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RESEARCH ARTICLE

Dysregulatory effects of retinoic acid isomers in late zebrafish embryos

Laia Navarro-Martín[1] · Eva Oliveira[1] · Marta Casado[1] · Carlos Barata[1] · Benjamin Piña[1]

Abstract All-*trans* retinoic acid (atRA) and 9-*cis* retinoic acid (9cRA) are two natural derivatives of vitamin A that contribute to the normal vertebrate development by affecting gene expression through the retinoic acid signalling pathway. We show transcriptomic effects of the ectopic addition of atRA or 9cRA to zebrafish embryos at the posthatching embryonic stage. Exposure for 24 or 72 h to sublethal concentrations of both isomers resulted in characteristic transcriptome changes, in which many proliferation and development-related genes became underexpressed, whereas genes related to retinoid metabolism and some metabolic functions became overrepresented. While short and long exposures elicit essentially the same set of genes, atRA specifically induced expression of a specific subset of proteases, likely acting at the extracellular level, and of elements of the response to xenobiotics. These results reflect the well-known antiproliferative activity of retinoids, and they suggest a dysregulation of the developmental process at final stages of embryogenesis. They also indicate a potential role of endopeptidases as markers of developmental alterations, as well as their possible control by the retinoic signalling pathway. We propose to monitor mRNA levels of *cyp16a*, *cyp16b*, and *cyp16c* in zebrafish embryos as a bioassay for retinoid disruption.

Keywords Retinoids · Embryos · Development · Microarrays · Endocrine disruption · Transcriptomics

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Introduction

Retinoids are naturally occurring isomers of retinoic acid (RA), a natural vitamin A derivative. They are essential morphogens in vertebrate development, regulating the formation of the body axes and the development of a number of organ systems, including the retina, the brain, the heart, the urogenital system, and the lungs (Eichele and Thaller 1987; Hu et al. 2008). In adults, they intervene in the regulation of different metabolic processes in many tissues, involving cellular growth, immune response, proliferation, differentiation, metabolism, and apoptosis (Blomhoff and Blomhoff 2006; Mark et al. 2006; Niederreither and Dolle 2008). The requirement for retinoids during embryogenesis has been long recognized, since vitamin A is an essential dietary requirement, and either the excess or a deficiency in vitamin A or RA disrupts the development of zebrafish embryos (Dobbs-McAuliffe et al. 2004).

The effects of retinoids are mediated through its binding to retinoid receptors, which are members of the nuclear receptor family. In vertebrates, there are two multigenic classes of retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (Dolle 2009). RARs are able to

bind both all-*trans* and 9-*cis* retinoic acid (atRA and 9cRA, respectively) with similar affinity, although the kinetics of binding can differ among RAR paralogs, whereas 9cRA is the natural ligand of RXRs (Heyman et al. 1992). However, the biological significance of 9cRA as the in vivo RXR ligand remains controversial, since no significant levels of this isomer has been detected in vivo in vertebrates (Costaridis et al. 1996; Wolf 2006). In addition, RXRs are heterodimerization partners for a considerable number of other nuclear receptors, and as such are often considered to loosen the specificity of the signalling pathway (Francis et al. 2003; Lefebvre et al. 2010). An extensive number of studies have tested the effects of exogenous retinoids in embryos from different vertebrate species and suggested possible divergences of their teratogenic effects (Kosian et al. 2003 397; Szondy et al. 1997; Zhang et al. 1996). RA has also been implicated in clinical applications, both as a potential anti-tumor agent and for the treatment of skin diseases (Theodosiou et al. 2010), but the possible deleterious effects of the ectopic presence of retinoids, either as a consequence of medical treatments or as environmental pollutants are still poorly understood (Blomhoff and Blomhoff 2006; Tabb and Blumberg 2006).

The aim of this study was to elucidate the effects of exogenous administration of atRA and 9cRA in zebrafish eleutheroembryos by transcriptomic analyses. Our target was to identify genetic markers of retinoid disruption and to investigate possible differential effects between both isomers. We focused in the period of development going from hatching at about 48 h post fertilization, or hpf, to the start of the self-feeding stage, at 120 hpf, avoiding the initial embryonic stages, but still previous to the independent larval stage. In the present study, we intend to set a bioassay to detect and quantify potential retinoic disruptors and their putative negative effects for human and environmental health.

Material and methods

Animals and rearing conditions

Zebrafish (*Danio rerio*) embryos were obtained by natural mating following standard procedures. At 2 h post fertilization (hpf), fertilized viable eggs were transferred to embryo water, 90 μg/ml of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4 \cdot_2 H_2 O$, dissolved in reverse osmosis purified water, under standard conditions (28.5 °C and 12 L:12D photoperiod). All procedures were conducted in accordance with the institutional guidelines under a license from the local government (DAMM 7669, 7964) and were approved by the Institutional Animal Care and Use Committees at the Research and Development Centre of the Spanish Research Council.

Zebrafish eleutheroembryos exposures to 9cRA and atRA

DMSO, all-*trans* retinoic acid (atRA) and 9-*cis* retinoic acid (9cRA) with ≥ 98% purity (HPLC powder) were purchased from Sigma-Aldrich (catalogue numbers, R4643 and R2625, respectively, St. Louis, MO). Stock solutions were prepared in DMSO on the day of the experiment. Experimental solutions with the same final concentration of DMSO (0.1%) were directly prepared in embryo water. This DMSO concentration is the upper limit at which no morphological or developmental changes are observed (Hallare et al. 2006). Stability of both atRA and 9cRA isomers were checked by HPLC analysis (see supplementary materials and methods). We verified that both compounds were stable and detectable in filtered embryo water, at least for 24 h (Supplementary Fig. 1a). For that reason, exposures were carried out under semi-static conditions, new water treatments were freshly prepared, and fish water was changed every day in all experimental conditions. All concentrations reported in the results are nominal.

