Comparative genomics identifies genes mediating cardiotoxicity in the embryonic zebrafish heart

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Comparative genomics identifies genes mediating cardiotoxicity in the embryonic zebrafish heart. Physiol Genomics 33: 148–158, 2008. First published January 29, 2008; doi:10.1152/physiolgenomics.00214.2007.—Retinoic acid (RA) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) activate distinct ligand-dependent transcription factors, and both cause cardiac malformation and heart failure in zebrafish embryos. We hypothesized that they cause this response by hyperactivating a common set of genes critical for heart development. To test this, we used microarrays to measure transcript changes in hearts isolated from zebrafish embryos 1, 2, 4, and 12 h after exposure to 1 μM RA. We used hierarchical clustering to compare the transcriptional responses produced in the embryonic heart by RA and TCDD. We could identify no early responses in common between the two agents. However, at 12 h both treatments produced a dramatic downregulation of a common cluster of cell cycle progression genes, which we term the cell cycle gene cluster. This was associated with a halt in heart growth. These results suggest that RA and TCDD ultimately trigger a common transcriptional response associated with heart failure, but not through the direct activation of a common set of genes. Among the genes rapidly induced by RA was Nrf2F5, a member of the COUP-TF family of transcriptional repressors. We found that induction of Nrf2F5 was both necessary and sufficient for the cardiotoxic response to RA.

We have found (1, 34) that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) also causes cardiovascular abnormalities in developing zebrafish embryos. TCDD produces pericardial edema, reduced heart chamber size, and heart failure in the zebrafish embryo (1).

As with RA, the effects of TCDD are mediated by a ligand-activated transcription factor, the aryl hydrocarbon receptor (AHR). Agonists such as TCDD bind to AHR, causing it to form a heterodimer with the related basic helix-loop-helix PAS protein, ARNT. The heterodimer then binds to DNA sequence motifs known as dioxin response elements to initiate transcription of target genes (11, 29).

One of the most important obstacles in understanding the mechanism by which AHR agonists such as TCDD affect the development of the heart has been identification of the genes targeted by the activated AHR/ARNT complex. In recent work we (6) developed methods for measuring changes in mRNA levels in embryonic hearts exposed to TCDD with time course microarray experiments. These experiments revealed a very rapid induction of putative AHR target genes within 1–4 h after TCDD exposure, followed by a striking downregulation of a large set of genes involved in cell cycle progression. This latter response coincides with heart failure and a halt in cardiomyocyte proliferation.

TCDD and RA exposure both produce malformations in embryonic hearts, presumably through the activation of their respective receptors. In both cases the receptors directly activate gene expression, but these receptors bind to distinct DNA sequence motifs. Whether or not there are important genes containing binding sites for both receptors, allowing regulation by both RA and TCDD, is not known. This raises a question: Do RA and TCDD activate a common set of genes that lead to heart malformation and failure? The recent development of techniques for global analysis of gene expression changes in hundreds of intact hearts isolated from synchronously developing embryos (5, 6) allows us now to address the hypothesis that TCDD and RA affect the developing heart through a common set of target genes.

We were also interested in the possibility that the hypothesis would be incorrect, that RA and TCDD would produce similar signs of cardiotoxicity without activating a common set of genes. In this case, we hypothesized that the progressive heart failure produced by the two agents would nonetheless be associated with a characteristic set of gene expression changes, diagnostic for the condition of the damaged hearts.

Finally, while the effects of RA on heart development have been widely studied, we do not yet know which gene targets lead to cardiotoxic responses in the embryonic heart. The use of microarray experiments offers the chance of identifying such gene targets activated by RAR/RXR.
Here we report the pattern of gene expression changes in embryonic zebrafish hearts following exposure to excess RA compared with the previously identified changes produced by TCDD. We found little overlap in early gene expression changes induced by RA and TCDD, indicating that RA and TCDD initiate cardiotoxicity through distinct mechanisms. However, we found that, as time progressed, the heart failure produced by either agent was preceded by a common set of gene expression changes. This was characterized by the marked downregulation of a set of genes associated with DNA replication and cell division. We found that the transcript with the strongest induction in response to RA exposure was Nr2f5, a member of the chicken ovalbumin upstream promoter-transcription factor (COUP-TF) family of nuclear receptors. Overexpression of Nr2f5 phenocopied cardiac defects produced by RA exposure, while blockade of Nr2f5 expression was sufficient to block RA-induced cardiotoxicity.

