glucose/sugar metabolism (shaded orange) were impacted to a higher degree $\geq$ 12 h as compared with $\leq$ 6 h. Model differences in RA response at the functional level were also apparent in a time-dependent manner. Similar to observations of peak response on the gene level, at the functional level, *in vivo* RA responses were greatest at 6 h for the majority of enriched GO biological processes (> 70%). Less apparent in time-specificity peak responses at the functional level varied in the WEC dependent on the enriched GO term. In comparison to *in vivo*, only 4% showed peak changes at the functional level at 6 h in WEC. Furthermore, in general, the majority of enriched GO biological processes consisted of RA targets, which were both up- or downregulated (red bar), with the exception of glucose/sugar metabolism genes (all upregulated).

Developmentally Related RA-Induced Gene Expression Alterations

Twenty-nine percent of all genes significantly impacted by RA were identified to be associated with the GO term Developmental Process (248 genes, data not shown). With additional criteria (FC $\geq$ 1.5 at least at one of the time points in either model), 106 genes were observed to be altered due to RA exposure in at least one of the two model systems (Fig. 5). In general, independent of time, genes were observed to be either upregulated or downregulated by RA in both model systems. Many genes showed time-dependent specificity in peak regulation (either up or down). For example, genes such as GBX2 and RND1 were observed to peak in response (upregulation) at 2 h as compared with ADM, which peaked at 48 h (upregulation). Multiple genes were associated with developmentally related GO subcategories, including anterior-posterior pattern formation, proximal/distal pattern formation (PDPF), NCD, and cell fate commitment (CFC).

RA significantly altered the expression of eight genes, PBX1, PBX2, GLI1, HOXA9, DLX1, HOXA10, HOXB10, and HOXC10 associated with PDPF (Fig. 6). Specifically, RA