Yolk-dependent stages of zebrafish embryo are a standard model for ecotoxicological studies and aquatic toxicity testing of chemicals (EC 2010; Strahle et al. 2012). For that reason, zebrafish embryos were exposed to each compound in two different windows of exposure: from 2 to 5 dpf and from 4 to 5 dpf, hereafter referred to as "long" and "short" treatments, for simplicity. Starting at 48 hpf avoids interferences with the early embryonic development, whereas the end of both exposures coincides with the start of self-feeding stage (Strahle et al. 2012) .

A preliminary acute toxicity test was carried out at 100 and 1000 nM of either atRA or 9cRA (6-well-plates, 2 embryos/ mL). Sub-lethal and teratogenic endpoints for the controls and treated samples were observed at the end of each assay, based on the criteria previously established (Lammer et al. 2009; Nagel 2002). Embryos were evaluated and images were taken in order to record phenotypic characteristics during the development using a Nikon digital Sight DS-Ri1 camera. Results determined 100 nM as the highest effective concentration with no observable macroscopic effects for both isomers (NOAEC, Supplementary Fig. 2).

Exposures for transcriptomic analysis were performed with a final concentration of 100 nM in 100-ml glass beakers (50 larvae/beaker) with 3 biological replicates per treatment. Final treatments include a control (0.1% DMSO), 100 nM 9-*cis* retinoic acid from 2 to 5 dpf (9cRA-L), 100 nM all-*trans* retinoic acid from 2 to 5 dpf (atRA-L), 100 nM 9-*cis* retinoic acid from 4 to 5 dpf (9cRA-L), and 100 nM all-*trans* retinoic acid from 4 to 5 dpf (atRA-S).

RNA extraction and microarray analysis

Total RNA was isolated from pools of approximately 50 whole zebrafish embryos. Homogenization was done in

Trizol (Invitrogen Life Technologies, Carlsberg); using Eppendorf-fitting RNase free pestles (Sigma-Aldrich); and purified using the RNeasy Kit (Qiagen) standard protocol. RNA concentration was measured by spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality checked in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RIN (RNA Integrity Number) values ranged between 9.5 and 10.

Microarray studies were performed using the commercial Agilent Zebrafish (v3) Gene Expression Microarray (4x44K) using a two-color strategy. The study included three biological replicates. Treated samples consisted in pools of 50 embryos independently treated, whereas control samples consist of a mixture of untreated pools corresponding to the same batches. Results were deposited at GEO, reference-GSE41335: GSM1015039, GSM1015040, GSM1015041, GSM1015042, GSM1015043, GSM1015044, and GSM1015045 for 9cRA exposures and GSM1015046, GSM1015047, GSM1015048, GSM1015049, GSM1015050, and GSM1015051 for atRA exposures. The microarray was carried out following the procedures described in (Oliveira et al. 2013). Microarray data quality was evaluated manually using the quality control report generated by Agilent Software. No statistical differences were observed between the biological replicates of each treatment. Raw data and feature extraction pre-proceeded data from the Agilent Microarrays were imported into the open-source software R statistical environment (http://www.r-project.org) using the limma package for normalization of two-color microarray data (Bioconductor platform, (Ritchie et al. 2005)). Values were background-corrected with the method *normexp*, which results in strictly positive adjusted intensities and normalized within arrays with the *loess* method.

Microarray validation by qRT-PCR

Total RNA was treated with DNAse I (Ambion, Austin, TX) to remove genomic DNA contamination and retro-transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (F. Hoffmann-La Roche, Basel, Switzerland). Aliquots of 50 ng of total RNA transcribed to cDNA were used to quantify specific genes in LightCycler® 480 Real-Time PCR System (F. Hoffmann-La Roche) using SYBR® Green Mix (Roche Applied Science, Mannheim, Germany). The selected gene primers used for qRT-PCR validation were designed from existing zebrafish nucleotide sequences.

Appropriate primers (Supplementary Table 1) for 17 test genes (*acox1, acsl1, apoa1, cyp26a1, dhrs3a, guca1c, hoxb1b, hoxb5a, hoxb5b, igfbp1b, klf2a, lpl, matn3a, pde6h, pdk2, ppiaa, rps25*) were designed using Primer Express 2.0 software (Applied Biosystems) and the Primer-Blast server (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). Amplification efficiencies were ≥

90% for all tested genes as described (Pfaffl et al. 2002). Housekeeping gene *ppiaa* was selected as reference genes (Morais et al. 2007; Pelayo et al. 2012), as mRNA levels did not change upon treatments (Pfaffl et al. 2002). PCR products (amplicons) were sequenced in a 3730 DNA Analyzer (Applied Biosystems), and compared to the corresponding reference sequences at NCBI server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Relative mRNA abundances were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated from technical triplicates). To minimize errors on RNA quantification among different samples, Cp values for target genes (Cp_{tg}) were normalized by Cp values for *ppiaa* for each sample (corr $Cp_{tg} = Cp_{tg} - Cp_{ppiaa}$).Changes in mRNA abundance in samples from different treatment were calculated by the $\Delta\Delta Cp$ method (Pfaffl 2001), using corrected Cp values from treated and non-treated samples ($\Delta\Delta Cp = $ corr $Cp_{tg\_untreated} - Cp_{tg\_treated}$).

Statistical analyses

Calculations for all microarray statistical analysis were done using different packages in the R environment. Differentially expressed genes (DEGs) were identified using binomial tests to strength the power of the assays. Genes that showed significant changes in their relative abundances upon exposure to either atRA or 9cRA were identified by one-sample *t* test, using the *graphics* package in R. Differences between short and long treatments were tested using two-sample *t* test analyses in the same package. FDR was used to correct for multiple testing, using a *p* value cut-off of 0.05. Only those DEGs showing at least a twofold change in at least one of the treatments were considered for further analyses. Hierarchical clustering and medoid pam clustering analysis were performed using the packages *gplots*, *fpc*, and *cluster* in R. Moreover, sample classification using the complete transcriptomic dataset with "compound" (atRA vs. 9cRA) and "time" ("long" vs. "short") as factors was done by partial least squares discriminant analyses (PLS-DA) using the MATLAB Toolbox (Eigenvector Research Inc., Wenatchee, WA). This multivariate analysis allowed us to determine which genes best describe the differences between groups using the selectivity ratio (SR) method, a reliable method for transcriptomic data sets (Farres et al. 2015).