MATERIALS AND METHODS

Zebrafish embryos. All zebrafish embryos were kept at 27°C in egg water (60 µg/ml Instant Ocean Salts; Aquarium Systems, Mentor, OH) with a 14:10-h light-dark cycle. Cmlc2::GFP embryos were used for heart extraction for microarray analysis (5). Albino embryos were used for imaging in the experiments to assay cardiovascular function. Cmlc2::DsRed2-nuc embryos raised in 0.003% phenylthiourea egg water were used for imaging in the experiments to assay cardiac myocyte number (1, 21).

All-trans retinoic acid and vehicle exposure. All-trans RA (≥98% purity, Sigma-Aldrich, St. Louis, MO) was kept at −80°C as a stock solution at 0.01 M in 100% DMSO. Before each experiment, RA was diluted to 1 µM/0.1% DMSO in egg water for embryo exposure; embryos were exposed to 0.1% DMSO as vehicle controls. RA solution was renewed every 24 h. For morpholino (MO) experiments, RA stock solution was diluted to 0.1 µM in 0.1% DMSO. All manipulations of RA were performed in subdued light.

Experimental design. For assays of stroke volume, cardiac output, and heart rate, six replicate experiments of 5 zebrafish embryos each were used per treatment group. For assays of peripheral blood flow, pericardial edema, and cardiac myocyte number, five replicate experiments of 5 zebrafish embryos each were used. For microarray analysis, three arrays were used for each sample time point, with each array using RNA samples collected from independently conducted experiments. Each array replicate used RNA collected from three arrays were used for each sample time point, with each array using RNA samples collected from independently conducted experiments. Each array replicate used RNA collected from three arrays were used per treatment group. For assays of peripheral blood flow, time-lapse frames lasting 10 s were identified, and the number of red blood cells passing a chosen landmark in the posterior intersegmental vein during 10 s was counted (6, 25).

Peripheral blood flow. Video images of blood flow in the posterior intersegmental vein in the trunk of either RA- or DMSO-exposed embryos were captured with a Princeton Instruments Micromax charge-coupled device camera mounted on an inverted microscope, and the number of red fluorescent nuclei was counted for each heart image (1, 6, 21).

Heart extraction. Before heart extraction, ~200 embryos were anesthetized with Tricaine-S (Aquatic EcoSystems, Apopka, FL), collected in a microcentrifuge tube, and immersed in 1 ml of Lebovitz’s L-15 medium-10% FBS. Hearts of zebrafish embryos were separated from the bodies by shear force generated by repelling the embryos through a 19-gauge syringe needle, followed by size fraction of the disrupted embryos and manual retrieval of the individual green fluorescent protein (GFP)-expressing hearts with a pipette under epifluorescence. The hearts were snap frozen in liquid nitrogen and stored at −80°C for further analysis (8).