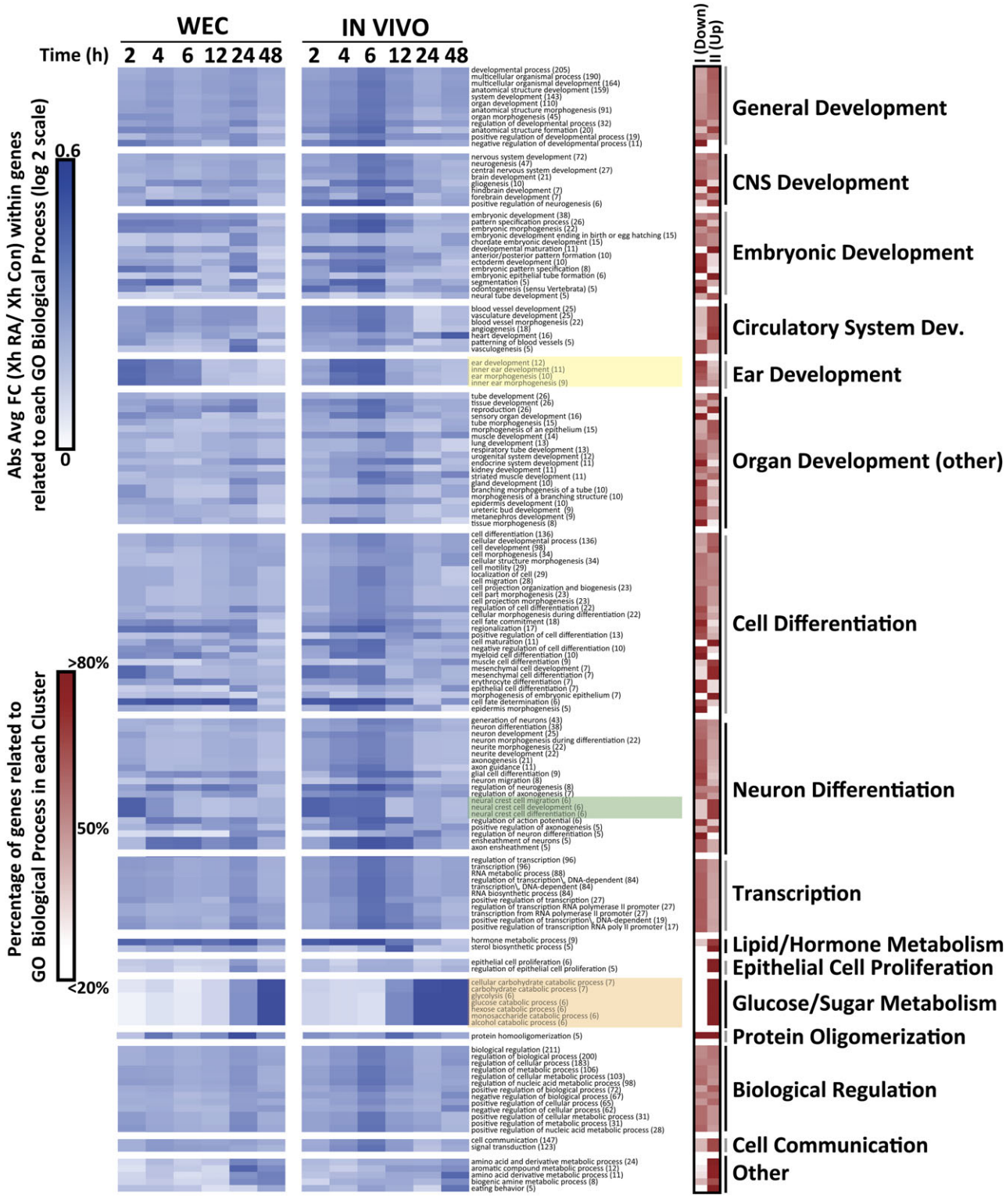

FIG. 4. Quantitative analysis of GO biological process altered by RA in WEC and *in vivo*. The color diagram represents the absolute average degree of change due to RA exposure within genes related to each enriched GO biological process across time in WEC and *in vivo*. In total, 154 GO biological processes were identified to be significantly enriched ($Z \geq 2.0$, $p \leq 0.05$, number changed ≥ 5) within the 845 genes altered by RA (FDR ≤ 0.01, absolute FC ≥ 1.2). General themes of enriched GO biological processes are to the right of the list of GO terms. Color gradient indicates the percentage of genes identified to be in cluster I (downregulated) or cluster II (upregulated).

exposure resulted in decreased of expression of DLX1, HOXA10, HOXC10, and HOXD10 in both WEC and *in vivo*. Differential RA-induced regulation in a time-dependent manner was observed between model systems. Greater reduction in DLX1, HOXA10, HOXC10, and HOXD10 was observed at 12 h *in vivo* as compared with WEC.

RA Metabolism Gene Expression Alterations

RA significantly altered seven genes associated with RA Metabolism (Fig. 7). In particular, RA induced upregulation of expression of three enzymes, CYP26A1, CYP26B1, and DHRS3, both *in vivo* and in WEC. Interestingly, differential temporal RA regulation for these three enzymes between *in vivo* and WEC was observed. *In vivo*, RA induced expression of all three enzymes > 4× (log 2 scale) at 2–6 h, with peak expression at 6 h (> 4×). In comparison, at 24 and 48 h, expression of all three enzymes was only slightly upregulated (< 1.5×) in RA-exposed embryos. In WEC, RA induced expression in a different time-dependent pattern as compared with embryos *in vivo*, varied peak response (2–12 h), overall lower peak expression, and prolonged upregulated expression until 24 h (> 2×) for all three enzymes.

DISCUSSION

In the current study, using a toxicogenomic approach, we investigated the comparability of RA-induced time-dependent effects on gene expression and morphology in embryos derived from WEC and *in vivo* using doses known to induce teratogenicity within the same time frame of development.

RA Induces Developmental Toxicity in WEC and In Vivo

RA induced significant adverse morphological effects in WEC and *in vivo*, including general growth retardation (somites, size) as well as alterations in cranial and caudal neural tube, branchial, and limb development (Figs. 1 and 2). Morphological changes due to RA observed in this study are supported by multiple studies associating RA exposure *in utero* with a myriad of teratogenic outcomes. Following RA exposure *in vivo* during a similar time window as the current study (Wistar rat, 50 mg/kg, oral, GD 10), significant increases in cases of spina bifida, cleft palate, mandibular hypoplasia, exopthalamus, anal atresia, and protruding tongue were observed in fetuses (GD 21) (Tembe *et al.*, 1996). Likewise, increased defects of the neural tube (exencephaly, spina bifida), cranium, eye, intestine, skeleton, limb, and cleft palate were observed after maternal exposure (Fu-albino rats, 120 mg/kg, oral) with the degree of incidence for each specific defect dependent on the time of exposure (GD 1–20, single exposure) (Kistler, 1981). In particular, alterations in the caudal neural tube due to RA exposure have been studied extensively (Alles and Sulik, 1990; Cai *et al.*, 2007; Danzer *et al.*, 2005). In Sprague-Dawley rats, RA (50 mg/kg, oral, GD10) induces caudal defects in 30% of GD 22 fetuses (Danzer *et al.*, 2005). Detailed pathologic investigations of the dorsal lumbosacral region of embryos exposed *in utero* to RA (Wistar rat, 40 mg/kg, oral, GD 10) suggest alterations directly to the neuroepithelium, resulting in open neural tube of the lumbar level (myeloschisis) accompanied by a disorganized mass in the lumbosacral region, morphologically similar to the human Currarino Triad condition (Cai *et al.*, 2007). Our results in both *in vivo* and WEC model systems support the extensive database associating the exposure of RA and other retinoids with developmental toxicity due to exposure *in utero*.