Gene enrichment and functional analysis

Functional analysis of selected genes was performed using DAVID Bioinformatic Resources 6.8 (Huang et al. 2009) and the Amigo Webpage (http://www.geneontology.org (Carbon et al. 2009)). Gene enrichment analysis was estimated in DAVID using the microarray set up as background gene set;

enrichment significance was set at a false discovery ratio (FDR) value of 0.05.

Results

Differentially expressed genes (DEGs) after exposures to retinoic acid in zebrafish embryos

In total, we identified 1035 differentially expressed genes (DEGs) out of the 15,818 unique genes present in the microarray. A heatmap including hierarchical clustering of the 1035 DEGs for all individual samples ($n = 12$) or for samples averaged by experimental group ($n = 4$) are shown in Supplementary Fig. 3 and Fig. 1a, respectively. Principal component analysis (PCA) showed that the first two components explained a total of 66% of the variance (Fig. 1b). PCA score plot of the first two components clearly disaggregate between samples exposed to the different compounds (9cRA vs atRA), but it is less clear differentiating exposure time (long vs short). Fold-change values (treated vs non-treated) calculated from the microarray data agreed with the corresponding results obtained from qRT-PCR analyses for 16 genes in all the four conditions tested (two isomers, two exposure times, $r = 0.785$, $n = 64$, $p < 10^{-4}$, Supplementary Fig. 4).

Medoid cluster analysis defined three clusters, which explained 81.6% of sample variability using two components (Supplementary Fig. 5). A complete list of DEGs showing their cluster ascription and fold change values is provided in Supplementary Table 2. Cluster A (350 DEGs) includes genes whose copy number increased in all treatments, although showing stronger effects in atRA-treated samples than in 9cRA-treated ones (Fig. 2a, d). Cluster B (355 DEGs) corresponds to genes underrepresented in all treatments, showing again the strongest effects in atRA-treated samples (Fig. 2b, e). Finally, we observed a time- and compound-dependent behavior in genes of cluster C (330 DEGs), in which relative abundances of most genes were downregulated for 9cRA-treated samples and upregulated for atRA-treated ones (Fig. 2c, f).

Functional analysis of DEGs after RA exposure

Genes included in the three clusters showed distinct functional profiles, as shown by DAVID functional analysis (summarized in Fig. 3, complete results in Supplementary Table 3). Cluster A included genes related to extracellular region, integral component of plasma membrane, sequence-specific DNA binding, intracellular membrane-bounded organelle, hormone activity, liver development, and retinol metabolism. The last category comprises all the *bona fide* markers for RA induction, including the three isoforms of *cyp26* present in zebrafish (*cyp 26a1*, *cyp26b1*, and *cyp26c1*) as well as other

components of the RA metabolic pathway, like *lrata and rdh5* (brown boxes in Fig. 4A). Cluster B included genes related to plasma membrane and other generic functional categories (DNA binding, transport, metabolism). DEGs from cluster C were included in functional categories such as proteolysis, oxidoreductase activity, peptidase activity, extracellular space, hydrolase activity, glycosyltransferase activity, glucuronosyltransferase activity, carboxylic ester hydrolase activity, steroid hormone biosynthesis, steroid hydroxylase activity, cellular response to xenobiotic stimulus, monooxygenase activity, serine-type peptidase activity, glyoxylate and dicarboxylate metabolism, sphingolipid metabolism, and drug metabolism. Some functional categories were shared between cluster A and cluster C, including oxidoreductase activity, liver development, hormone activity, iron ion binding, heme binding, and retinol metabolism. Retinol metabolism-related genes from cluster C include *cyp1a, cyp3a65, and ugt1a1* (pink boxes in Fig. 4A).

Identification of biomarkers of exposure to RA isomers

Based on the results obtained from the DAVID analysis of the identified DEGs ("Functional analysis of DEGs after RA exposure"), we have identified genes related to retinol metabolism as biomarkers of exposure to RA (regardless of the isoform). Fold change is shown in Fig. 4B for *cyp26a1, cyp26b1 and cyp26c1*, *rdh5*, *lrata*, *cyp3a65*, *rdh1*, *cyp1a*, and *ugt1a*.

Sample classification by PLS-DA was performed using the identified DEGs (X-block) and compound or time as the categorical variable (Y-block) to identify biomarkers (genes) that differentiate between exposures to both RA isomers (9cRA vs atRa) or between the two exposure times (short vs long). When looking at the categorical variable "compound", results revealed that one latent variable (LV) explained 84% of the variability of the Y-block using 59% of the X-block (Figure Supplementary 6A). Classification rates were of 100% (calibration) and 83.4% (cross-validation, with only one sample misclassified). A total of 345 genes were identified to best discriminate between RA isomers using a cut-off of 3.16 (given by default) of the selectivity ratio score (SR). On the other hand, results from the categorical variable time revealed that two latent variables were needed to explain a total of 86.4% of the variability of the Y-block using 64.6% of the X-block (Figure Supplementary 6B). Classification rates were of 100% (calibration) and 58.4% (cross-validation, with 5 samples misclassified). These results indicate that samples were not well discriminated between the variable time. Moreover, we were not able to identify biomarkers (genes) to discriminate between exposure times using a cut-off of 3.16 (given by default) of the selectivity ratio (SR).