Microarray analysis and data processing. Total RNA (1 µg/500 hearts) was extracted with a Qiagen RNeasy mini kit according to the manufacturer’s protocol (Qiagen, Valencia, CA). Biotinylated cRNA was generated with Affymetrix One-cycle Target Labeling and Control Reagents according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA), followed by fragmentation and hybridization to Affymetrix GeneChip Zebrafish Arrays. Hybridized arrays were scanned on an Affymetrix Fluidics Station 400 and scanned in an Agilent Gene Array Scanner. All data have been deposited in the GEO database with accession number GSE9020. All .cel images were preprocessed with the GC-RMA function in ArrayAssist Express version 4.0 software (Stratagene, La Jolla, CA). Briefly, GC-RMA (39) is an array preprocessing function similar to robust multiarray analysis (RMA) (17) but incorporating mismatch probe sets by using a model based on GC content of the probe. The method is composed of three steps: background correction based on the theory that non-specific binding tends to be directly related to GC content of the probe; quantile normalization; and summarization by the mean method. All summarized GC-RMA output signals were then log2 transformed. Transcripts that were altered by twofold or more compared with the control were selected for analysis. The raw .cel data were also preprocessed by Affymetrix Microarray Suite (MAS) 5.0 software in order to obtain information about whether the signal was present (P) or absent (A) for a particular probe set in a given sample. A probe set was determined as “A” if its MAS 5.0 signal was “A” in at least three of six samples from a given time point, unless all three “A”’s were confined solely to either the DMSO or the RA group. This decision was made as an attempt to include samples in which one of the treatments brought the transcript levels above background, while excluding samples with questionable expression under both conditions. After removal of absent signals, the transformed GC-RMA signals of all probe sets with twofold changes were input into the TIGR MultiExperiment Viewer (TMEV) software (27) from the Institute for Genomic Research (TIGR), and the probe sets with significant changes were selected by two-class unpaired significant analysis of microarray (SAM) (33) with a ≤10% false discovery rate (FDR). Data from the RA experiment shown in Fig. 5 were organized with the self-organizing map (SOM) program from ArrayAssist Expression software, using transcripts altered significantly by twofold or more by RA exposure (31). Hierarchical clustering (HCL) (14) with euclidean distances and average linkage was used in Fig. 8 to organize the combined results from the RA exposure experiment described in

Physiol Genomics • VOL 33 • www.physiolgenomics.org
this work and the TCDD experiment from Carney et al. (6). Again, the analysis was limited to transcripts altered significantly by twofold or more.

**Real-time PCR.** First-strand cDNA synthesis was performed with total RNA isolated from 100 hearts per replicate with a SuperScript III First-Strand Synthesis System for RT-PCR kit from Stratagene according to the manufacturer’s protocol. Real-time PCR was performed with 1 μl of cDNA from each replicate and gene-specific primers with a LightCycler FastStart DNA Master SYBR Green I kit (Roche, Indianapolis, IN) in the Light Cycler (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s protocol. The signal output for each gene was normalized to the level of β-actin to generate a relative expression ratio. Three replicate experiments were repeated at each time point for each gene, and the fold changes were averaged for a given sample. The change in gene expression was expressed as log2[fold change (RA/DMSO)]. Primer sequences were β-actin: forward (F): 5’-aagcagagacagtgtgctgtg-3’, reverse (R): 5’-ggaagacggtcctcctggtg-3’; cyp26b1: F: 5’-atgagctctgaaacttgcctgac-3’, R: 5’-ggagtggctttctgctctgtg-3’; Nr02b: F: 5’-ataaactccgctggcccgtg-3’, R: 5’-gattacacagctcagtcaagcag-3’. Nr2f5: F: 5’-gagcagagagttgctctgctgtg-3’, R: 5’-gagcagagagttgctctgctgtg-3’. BCL2-antagonist of cell death: F: 5’-gagcagtctttctgctctgctgtg-3’, R: 5’-gagcagtctttctgctctgctgtg-3’. Treatment groups was determined by two-way ANOVA followed by Levene’s test or the Cochran C, Hartley, Bartlett test for homogeneity.

**RESULTS**

**Experimental design.** Our immediate goal was to test the hypothesis that RA and TCDD both cause cardiotoxicity through the activation of a common set of target genes in the heart. Our intent was to take advantage of the previously published TCDD array data and collect a matching set of data from RA-exposed hearts. This goal set several constraints on the design of the experiment, which is outlined in Fig. 1.

Each embryonic heart is composed of ~300 cells, and we have found that successful hybridization of Affymetrix zebrafish arrays requires the RNA from ~400 hearts. Heart extraction is optimal at ~72 hpf, a factor that had previously led us to start our TCDD exposure experiment at 72 hpf (6). RA exposure was therefore initiated at 72 hpf, and hearts were collected at 1, 2, 4, and 12 h after exposure. This allows identification of the early changes produced by direct activation via RAR, as well as later changes associated with cardiotoxicity. This time course also corresponds to the time points previously used in the TCDD exposure experiment.