RA Induces Common Alterations in Developmental Pathways in WEC and In Vivo

Correlated with alterations in morphological development, we observed high similarity in RA impacts on RNA expression in terms of directionality (up/down) and significance at the gene and functional level (Fig. 3). Enriched processes represented genes involved in early embryonic development (i.e., embryonic development and cell/neuron differentiation) and functional processes critical for neurulation and early organogenesis (Copp and Greene, 2010), supporting the known involvement of RA on processes critical for early embryogenesis, patterning, and neural induction (Morriss-Kay and Sokolova, 1996). Interestingly, approximately 90% of genes impacted by RA in this study were also observed to be significantly changing across time ($p \leq 0.01$, ANOVA) in control embryo assessments (Robinson *et al.*, 2012). At the gene level, we observed early overlapping changes in genes related to neural crest development in WEC and *in vivo* (Fig. 4). Controlled by localized gradients, NCD and migration are normally regulated by RA in the embryo (Martinez-Morales *et al.*, 2011; Vieux-Rochas *et al.*, 2010). Significantly altered genes included upregulation of the homeobox gene, GBX2, required for mid-hind brain identity in the developing mouse brain (Li and Joyner, 2001) and a target of RA in anterior posterior specification (von Bubnoff *et al.*, 1996). In terms of specificity and direction of regulation, our results suggest RA induces similar aberrations in gene expression in both WEC and embryos *in vivo*, which correlate with commonly altered morphological effects. Furthermore, our results support RA to impact developmental pathways critical for early embryogenesis in relation with developmental toxicity outcomes.

RA Induces Unique Developmental Toxicity Outcomes in WEC and In Vivo

Interestingly, while in general, common RA-induced adverse morphological effects were observed, differences in the severity of effects as well as unique effects were observed between models systems. For example, significant midbrain, hindbrain, eye and ear adverse effects were primarily observed in WEC, while greater effects on caudal development and somitogenesis were observed *in vivo* (Figs. 1C–E and 2). Although differential effects could be simply a matter of more RA distributing to the embryo, a similar reduction in TMS (25%, WEC vs. 19%, *in vivo*) in RA-exposed embryos (Fig. 1B) and no significant differences in total RNA in