DAVID functional analysis of the biomarkers selected to discriminate between RA isomers (Supplementary Table 4) showed that the selected genes are involved in the following
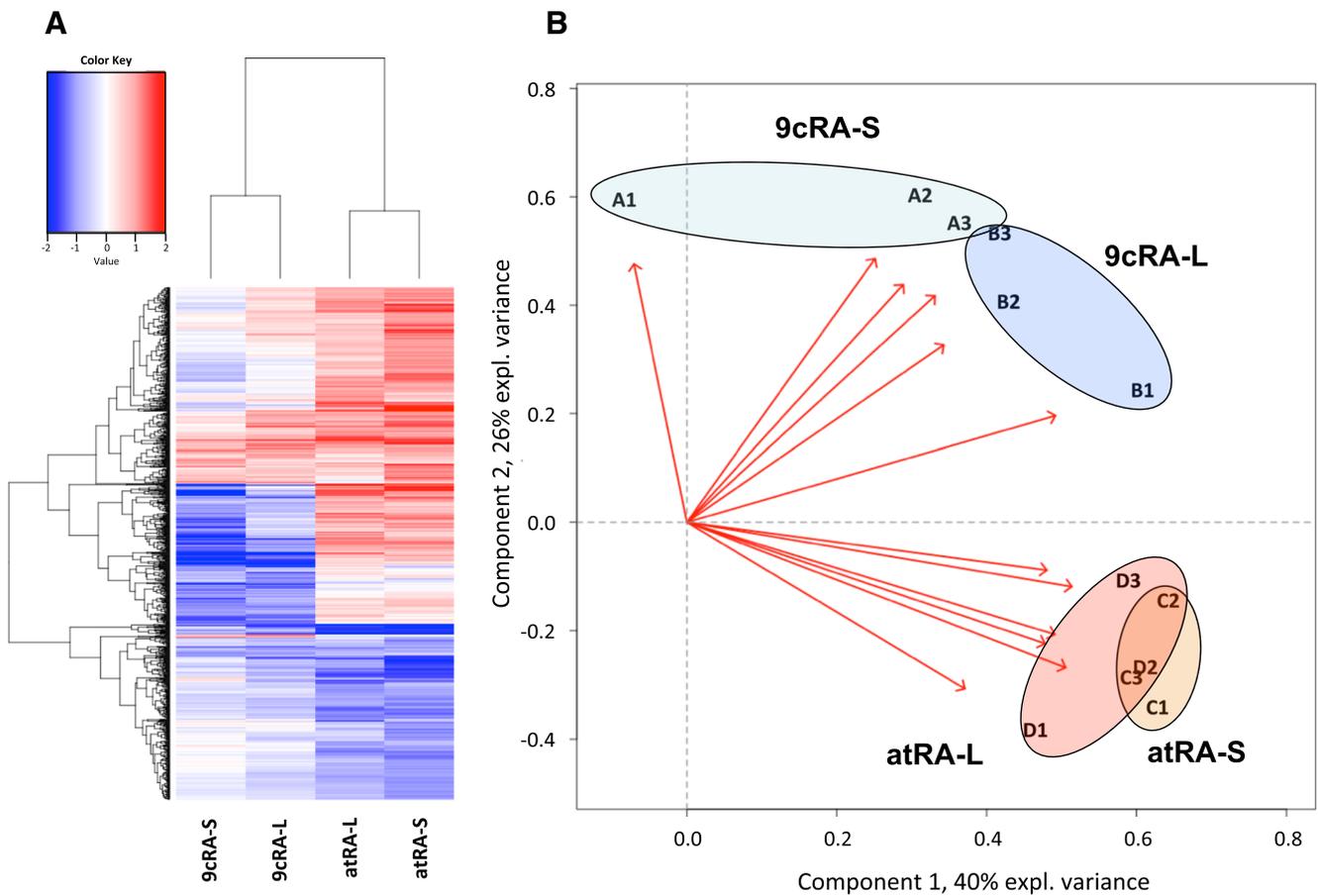
Fig. 1 **a** Heatmap representing differentially expressed genes at 5 dpfs after RA exposures in zebrafish. Zebrafish eleutheroembryos were exposed to 9-*cis* retinoic acid (9cRA) and all-*trans* retinoic acid (atRA) during two different windows of exposure: (1) a longer period from 2 to 5 dpf (L) and (2) a shorter period from 4 to 5 dpf (S). Dendogram represents a hierarchical clustering of the 1035 differentially expressed genes (DEGs) for all samples averaged by experimental group. Colors represent overexpressed (red) and underexpressed (blue) DEGs. Experimental groups include: 100 nM 9-*cis* retinoic acid from 4 to 5 dpf (9cRA-S), 100 nM 9-*cis* retinoic acid from 2 to 5 dpf (9cRA-L), 100 nM all-*trans* retinoic acid from 4 to 5 dpf (atRA-S), and 100 nM all-*trans* retinoic acid from 2 to 5 dpf (atRA-L). **b** PCA score plots for the first two components of the individual samples ($n = 12$, three biological replicates per experimental group)

biological processes: oxidation-reduction process, proteolysis, transport, chitin metabolic process, phosphorylation, cellular response to xenobiotic stimulus, cell adhesion, transmembrane transport, and regulation of transcription. Moreover, we observed that biomarker genes belong to protein families such as cytochrome P450, peptidases, troponin, UDP-glucuronosyl/UDP-glucosyltransferase, chitins, serpins, and sulfotransferases. All the exposed above denotes that genes related to the detoxification pathway are overrepresented in this set of genes. Figure 5 shows genes selected as biomarkers of RA isomer exposure related to different phases of the detoxification pathway: (1) phase I (*cytochrome p450* family), (2) phase II (*ugt* and *sult* families), and (3) transport genes (ABC transporter family). These results indicate that the observed differences between the isomers could be related to a general higher toxicity of atRa relative to 9cRA.