We also needed to demonstrate that RA still produced a cardiotoxic response in 72 hpf zebrafish. Therefore, Fig. 1 also outlines gathering of cardiotoxicity data to confirm this. It should be emphasized that the effects of RA on heart development have been well characterized, and the purpose of these experiments was not to replicate work characterizing the effects of RA on the developing heart.

We expected that RA activation of RAR would produce transcriptional responses relatively rapidly. To follow this, we collected samples for microarray analysis at 1, 2, 4, and 12 h after initial exposure. We also wanted to relate changes in gene expression to cardiotoxic effects produced by RA. Since this takes time to be manifested, these data were gathered at 4, 8, 12, 24, 36, and 48 h after initial RA exposure. Finally, to collect data about heart cell number, cardiac myocytes were counted at 12 and 36 h.

**Retinoic acid causes cardiac toxicity in embryonic zebrafish.** We found that RA exposure beginning at 72 hpf caused numerous cardiovascular defects, including pericardial edema, heart elongation, and reduced heart size (Fig. 2). In the lateral view, pericardial sac edema is clearly evident, along with an elongated atrium and a compacted ventricle. In the ventral view, pericardial edema is also evident, along with the reversal of the normal looping and elongated atrium. These defects, indicated by arrows in Fig. 2, were first evident at 24 h after exposure and became progressively more severe with time. This severe pericardial edema and decreased heart size was similar to the cardiac abnormalities observed in zebrafish embryos exposed to RA at the nine-somite stage (30).

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**Physiol Genomics • VOL 33 • www.physiolgenomics.org**

**Fig. 1. Schematic of experimental design.** The timing of microarray analysis and assessments of cardiac function and heart morphology in zebrafish embryos is shown. RA, retinoic acid.
We used a computer program to outline and calculate the change in pericardial area caused by RA exposure (Fig. 3). This confirmed an increase in pericardial edema by 30 h after initial RA exposure.

RA exposure also altered heart function, reducing ventricular stroke volume to nearly zero by 48 h after dosing (Fig. 4). Since RA had little if any effect on heart rate (Fig. 4B), the altered stroke volume produced a parallel decrease in cardiac output (Fig. 4C). Reduced cardiac output was clearly evident by subjective observation 30 h after exposure, with halted blood flow throughout the embryo. This was confirmed by measurements of blood blow in the posterior intersegmental vein (Fig. 4D).

Changes in transcript abundance in hearts of RA-exposed zebrafish embryos. We used DNA microarrays to identify transcripts that were altered in embryonic hearts exposed to RA. The number of affected transcripts increased from 30 at the first hour to a total of 278 affected by 12 h. Altogether, a total of 309 transcripts were altered by at least twofold for at least one time point. In general, the number of genes that were upregulated was similar to the number of genes downregulated by RA exposure.

We used a SOM algorithm to divide the transcripts into sets of genes with similar temporal patterns of expression (Fig. 5, B and C, and Supplemental Table S1). Cluster 1 was composed of genes that were immediately upregulated and remained up at all time points. This cluster consisted primarily of transcripts encoding metabolism-related enzymes (5 genes) and transcription factors. The online version of this article contains supplemental material.
(3 genes) and cell signaling transcripts (4 genes). Cluster 2 was composed of transcripts that increased in abundance steadily over time. This cluster was enriched in cell signaling genes (9) and transcription factors (15). Cluster 3 was composed of transcripts that in general were not upregulated until the latest time point at 12 h. This cluster included transcripts involved in cell signaling (8), metabolism (12), and cytokine and immune functions (7) as well as encoding structural proteins (6) and...
transcription factors (5). Transcripts in cluster 4 had no clear pattern of regulation across the time course but were in many cases downregulated. These transcripts tended to return to normal levels by the 12 h time point and encoded cell cycle/DNA replication components (6), structural proteins (5) and metabolism-related enzymes (6). Cluster 5 was composed of genes that were sharply downregulated at the 12 h time point. This was the largest single cluster, containing primarily transcripts related to cell cycle progression/DNA replication (33), encoding metabolism enzymes (33), and cell signaling transcripts (12).