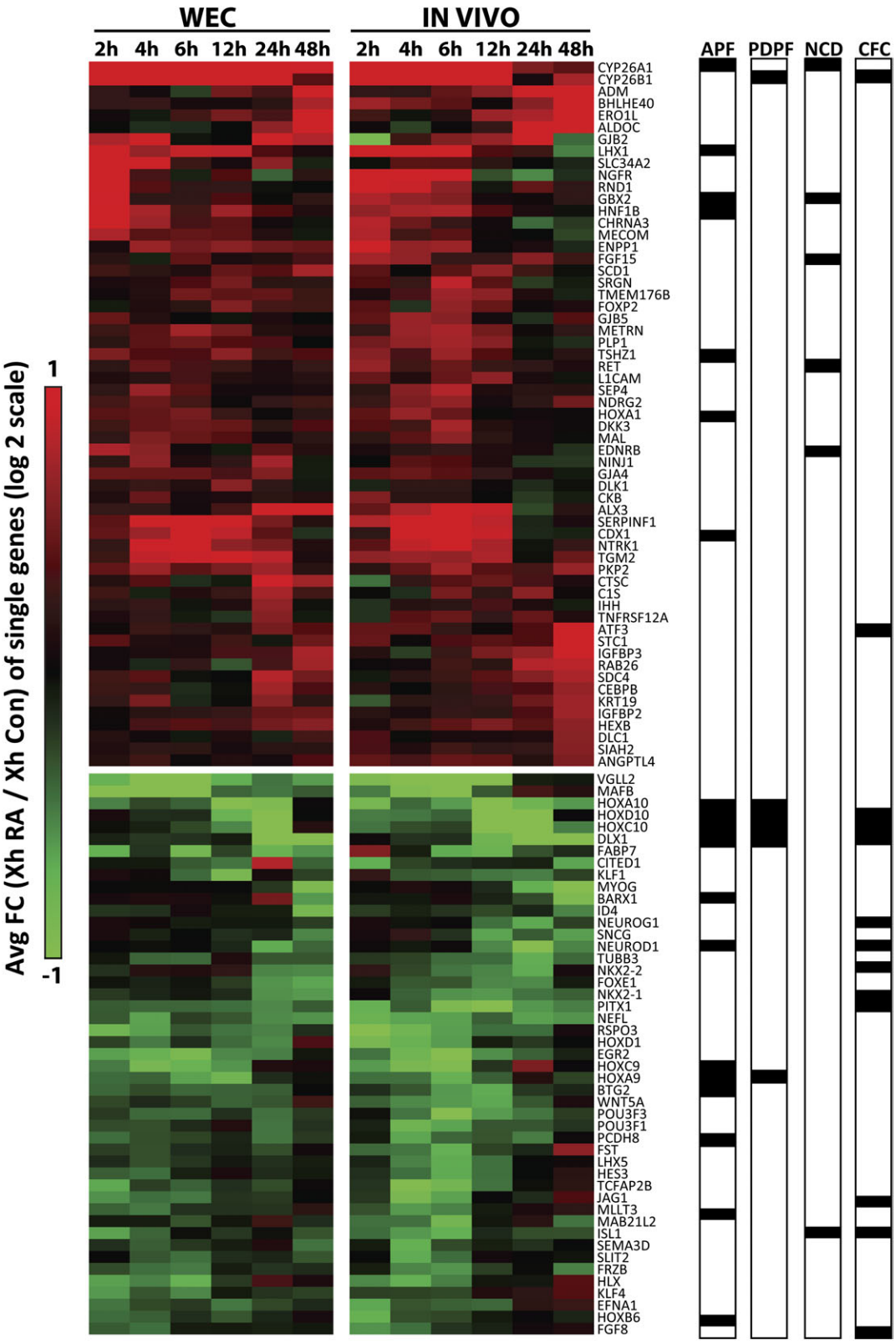

FIG. 5. Developmental genes altered by RA in WEC and *in vivo*. In total, 106 genes associated with the GO biological process ''Developmental Process'' were identified to be significantly altered by RA in WEC and/or *in vivo* (FDR $\leq$ 0.01, absolute FC $\geq$ 1.5). Specific genes related to specific developmentally related subcategories: Neural Crest Development (NCD), Cell Fate Commitment (CFC), and Proximal/Distal Pattern Formation (PDPF) are shown (black bar).

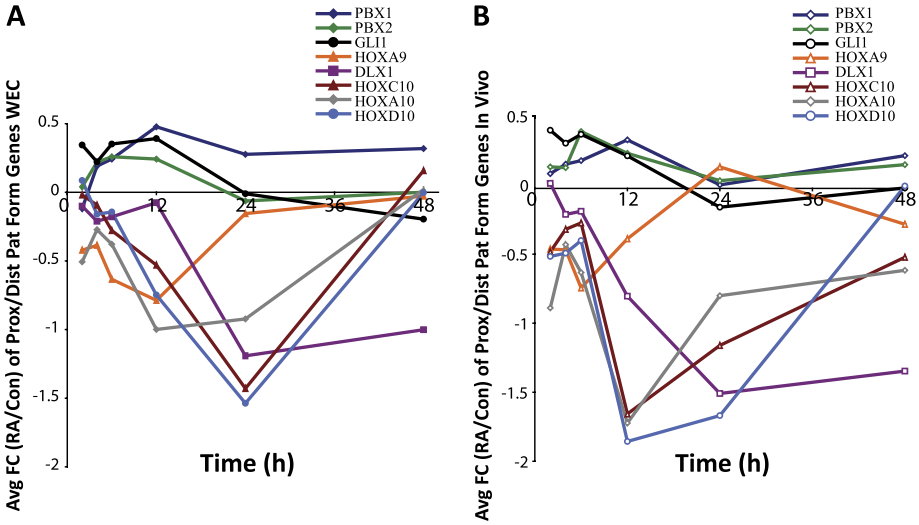

FIG. 6. Time-dependent expression of PDPF genes in WEC and i*n vivo*. RA-induced changes in gene expression (average FC) in genes associated with PDPF in WEC (A) and *in vivo* (B) (log 2 scale).