Discussion

The characterization of specific changes in the transcriptome ("transcriptomic footprints") associated to the ectopic activation or deactivation of physiological signalling pathways (hormonal, nervous, immune) in model organisms is of paramount interest in medicine, drug development, regulatory toxicology, and ecotoxicology (Pina and Barata 2011; Raldua and Piña 2014; Raldua et al. 2012). In the present study, we attempt to define genetic markers linked to retinoic ectopic signalling during ZF early development and to evaluate their response by studying the two main RA isomers in two different times of exposure. We adjusted the concentration to prevent major morphological changes that could add a confounding factor to the results. For the same reason, we excluded the early embryonic stages, in which earlier cell type commitment takes
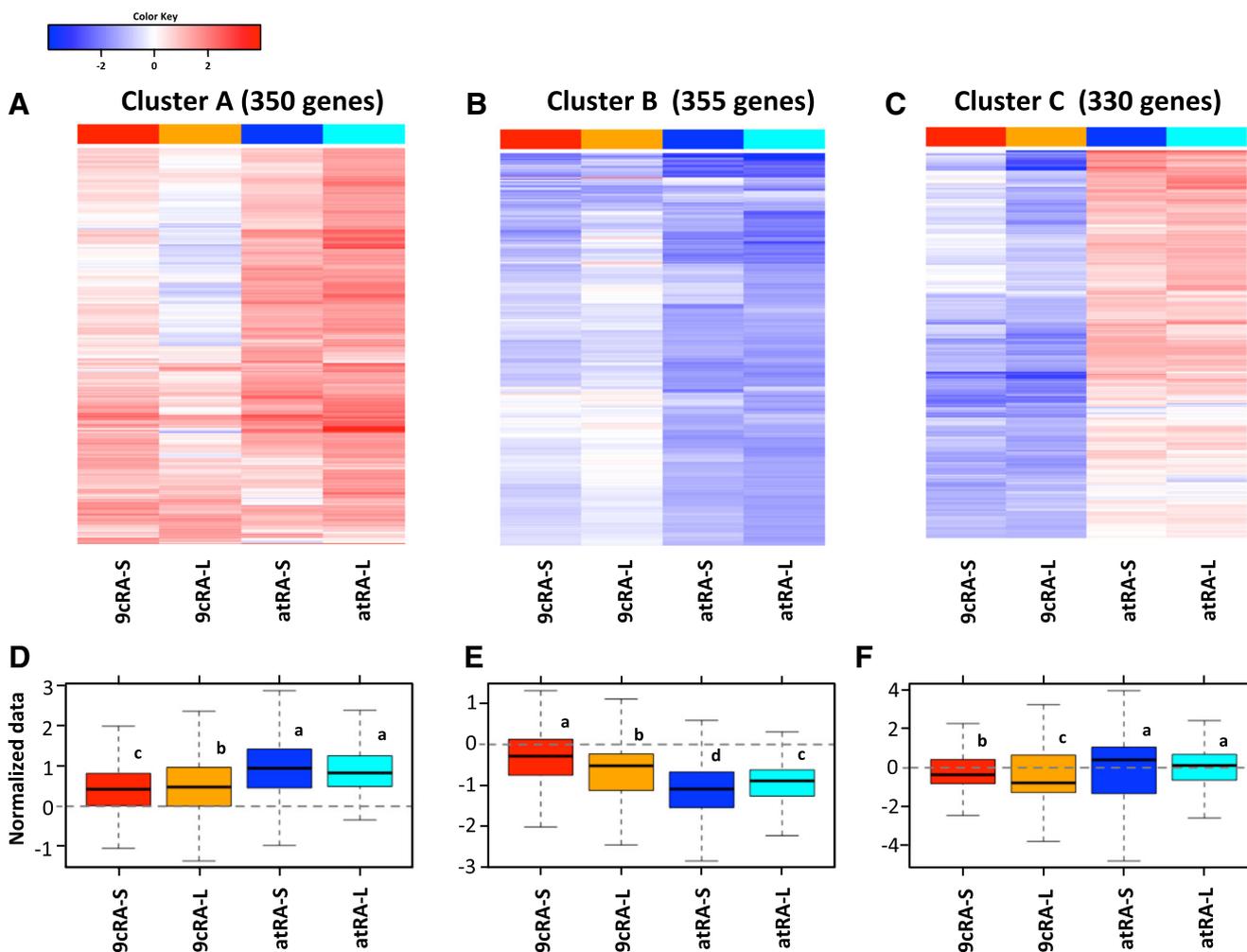
Fig. 2 Expression patterns of differentially expressed genes (DEGs) belonging to the three clusters identified by the medoid cluster analysis (see text). The total number of genes in each cluster is indicated at the top. Panels (**a**–**c**) show hierarchical clustered heatmaps; colors indicate overexpressed (red) or underexpressed (blue) DEGs relative to the controls. Panels (**d** and **e**) show the distribution of the corresponding expression values. Boxes i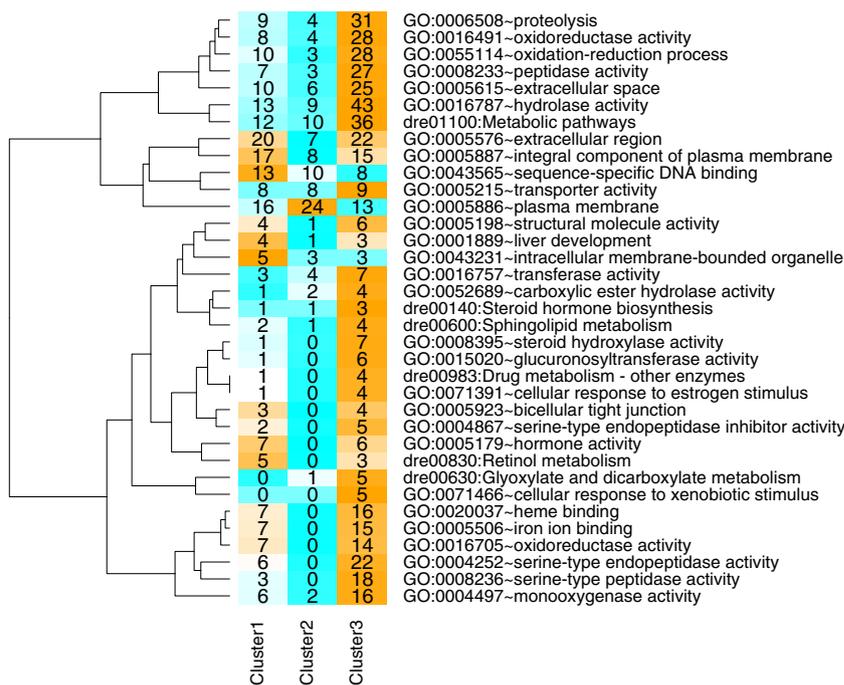ndicate ranges from the first to the third quartile, the thick lane indicates the media, and the whiskers expand the 95% confidence interval. Letters in the boxplots represent significantly different sets of values (ANOVA + Tukey's). Experimental groups include: 100 nM 9-*cis* retinoic acid from 4 to 5 dpf (9cRA-S), 100 nM 9-*cis* retinoic acid from 2 to 5 dpf (9cRA-L), 100 nM all-*trans* retinoic acid from 4 to 5 dpf (atRA-S), and 100 nM all-*trans* retinoic acid from 2 to 5 dpf (atRA-L)