Cluster 1 is notable because it contains the most rapidly induced transcripts (Supplemental Table S1). As might be expected, many of these transcripts encode proteins involved in negative feedback control of RA levels, thus affording a homeostatic response to the sudden increase in RA concentration. Cluster 1 includes cyp26a1 and cyp26b1, encoding cytochrome P-450s that inactivate RA by converting it to 4-oxo-RA and 4-hydroxy-RA (35, 36). This group also contained Dehydrogenase/reductase (SDR family) member 3, which belongs to the short-chain dehydrogenase family that catalyze the reduction of all-trans retinal to all-trans retinol (13). The most prominent member of cluster 1 was Nr2F5, a member of the COUP-TF family that functions to repress transcription of steroid-thyroid hormone receptor target genes. Thus Nr2F5 acts antagonistically to RAR (9, 19, 32). Nr2F5 was among the most prominently altered transcripts in the experiment; it was upregulated at the earliest time point, was increased >55-fold by 4 h after RA exposure, and was persistently upregulated throughout the time course.

Cluster 5 was remarkable in that a large fraction of this group was made up of transcripts involved with promoting cell division and growth. These included transcripts that function in DNA replication, DNA repair, cell cycle progression, cellular proliferation, cell division, transcription, and chromosome assembly and maintenance (Supplemental Table S1). The marked downregulation of these messages occurred at a time when RA was causing the normal increase in cardiomyocyte numbers to cease (see Fig. 7).

To validate the results obtained with the microarrays we collected hearts for quantitative PCR (Fig. 6). Triplicate independent replicates showed very close matches to the data obtained with the microarray experiments. This lends confidence to the array results.

The apparent reduction in heart size coupled with the down-regulation of cell cycle genes contained in cluster 5 suggested the possibility that RA was producing a response in which heart cells were no longer increasing in number. To test this, we used RFP-labeled cardiomyocytes to count the number of myocytes in the RA and control hearts. While in the control hearts the number of cells increased over time, growth slowed in the RA-exposed hearts, and at 30 h after initial RA exposure we observed a significant reduction in the number of cardiac myocytes compared with the controls (Fig. 7).

Comparison of RA and TCDD transcript changes. Overall, RA exposure produced cardiotoxicity similar to that induced by TCDD (Fig. 8B). Both compounds caused severe pericardial edema, heart elongation, reduced heart size, decreased cardiomyocyte number, and circulatory collapse (23). In addition, both RA and TCDD are thought to produce their effects through activation of transcriptional regulators. Therefore, we reasoned that the similarities in cardiac toxicity produced by TCDD and RA might be due to a common set of transcriptional events. To test this, we combined the microarray data from this report with previously published microarray data examining the effects of TCDD on heart transcripts (6). We selected and combined data for transcripts that were altered by twofold or more in either of the experiments and used HCL to look for similarities between the patterns of transcriptional changes produced by the two agents (Fig. 8, A and C).

We could find little if any overlap between the initial responses produced by RA and TCDD at 1, 2, or 4 h. This is revealed in the heat map in Fig. 8A, where transcripts shown as red or green are strongly regulated and unchanged transcripts are indicated by shades of black. In most cases at the early time points, the transcripts showing strong induction or repression in response to one treatment showed little response to the other treatment. However, we did find a cluster of overlapping transcripts at the 12 h time point, a point at which the hearts from both treatment types showed distinct signs of failure. This relatively large group of downregulated genes consists of transcripts involved in cell cycle progression (Fig. 8C, Supplemental Table S2). This overlapping group of transcripts corresponds to cluster 5 from Fig. 5 and cluster 9 from Carney et al. (6).