RA-exposed and nonexposed embryos (data not shown), another measure of fitness (Zhou *et al.*, 2011), suggests "similar" overall toxicity. Detailed examination at the gene level indicates RA-regulated expression of genes in a similar manner (directionality) and, to some degree, peak magnitude. However, temporal aspects of regulation differed between WEC and *in vivo* (Figs. 3C and 4 and Supplemental Fig. 2), suggesting that the timing of RA effects differed between models for particular genes and complimentary pathways. We hypothesize that differing (1) toxicokinetics and (2) developmental progression may influence the specificity and timing of RA gene targets, resulting in differences in RA-induced morphological outcomes between *in vivo* and WEC.

RA Induces Unique Developmental Toxicity Endpoints in Relation With Differing Regulation of Biomarkers for Excess RA

The toxicokinetics of RA has been described previously in rodent models *in vivo*. In Wistar rats (single, oral, GD 12, 6 mg/kg, RA), RA exposures result in maternal plasma levels (Cmax) and embryonic concentrations peaking $\leq$ 2 h, followed by an exponential decline, with RA levels at 8 h already ~100× less than concentrations at 2 h (Collins *et al.*, 1995). In a similar manner, in maternal plasma of rats exposed to RA (single, oral, GD 10, 50 mg/kg, RA), concentrations reached maximum levels at ~2 h (1.88 ± 0.42 µg/ml) and then declined, where by 8 h, RA concentrations were at background levels (Tembe *et al.*, 1996). We are unaware of any published

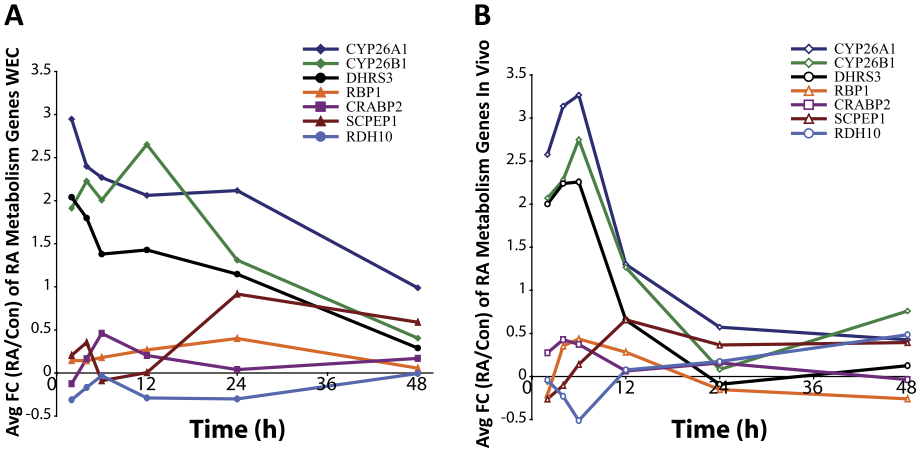

FIG. 7. Time-dependent expression of RA metabolism genes in WEC and i*n vivo*. RA-induced changes in gene expression (average FC) in genes associated with RA (or retinol) metabolism in WEC (A) and *in vivo* (B) (log 2 scale).

toxicokinetics RA exposure studies in WEC. In the absence of toxicokinetic data in this study, the induced expression of RA-induced metabolic enzymes provides clues into the kinetics of RA in the whole embryo. RA is well known to induce its own metabolism, including several enzymes involved in the biotransformation and elimination of RA (Collins *et al.*, 1995; Gudas, 1992; Muindi *et al.*, 1992). Specifically, CYP26A1 and CYP26B1 are known p450 enzymes involved in the breakdown of RA and critical in responding to excess levels of RA during embryonic development (Perlmann, 2002). Additionally, the retinal short-chain dehydrogenase-reductase, DHRS3, also responsive to excess RA and involved in the regulation of RA levels in the central nervous system, is an RA-induced feedback inhibitor of the production of RA (Cerignoli *et al.*, 2002; Feng *et al.*, 2010; Thatcher and Isoherranen, 2009). These studies suggest that the induced expression of these enzymes may be reliable markers of excessive RA levels in the embryonic system. In this study, these three genes represented some of the most responsive RA targets (FC and significance), upregulated both *in vivo* and WEC across all time points (Fig. 7). Differential regulation of CYP26A1, CYP26B1, and DHRS3 between WEC and *in vivo* suggest continued presence of RA in WEC, as opposed to the peak induction at 6 h and rapid decline in upregulated expression over time *in vivo* (Fig. 7). Kinetic and corresponding transcriptomic differences may underlie the higher specificity of morphological effects observed *in vivo* as opposed to a wider array of malformations observed in WEC (Fig. 1), reminiscent of relative peak exposure *in vivo* but relatively continuous exposure throughout WEC culture duration.

