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TRANSCRIPTIONAL AND HISTOLOGICAL ALTERATIONS IN GONAD OF ADULT ZEBRAFISH AFTER EXPOSURE TO THE SYNTHETIC PROGESTIN NORGESTREL

Wen-Jun Shi,[a,b] Jian-Liang Zhao,[a,b] Yu-Xia Jiang,[a,b] Guo-Yong Huang,[a,b] You-Sheng Liu,[a,b] Jin-Na Zhang,[a,b] and Guang-Guo Ying[a,b,c,*]

[a]State Key Laboratory of Organic Geochemistry, CAS Research Centre of PRD Environmental Pollution and Control, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou, China
[b]University of Chinese Academy of Sciences, Beijing, China
[c]The Environmental Research Institute, MOE Key Laboratory of Environmental Theoretical Chemistry, South China Normal University, Guangzhou, China

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Abstract: The aim of the present study was to investigate the effects of norgestrel (NGT) on gonadal development in adult zebrafish. Adult zebrafish were exposed to NGT for 14 d at 871 ng L^{-1} for microarray analysis, and a follow-up experiment was conducted to further study the targeted pathway in adult zebrafish after exposure to NGT at 6.7, 83, and 912 ng L^{-1} by quantitative polymerase chain reaction (qPCR) and histological analysis. The microarray analysis revealed that 11 545 transcripts were identified. Gene ontology analysis showed organ development, system development, multicellular organismal development, single-organism developmental process, and developmental process were significantly enriched. A Venn diagram displayed 434 target genes involved in organ development, and these genes were common in these 5 development-related processes. Kyoto Encyclopedia of Genes and Genomes analysis showed that the notch signaling pathway was the top toxicity pathway, and it was selected as the target pathway for further qPCR analysis. The qPCR analysis revealed significant and dose-dependent alterations of most target genes involved in the notch signaling pathway in the gonads, even at an environmentally relevant concentration of 6.7 ng L^{-1}. The transcriptional patterns were consistent with the notch signaling cascade. In addition, NGT significantly increased the frequency of mature sperm and decreased the frequency of immature sperm at all concentrations. Meanwhile, NGT treatment increased the percentage of mature vitellogenic oocytes and atretic follicles at 912 ng L^{-1} but decreased the percentage of immature vitellogenic oocytes. Thus, the present study demonstrated significant developmental toxicity in the gonad of adult zebrafish even at environmentally relevant NGT concentrations. *Environ Toxicol Chem* 2017;36:3267–3276. © 2017 SETAC

Keywords: Norgestrel Microarray Notch signaling pathway Histology Gene transcription Zebrafish

INTRODUCTION

Synthetic progestins are widely used in human and veterinary medicine and finally end up in the aquatic environment via wastewater discharge and agricultural runoff [1–3]. The total consumption of synthetic progestins was 219 kg in Switzerland in 2010 [1]. The consumption of major synthetic progestins had reached 1563 kg in the United Kingdom and 1919 kg in France [2,3]. Meanwhile, progestins such as progesterone (P4), norgestrel (NGT), medroxyprogesterone acetate, and dydrogesterone have been detected in wastewater-treatment plant effluents and surface water at concentrations ranging from a few to several hundred nanograms per liter [1–3]. Environmental progestins have been known to be one of the most active pharmaceutical classes and could interact with target receptors that are highly conserved between species [4]. Recently, there has been growing interest in potential adverse impacts of progestins in aquatic organisms. For instance, several studies have reported the progestins could decrease egg production, alter the transcription of reproduction-related genes, affect sex differentiation, and change gonadal development of fish at environmentally relevant concentrations [5–8]. Moreover, toxicogenomic analyses showed that, besides the reproductive effect, P4 and drospirenone (DRSP) could alter cell cycle regulation and the circadian rhythm pathway in the brain of zebrafish [8].

Norgestrel is one of the frequently used progestins in oral contraceptives, contraceptive implants, intrauterine devices, and vaginal rings and as an active pharmaceutical ingredient for clinical use in humans. For instance, the consumption of NGT in France was 11.92 kg in 2004 [3]. Furthermore, the consumption of levonorgestrel reached 19.59 kg in the United Kingdom in 2006 [2]. Although the consumption was not very high compared with P4, it has been widely detected in the aquatic environment [1,9,10]. Measured concentrations of NGT were up to 22 ng L^{-1} in surface waters. In municipal wastewater-treatment plant effluents, NGT was detected at a concentration of 11 ng L^{-1} [9]. In addition, NGT was found in the receiving surface waters of swine farms at much higher concentrations of up to 465 ng L^{-1} [10]. Furthermore, NGT was also detected in flush waters of swine and dairy cattle farms with relatively high concentrations up to 10 800 to 646 022 ng L^{-1} because it is often used to control the pregnancy time of animals in the livestock industry [10]. Norgestrel is a racemic mixture of 2 optical isomers called enantiomers: levonorgestrel and dextronorgestrel. Furthermore, levonorgestrel is the only bioactive isomer in the racemic mixture NGT. Several studies have reported that NGT showed adverse effects in fish [6,11]. Similar to levonorgestrel, NGT has a strong androgenic activity and can affect sex