place. By the beginning of the exposure window at 24 hpf, major embryogenetic processes (gastrulation, blastula formation, axial patterns) are completed, and by the end of exposure at 120 dpf, many of the major organic systems (brain, eyes, muscle, heart, digestive track) are already defined and at least partially operative. However, the embryo is still not capable to self-feeding at this stage, and is therefore excluded from the current animal welfare regulations, a very important aspect for regulatory toxicology and drug screening (EC 2010; EFSA 2005; Scholz 2013; Strahle et al. 2012). By using this approach, we consider that the observed transcriptomic effects are in fact related to the activation of the retinoid signalling pathway and to the subsequent secondary responses, and not to a modification of the body plan of the developing embryo, precisely the pattern of toxicity one should expect in vertebrates, from fetal (as opposite to embryonic) to adult stages.

The coordinate upregulation of retinol-metabolizing genes likely represents the hallmark of the exposure of retinoids. This response constitutes a negative feedback mechanism that regulates and balances RA levels in embryos up to adulthood (Dobbs-McAuliffe et al. 2004). Such regulation not only maintains the endogenous RA level within a normal range, but also allows the organism to respond to exogenous RA fluctuations. Another coordinate response to RA exposure was the decrease in relative abundances of mRNAs related to plasma membrane, transport, and metabolism, an effect likely linked to the known antiproliferative effect of retinoids, which lies in the basis for many of their pharmacological applications (Altucci and Gronemeyer 2001) .

In addition to the retinoid- and proliferation-related functional classes, we observed a dysregulation of several genes involved in metabolic pathways, particularly for redox

Fig. 3 Distribution of genes belonging to GO or KEGG functional categories, calculated by DAVID. Only categories with significant enrichment in at least one of the treatments are shown. Colors correspond to the relative importance of each cluster in the total distribution of the genes belonging to each category. Orange and cyan correspond to genes over- or under-represented in each particular cluster; the actual number of hits for each cluster and categories is indicated with black figures

| Cluster1	Cluster2	Cluster3	
9	4	31	GO:0006508~proteolysis
8	4	28	GO:0016491~oxidoreductase activity
10	3	28	GO:0055114~oxidation-reduction process
7	3	27	GO:0008233~peptidase activity
10	6	25	GO:0005615~extracellular space
13	9	43	GO:0016787~hydrolase activity
12	10	36	dre01100:Metabolic pathways
20	7	22	GO:0005576~extracellular region
17	8	15	GO:0005887~integral component of plasma membrane
13	10	8	GO:0043565~sequence-specific DNA binding
8	8	9	GO:0005215~transporter activity
16	24	13	GO:0005886~plasma membrane
4	1	6	GO:0005198~structural molecule activity
4	1	3	GO:0001889~liver development
5	3	3	GO:0043231~intracellular membrane-bounded organelle
3	4	7	GO:0016757~transferase activity
1	2	4	GO:0052689~carboxylic ester hydrolase activity
1	1	3	dre00140:Steroid hormone biosynthesis
2	1	4	dre00600:Sphingolipid metabolism
1	0	7	GO:0008395~steroid hydroxylase activity
1	0	6	GO:0015020~glucuronosyltransferase activity
1	0	4	dre00983:Drug metabolism - other enzymes
1	0	4	GO:0071391~cellular response to estrogen stimulus
3	0	4	GO:0005923~bicellular tight junction
2	0	5	GO:0004867~serine-type endopeptidase inhibitor activity
7	0	6	GO:0005179~hormone activity
5	0	3	dre00830:Retinol metabolism
0	1	5	dre00630:Glyoxylate and dicarboxylate metabolism
0	0	5	GO:0071466~cellular response to xenobiotic stimulus
7	0	16	GO:0020037~heme binding
7	0	15	GO:0005506~iron ion binding
7	0	14	GO:0016705~oxidoreductase activity
6	0	22	GO:0004252~serine-type endopeptidase activity
3	0	18	GO:0008236~serine-type peptidase activity
6	2	16	GO:0004497~monooxygenase activity

metabolism (36 transcripts, mostly from the cytochrome P450 family), peptidases (37 transcripts, with a high proportion of serine-type peptidase) and in membrane-associated and extracellular proteins (Supplementary Table 2). These functional categories were particularly enriched in cluster C, suggesting a preferential induction of these general processes by atRA when compared to 9cRA. The molecular mechanisms underlying these responses may be related to general embryotoxic effects. On one side, metalloprotease-codifying genes have been involved in bone formation and in digestive tract maturation, two processes that are probably ongoing during the late embryonic developmental stages of zebrafish, and for which there are some indications that the RA signalling pathway is implicated (Guerrera et al. 2015; Jimenez et al. 2001; Spoorendonk et al. 2008). On the other side, different cytochrome P450 members have been related to the xenobiotic response, a regulatory pathway that also includes most of the glucuronosyltransferases and other phase II response enzymes (sulfatases, hydrolases), which also appeared in cluster C (Fig. 3, Supplementary Table 2) (Kohle and Bock 2007).