Overall, our results are consistent with a model in which the divergent RA and TCDD signaling pathways do not initially produce a common transcriptional response. However, both agents produce effects that ultimately trigger a heart failure response that appears to be similar for both compounds. Once initiated, this response appears to proceed in parallel with both agents, producing a downregulation of a cell cycle gene cluster (CCGC) and a halt in myocyte growth.

![Figure 6](http://physiolgenomics.physiology.org/)
**Significant difference between an RA point and its respective vehicle control (P ≤ 0.05).**

**Fig. 7**. RA exposure causes a reduction in cardiomyocyte numbers. Cmlc2::dsRed-nuc embryos were exposed to 1 μM RA or 0.1% DMSO control starting from 72 hpf. Embryos were flat mounted, and the number of cardiomyocytes expressing the nuclear marker was counted under epifluorescence at 12 and 30 h after dosing. Values are means ± SE of 6 replicates for each group.

**Nr2F5 mediates RA-induced cardiac toxicity in 72 hpf zebrafish embryos.** As mentioned above, Nr2F5 was massively upregulated after RA exposure. Nr2F5 is a member of the COUP-TF family, a group of orphan steroid hormone receptors. These proteins function as transcriptional repressors, countering the activation produced by steroid-thyroid hormone receptors such as RAR.

We hypothesized that the rapid increase in Nr2F5 expression produced by RA exposure plays an important role in causing heart malformations. An alternative hypothesis is that changes in Nr2F5 levels are just one part of the overall RA response, and do nothing to cause the cardiotoxic effects of RA. If the first hypothesis is true, then artificially increasing Nr2F5 expression should phenocopy the heart malformations produced by RA exposure. To test this we microinjected Nr2F5 mRNA into embryos at the one-cell stage to increase Nr2F5 expression. This recapitulated the cardiac response to RA, producing a small, stretched heart with pericardial edema (Fig. 9). Because of the mosaic expression of injected mRNA in zebrafish embryos, we expected that a limited percentage of the injected embryos would have sufficient expression levels in the right location in the fish to produce the full response. Accordingly, we found that 17% of embryos injected with Nr2F5 mRNA (11 of 63) exhibited clearly apparent signs matching RA-induced cardiac malformations by 72 hpf. In sharp contrast, 0% of the embryos microinjected with control mRNA exhibited cardiac malformations.

If the induction of Nr2F5 mRNA by RA exposure plays an important part in producing cardiac malformation, then we should be able to protect embryonic hearts from RA toxicity by blocking the induction of Nr2F5. We tested this using morpholino oligos (MOs) that specifically block Nr2F5 translation by hybridizing to the mRNA. This allowed us to determine whether knocking down Nr2F5 translation could rescue RA-treated embryos from cardiotoxicity. Zebrafish embryos at the 1- to 2-cell stage were injected with either a control MO or a Nr2F5 MO and then starting at 72 hpf were continuously exposed to either RA or vehicle. We found that the specific Nr2F5 MO targeted to the Nr2F5 translational start site blocked the cardiotoxicity produced by RA exposure (Fig. 10A). The hearts of the RA-exposed embryos treated with the Nr2F5 MO appeared normal and were indistinguishable from the hearts of control embryos not exposed to RA. In sharp contrast, control MO had no ability to protect the heart from RA exposure.

We used lateral-view digital images to measure the protection afforded by the Nr2F5 MO against pericardial edema in fish exposed to RA (Fig. 10B). This clearly showed that while control MO provided no protection, the Nr2F5 MO was effective in rescuing the developing zebrafish from signs of pericardial edema. Taken together, our results indicate that induction of Nr2F5 by RA exposure plays a critical part in producing cardiac toxicity.