RA Induces Unique Developmental Toxicity Endpoints in Relation With Background Expression Differences Between In Vivo *and WEC*

Small variations in embryonic developmental stage may significantly influence toxicity and phenotype specificity as shown in studies comparing developmental stage sensitivity of embryos to ethanol (Clode *et al.*, 1987) and inorganic arsenic (Tabocova *et al.*, 1996). In our complementary study (Robinson et al., 2012), we identified significant retardation in morphological progression in WEC as compared with embryos *in vivo* as early as 24 h in culture (GD 10 + 24 h) under control conditions. Corresponding or preceding these differences in morphological events, differences in baseline expression of multiple genes were observed, many associated with developmental pathways between *in vivo* and WEC. Genes, such as HOXA10, HOXC10, and HOXD10 normally expressed in the caudal region of the neural tube and critical for regulating motor neuron development (Choe *et al.*, 2006), were identified to be expressed at higher levels *in vivo* versus WEC, especially at time points $\geq$ 12 h, associated with significantly greater development in the caudal region *in vivo* at 24 and 48 h. These results suggest that later time points in WEC may not correspond exactly with the same developmental window *in vivo*. In the current study, we observed RA to significantly impact HOXA10, HOXC10, and HOXD10 expression in both WEC and *in vivo* (Fig. 6) associated with negative impacts on caudal development, including somitogenesis and neural tube closure (Figs. 1 and 2). Moreover, earlier and greater effects on these three genes were observed *in vivo* versus WEC, associated with greater effects on caudal development *in vivo*. These results may suggest that differences in the underlying rate of developmental progression (morphologically and underlying gene expression) in combination with RA exposure may also play a role in differential toxicity outcomes on the gene and morphological level.

Differences between culture conditions and the *in vivo* environment may cause differences in developmental progression and may influence toxicity outcomes. In our previous WEC transcriptomic study (Luijten *et al.*, 2010) in which we compared RA-exposed and nonexposed embryos cultured in 100% rat serum versus embryos cultured in a serum mixture (90% bovine, 10% rat) similar to this study, we observed higher toxicity in WEC in the latter medium. Moreover, specific genes such as CRABP2, EGR2, DLX5, and NAB2 were identified to be differentially regulated by RA, dependent on culture medium composition. In the present study, CRABP2 and EGR2 were both identified to be regulated by RA *in vivo* and in WEC, whereas DLX5 and NAB2 were not significantly regulated in either model system. Interestingly, despite the use of different sera, differential sensitivities of each model, and different microarray platforms, comparisons within the present study as well as between the current study and the Luijten *et al.* (2010) study suggest a general similar directionality of regulation in RA effects (Supplementary fig. 3).

CONCLUSIONS

Using the classical teratogen, RA, we show strong similarities in transcriptomic effects in relation with common and unique developmental toxicity endpoints between WEC and *in vivo* model systems on a global scale. Despite these general similarities at the molecular level, subtle differences are apparent due to possible differential developmental progression and toxicokinetics, which associate with differences in morphological alterations and time specificity of gene expression impacts. Obvious challenges are presented due to the complexity of comparing *in vitro* and *in vivo* model systems; however, predictive models that incorporate kinetics of a compound, including both dynamic changes as well as the maximum peak concentrations (Daston *et al.*, 2010) may improve the applicability of alternative models for developmental toxicity assessment and prediction. Additionally, better understanding of factors such as developmental timing (e.g., background expression) and other functional characteristics may improve correlations between *in vivo* and alternative models. This dataset serves as a valuable resource for WEC-*in vivo* comparisons investigating

similarities and differences in characterizing mechanisms of action and predicting developmental toxicity potential.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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