In this study, both RA isomers affected a very similar subset of functional categories (Fig. 3, Supplementary Table 2). The only real difference between the two treatments was the higher number of putative toxicity- and/or xenobiotic response-related genes induced by atRA. This is somewhat surprising when considered the number of cell promoters for which RXR acts as a necessary cofactor, mostly by heterodimerizing with other nuclear factors, including the peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXRs), farnesol X receptor (FXR), the pregnane X receptor (PXR)/steroid and xenobiotic receptor (SXR), and the Vitamin D receptor (Francis et al. 2003; Lefebvre et al. 2010). The ectopic activation of some of these signalling pathways may explain the observed changes in genes related to xenobiotic response, like some cytochrome P450 or to phase II-related enzymes, although it is difficult to explain why these effects are weaker (or simply not observed) in animals treated with 9cRA. This observation adds to the current controversy on the physiological role of 9cRA, whose levels are very low or undetectable in zebrafish and other vertebrates, both as embryos or as adults (Costaridis et al. 1996; Rhinn and Dolle 2012; Wolf 2006). Retinoic acid isomer concentrations are tightly regulated in vitro (Dawson and Xia 2012; Kane 2012), and there are evidences for their interconversion one into another (Chen et al. 2001; Urbach and Rando 1994). The easiest explanation of our results is that 9cRA has relatively low biological effects in the zebrafish embryo (at least at the stage we are studying), and that the observed changes were elicited by its partial interconversion to atRA in the animal.

The other tested parameter, the window of exposure, showed essentially quantitative, rather than qualitative effects on the transcriptome, in the sense that no mRNA (or at least, very few of them) appeared to be specifically enriched or depleted by longer or shorter exposures. This behavior is accompanied by the chemical stability we observed for both isomers. In practical terms, these results suggest that 24 h of
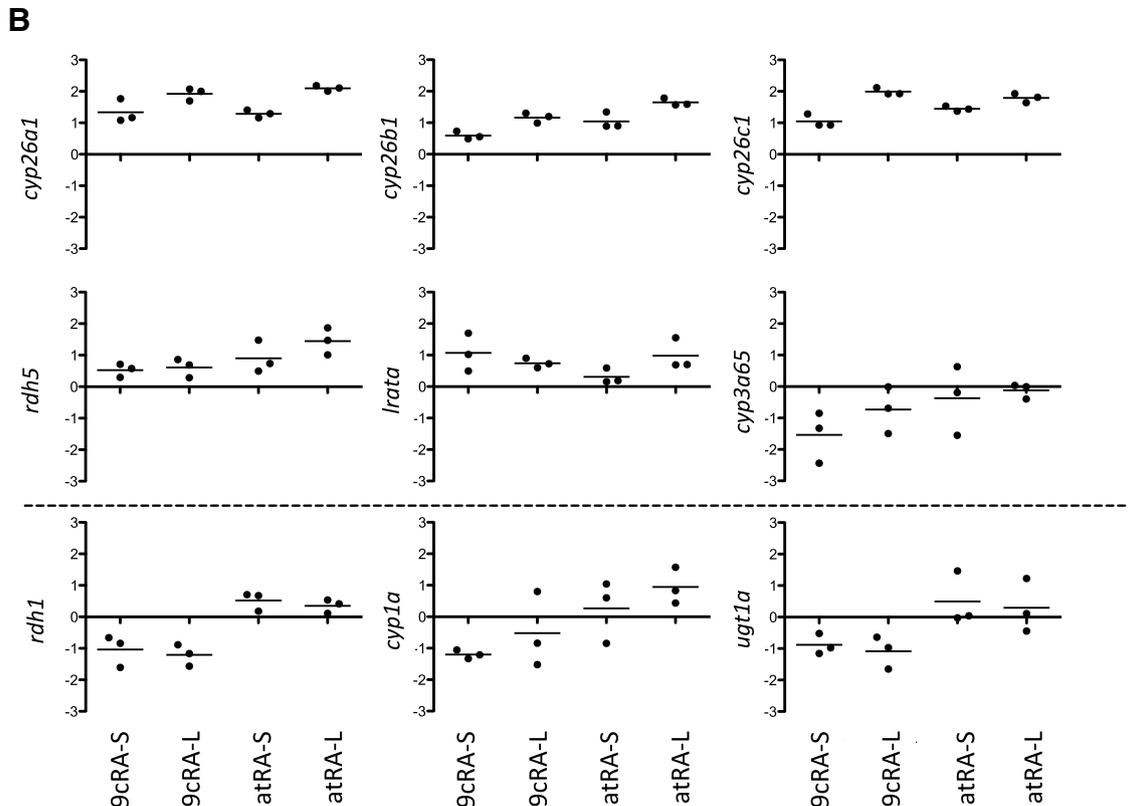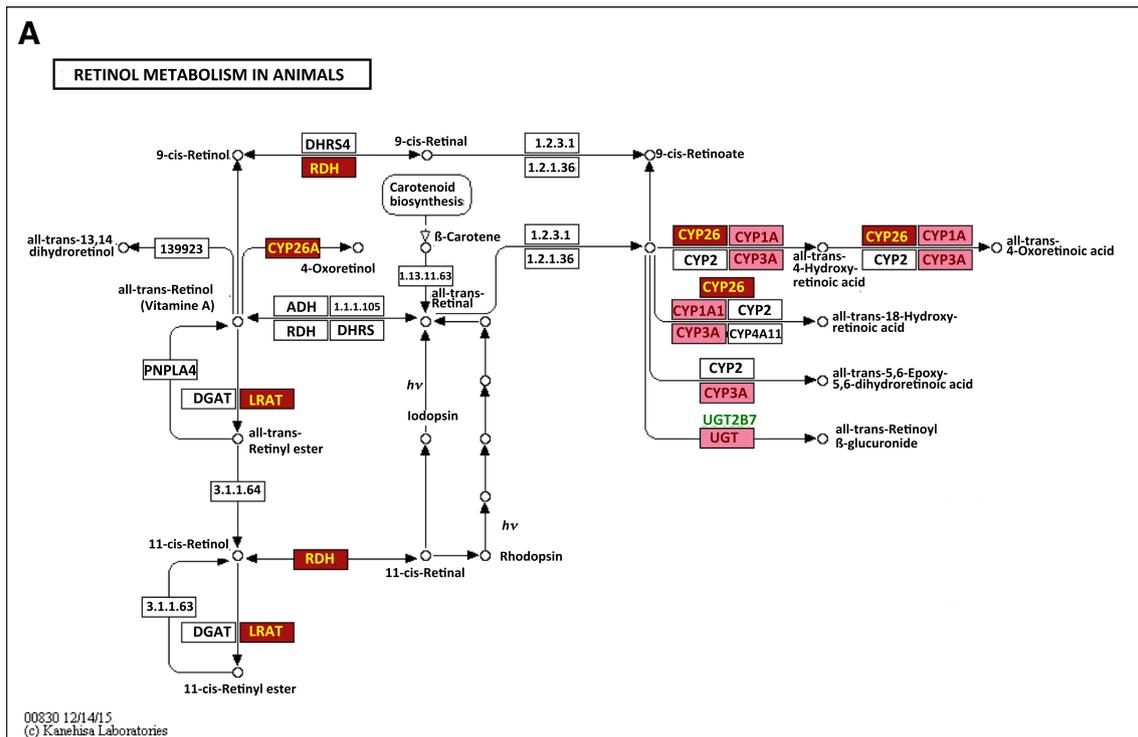
Fig. 4 **a** Kyoto Encyclopedia of Genes and Genomes (KEGG) diagram representing the retinol metabolism pathway. Differentially expressed genes (DEGs) from cluster A are indicated in brown and from cluster B indicated in pink. **b** Expression profiles of DEGs related to retinol metabolism extracted from the microarray dataset. Experimental groups include: 100 nM 9-*cis* retinoic acid from 2 to 5 dpf (9cRA-L), 100 nM all-*trans* retinoic acid from 2 to 5 dpf (atRA-L), 100 nM 9-*cis* retinoic acid from 4 to 5 dpf (9cRA-L), and 100 nM all-*trans* retinoic acid from 4 to 5 dpf (atRA-S). Doted lines (bottom panel) separate identified genes (*rdh1*, *cyp1a*, *ugt1a*) that differentiate between RA isoforms