**DISCUSSION**

**RA cardiotoxicity at 72 hpf.** Many studies of RA cardiotoxicity have concentrated on early events such as precardiac field formation, chamber morphogenesis, looping, and cardiac myocyte differentiation (8, 12, 18, 30). However, our experiments examined developmental stages that occur after these basic pattern formation events are completed. By 72 hpf the zebrafish heart has fully formed, functional, well-looped chambers. This did not prevent RA from producing a cardiotoxic effect. Because the cardiotoxicity produced by RA in our study occurred after basic heart formation was completed, the cardiotoxic response that we observed could not be due to the blockade of events occurring before the RA exposure and must instead be due to interference with processes ongoing at 72 hpf. This raises the question of whether heart patterning events are irreversible after completion, or whether some aspects of heart patterning or organogenesis must be actively maintained even after completion. In our experiments, RA exposure appeared to elongate the heart, reducing looping and leaving a heart in which the atrium lies almost directly posterior to the ventricle. This suggests that an active process maintains the heart pattern after formation, at least at 72 hpf, and that this process involves RA signaling. Recent work examining the ability of adult heart cells to proliferate in response to wounding or growth signals indicates that proliferative signals are involved in maintaining the structure of the heart throughout zebrafish life (37).

**Transcriptional response to RA in embryonic zebrafish heart.** By rapidly isolating hearts from RA-exposed zebrafish embryos we were able to follow the transcriptional response to RA as it progressed to cardiotoxicity. This yielded a group of genes that could be divided in terms of time course and known function. Our expectation was that the direct targets for the activated RAR complex would be found among the genes affected soon after RA addition. Not surprisingly, the transcripts affected earliest included mRNAs encoding proteins known to antagonize RA action, indicating that RA activation is linked to a negative feedback loop maintaining RA homeostasis.

While it might be expected that direct targets of RA-activated RAR/RXR would be revealed as rapidly upregulated transcripts, we observed an almost equal number of downregulated transcripts at 1 h after RA exposure. This suggests either that RA-induced transcripts are rapidly translated to produce transcriptional inhibitors that act within this first hour or that RA-activated RAR/RXR can in some manner cause the repression of specific target genes.

There is no doubt that RA treatment produced effects in the developing zebrafish that were not limited to altered mRNAs in...
the heart. These effects would be expected to contribute to the overall response to RA, but an intrinsic limitation of our focus on the heart is that these effects would not be identified with our approach.

Comparison of transcriptional responses to RA and TCDD. Both RA and TCDD exposure rapidly altered transcript levels in the zebrafish heart. While the compounds produced similar types of cardiotoxicity, we found little evidence that the two different compounds cause cardiotoxicity by induction of the same set of target genes. The transcriptional changes produced by the two agents were almost entirely nonoverlapping during the first three time points, a period during which we expected the receptors to be directly regulating transcription.

Nonetheless, as toxicity progressed into the early stages of heart failure at the 12 h point, we found a cluster of downregulated transcripts that was common to both treatments. This corresponded to a CCGC that had been earlier identified as a late response to TCDD treatment (6). This cluster is made up of genes known to play important roles in cell cycle progression. Furthermore, many of these genes were downregulated substantially after RA exposure, suggesting that this produces a halt in cardiomyocyte proliferation. However, the degree to which these genes must be repressed in order to halt heart cell growth in the developing zebrafish has not been determined, so we cannot say with certainty that the drop in CCGC expression causes a halt in heart growth. Nevertheless, our results suggest a
model in which different cardiotoxic agents can trigger a response that represses cell proliferation genes and halts the growth of the heart in the developing zebrafish. We have not identified any common pathway that could lead to this; however, it is possible that the response is triggered by a common hemodynamic failure caused by divergent RA and TCDD signaling events.

The COUP-TF protein Nr2F5. An obvious goal for microarray studies is to identify the important downstream transcripts that control biological responses. Among the more interesting potential RA targets found in cluster 1 was Nr2F5, a member of the COUP-TF family. This transcript stands out because of the rapidity and degree of induction by RA. The sequence of a canonical RARE is described as two direct PuG(G/T)TCA repeats separated by \( n \) number of spacers, PuG(G/T)TCA(X)\( n \)PuG(G/T)TCA, summarized by Chambon (7). The most frequent number of spacers is 5, 2, or 1; however, wider spacers and more complex organizations of the motifs have been observed (3, 7). For Nr2F5 we found two TGGTCA motifs with a spacer of \( n = 13 \) at the \(-1410 \) to \(-1433 \) position, indicating that the rapid induction by RA could reflect a direct interaction between RAR and the Nr2F5 promoter.