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* Address correspondence to guang-guo.ying@gmail.com; guangguo.ying@gig.ac.cn

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differentiation and gonadal development in fish at environmentally relevant concentrations [6]. However, those studies mainly focused on the transcription of some target genes involved in the hypothalamic–pituitary–gonadal (HPG) axis and sex differentiation [5,6,11]. Little attention was paid to the key signaling pathways that regulate gonadal development. Therefore, it is necessary to understand the effects of NGT on the signaling pathways related to gonadal development in fish.

Development-related signaling pathways represent a complex network which contains the notch signaling pathway, the wnt signaling pathway, and the transforming growth factor-β signaling pathway [12–14]. The notch signaling pathway decides the cell fate, such as cell growth, proliferation, and apoptosis, in many different tissues in multicellular organisms [15]. Alteration of the notch signaling pathway affected gonad development, brain development, and embryonic development [16–18]. Moreover, disruption of the notch signaling pathway also disturbed the cell cycle, cell proliferation, and even energy metabolism [19,20]. The wnt and transforming growth factor-β signaling pathways are also key developmental signaling pathways and play a vital role in embryonic and skeletal development [13,21]. However, the effects of environmental NGT on the development-related signaling pathways in aquatic organisms are still not known.

It is the overarching goal in ecotoxicology to predict and then prevent the harmful effects of pollutants in the environment. Traditional techniques fall short of predicting new toxicity because of their overdependence on phenotype observation. Microarray analysis provides an attractive tool for toxicological study. Several studies have reported transcriptomic alteration after exposure to progestins by microarray analysis [8,22]. For instance, microarray analysis was used to investigate the effect of P4 and drospirenone on reproductive development and the circadian rhythm in zebrafish [8,22]. In addition, high-throughput expression studies using microarray are effective for predicting the potential toxic effect and identifying biomarkers [23,24].

The objective of the present study was to investigate the effects of NGT on gonadal development in adult zebrafish at the transcription and histology levels. Adult zebrafish were exposed to NGT for 14 d, followed by microarray analysis and quantitative polymerase chain reaction (qPCR) analysis. The microarray analysis was aimed at identifying target genes involved in the development-related pathways, so a physiological concentration higher than environmentally relevant concentrations was applied to initiate robust molecular responses in short exposure duration. Then the transcription of development-related genes was further validated at environmentally relevant concentrations using qPCR analysis. In addition, the status of gonadal maturity stages was selected as the mark for gonadal development in both adult and juvenile fish [7]. The results can facilitate a better understanding of toxicological effects of NGT in fish.

MATERIALS AND METHODS

Test organisms and test chemical

Juvenile zebrafish (*Danio rerio*) were purchased from a local supplier and acclimated in the laboratory for at least 2 mo prior to the experiment. The fish were held in aerated freshwater (pH 7–8) in flow-through holding tanks and fed with brine shrimp (Tianjin, China) once a day. The water temperature was 26 ± 1 °C, with a 14:10-h light:dark cycle. Norgestrel (Chemical Abstracts Service no. 6533-00-2, purity 100%) was obtained from US Pharmacopeia. The stock solution ($1\ mg\ mL^{-1}$) of NGT was prepared in dimethylsulfoxide (DMSO) and then stored at –20 °C in the dark.

Exposure experiment

To obtain the significant biological pathways by microarray analysis, 4 female zebrafish (5 mo old) per replicate were selected for microarray analysis. Previous studies showed effects on the transcription and histology after 14-d exposure in fish [8,25]. Therefore, adult zebrafish were exposed to NGT for 14 d in 5-L glass tanks with 3 L of charcoal-filtered tap water (pH 7.0–7.4) in each tank. To ensure water quality, the water used in the experiment was aerated (oxygen level ≥75%) and renewed daily. Four replicate tanks were included for each treatment. The experimental setup consisted of the following exposure treatments: solvent control (containing 0.001% [v/v] DMSO) and NGT ($1000\ ng\ L^{-1}$). All treatments were performed in a semistatic system at 26 ± 1 °C with a 14:10-h light:dark cycle. After 14-d exposure, all fish were anesthetized with 0.01% tricaine methane-sulfonate. All fish were dissected. Ovaries from 3 fish per replicate ($n = 4$) were pooled and stored at –80 °C for microarray analysis. Ovaries from the other fish per replicate were stored at −80 °C as backup.