Fig. 5 Heatmap representing differentially expressed genes (averaged by experimental group) related to different phases of the detoxification pathway and selected as biomarkers of RA isomer exposure. This includes genes of phase I (*cytochrome p450* family), phase II (*ugt* and *sult* families), and transport genes (*abc* transporter family). Colors represent overexpressed (red) and underexpressed (blue) DEGs
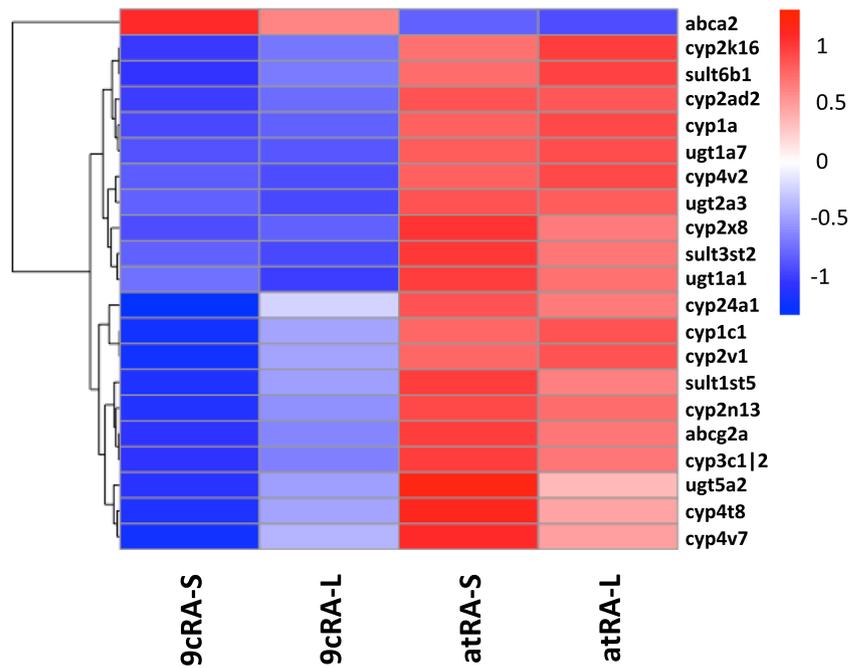
exposure from 96 to 120 hpf is sufficient to elicit all major transcriptomic responses of zebrafish to retinoids.

Aside of the intrinsic interest on elucidating the molecular mechanisms underlying retinoid signalling, our results are particularly relevant for the identification and assessment of putative exogenous retinoids that can enter into the organisms either as diet, as prescription drugs, or as pollutant. There is a growing concern of the presence of the so-called retinoid disrupters, molecules able to alter physiological processes that depend on retinoids, either by altering their physiological levels or by binding to the corresponding receptors. Bisphenol A, brominated flame retardants, or organotin molecules, have been suggested as putative retinoid disrupters, although the effects have not been thoroughly confirmed, at least in vertebrates (Li et al. 2016; Lima et al. 2015; Tabb and Blumberg 2006; Zhao et al. 2015). Even high doses of the natural precursor, vitamin A, have been linked to negative health effects in humans (Blomhoff and Blomhoff 2006; Tanumihardjo et al. 2016). We propose here that expression levels of the CYP26 genes in zebrafish embryos as a sensitive marker of exposure to retinoids, suggesting that examination of these genes can be developed into a bioassay for monitoring retinoid endocrine disruption.

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