Previous reports have shown that COUP-TF proteins are essential for the function of RA in growth inhibition and apoptosis in cancer cell lines (20). Targeted deletion of COUP-TF II in mouse embryos led to defects in heart development and angiogenesis (24). This known association with RA and the heart, taken with the prominent induction by RA in our experiments, made Nr2F5 an attractive candidate for a mediator of RA-induced cardiotoxicity. We found that overexpression of Nr2F5 could phenocopy and thus explain the cardiotoxicity produced by RA. While it can be argued that the production of heart failure and pericardial edema could be due to nonspecific effects of mRNA injection or protein overexpression, we also found that Nr2F5 MOs blocked the cardiotoxicity produced by RA exposure. It is difficult to imagine how nonspecific mechanisms could lead to such a dramatic rescue. The simplest explanation for our results is that Nr2F5 plays a role in mediating the cardiotoxic response to RA in the zebrafish heart.

Fig. 9. Nr2F5 as a critical target in RA-induced cardiac toxicity in zebrafish embryos. One-cell-stage embryos were injected with either control or Nr2F5 mRNA as described in MATERIALS AND METHODS, and images of the hearts were taken at 72 hpf. Top: typical appearance of embryo after injection of 100 pg of control RNA in 1-cell-stage embryo showing no effect on heart development. Middle and bottom: range of appearance of embryos injected with 100 pg Nr2F5 mRNA, showing small and elongated hearts with mild to severe pericardial edema. Arrows point to the hearts.

Fig. 10. Nr2F5 morpholino (MO) blocks RA-induced cardiac toxicity in zebrafish. A: 1-cell-stage embryos were injected with either Nr2F5 MO or control MO as described in MATERIALS AND METHODS. At 72 hpf the embryos were exposed to 0.1 \( \mu \text{M} \) RA or 0.1% DMSO as the vehicle control, and images of the hearts were recorded at 120 hpf. Top: Nr2F5 MO- and control MO-injected embryos exposed to DMSO, showing normal heart development; bottom, Nr2F5 MO- and control MO-injected embryos exposed to RA, showing rescue from the effect of RA for the Nr2F5 morphants, but not for the embryos receiving the control MO. Arrows point to the hearts. B: lateral views of embryos from the experiment described in A were photographed and the area of the pericardial sac was measured for each embryo with NIH Image. Gray bars, embryos treated with RA; open bars, control embryos exposed to DMSO. Values for edema are means ± SE from 4 separate experiments with \( n = 3-5 \) embryos/group in each experiment. *Significant difference between Nr2F5 MO and control MO (\( P \leq 0.001 \)).
Nr2F5 is one of three COUP-TF family members found in zebrafish (15). COUP-TF proteins act as repressors at hormone-responsive elements (9, 19, 32) and are needed for RA-induced growth inhibition and apoptosis (20). It is interesting that the level of nuclear COUP-TF has been found to be elevated in heart cells of mice with cardiac hypertrophy (26), while loss of COUP-TF II in knockout mouse embryos produces underdevelopment of the atria and sinus venosa (24). COUP-TF II has been reported to interact with MyoD, the master transcription factor required for skeletal muscle myogenesis. COUP-TF II tethers MyoD away from the activation complex, suppressing MyoD-mediated myogenesis in pluripotency (23). COUP-TF II has been reported to interact with MyoD, the orphan receptor COUP-TF II, inhibits myogenesis by post-transcriptional regulation, providing a step forward in understanding the effects of RA on the developing heart at the molecular level.

RA-induced cardiac toxicity and teratogenic effects on the heart have been widely reported at the anatomic, pathological, and pathophysiologic levels. Our findings provide a step forward in understanding the effects of RA on the developing heart at the molecular level.

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REFERENCES


GRANTS

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