A follow-up experiment was conducted to study the target pathway in zebrafish. Four pairs of fish (5 mo old; 4 female and 4 male) were selected for 14-d exposure. The experimental setup consisted of the following exposure treatments: solvent control (containing 0.001% [v/v] DMSO) and NGT (10, 100, and $1000\ ng\ L^{-1}$; referred to as low, medium, and high concentrations, respectively). The experimental process and conditions were the same as the above experiment with a slight difference. Briefly, each fish was measured for body length (centimeters) and weight (grams) to determine the condition factor. Eight fish (4 female and 4 male) per replicate ($n = 4$) were dissected. According to previous studies [22,25], 1 male and 1 female fish per replicate tank (total of 4 fish/treatment) were randomly taken, and the gonads were fixed in Bouin's solution for 24 h prior to histological analysis. The gonads of 3 female fish and 3 male fish per replicate (total of 12 fish/treatment) were pooled and then transferred to RNAlater. All samples were stored at −80 °C for real-time qPCR analysis.

Chemical analysis

The extraction of water samples and chemical analysis of NGT were described in our previous publication [9]. Briefly, the water samples (100 mL) from replicates ($n = 4$) of the adult zebrafish exposure experiment were randomly collected in 1-L amber glass bottles at 9 d postfertilization from the NGT treatment and solvent control groups at the beginning of exposure (0 h) and prior to water renewal (24 h). The water samples from 2 replicates were pooled at 0 and 24 h (total 200 mL each for solvent control, $10\ ng\ L^{-1}$ NGT, $100\ ng\ L^{-1}$ NGT, and $1000\ ng\ L^{-1}$ NGT). For water samples, about 10 mL of methanol was added to each bottle (total 200 mL). Then, the water samples were filtered through glass fiber filters (Whatman GF/F, 0.7 μm effective pore size). To extract the water samples, CNWBOND LC-C18 solid-phase extraction cartridges (200 mg, 3 mL) were used. The NGT in the exposure solutions was analyzed by an Agilent 1200 series 190 ultrahigh-performance liquid chromatograph (Agilent) coupled to an Agilent 6460 triple quadrupole mass spectrometer. The recovery determined by spiking surface water with known amounts of NGT (5 and $100\ ng\ L^{-1}$) was 87.5 to 96.8% [9]. The limit of detection of NGT was $0.04\ ng\ L^{-1}$ [9].

Microarray labeling and hybridization

Microarray analysis was performed to investigate the global transcription following the exposure of fish to NGT. The ovary samples stored in RNAlater were sent to CapitalBio for microarray analysis. A single-color zebrafish oligo microarray in a 4×44 K slide format (Agilent; design ID 026437) was used in the microarray experiments. Samples were labeled with a fluorescent dye (Cy5 and Cy3-dCTP) by using the CapitalBio cRNA Amplification and Labeling Kit (CapitalBio) for producing yields of labeled cDNA. Hybridization was performed at 42 °C overnight in an Agilent hybridization oven with a rotation speed of 20 rpm. The arrays were washed and then scanned by Agilent G2565CA Microarray Scanner (Agilent) [26,27].

Quantitative real-time PCR assay

The qPCR analysis was used to validate the microarray data and further study the target pathway at environmentally relevant concentrations. The ovaries of 3 females per replicate ($n = 4$) were pooled as a sample. Similarly, the testis of 3 males per replicate ($n = 4$) were pooled as a sample. Total RNA extraction, reverse-transcription of RNA into complementary DNA (cDNA), and quantitative real-time PCR were performed as described [6]. Briefly, total RNA was extracted from the gonad samples by using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The quality of total RNA was assessed by gel electrophoresis. Concentrations of RNA were analyzed and quantified with absorbance measurements at 260 and 280 nm by using a SmartSpec™ Plus Spectrophotometer (Bio-Rad). The OD260/OD280 ratio was calculated for the purity of the RNA sample (1.83–2.03 for all RNA samples). Synthesis of cDNA was carried out from 500 ng of total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo) in a total volume of 50 μL following the manufacturer's protocol. The first-strand cDNA was diluted with nuclease-free water to 40 ng μL^{-1} and then stored at −20 °C for subsequent qPCR analysis.

The qPCR analysis was conducted on the Applied Biosystems ViiATM 7 Dx (ABI) using the THUNDERBIRD SYBR® qPCR Mix (Toyobo). The final volume of 20 μL SYBR reaction mixture contains 10 μL of THUNDERBIRD SYBR qPCR Mix, 6.7 μL of nuclease-free water, 2.5 μL of cDNA sample, and 0.4 μL of specific primers. All primers were designed (batch primer 3) and synthetized by Biotechnology. The length of primers was usually 18 to 20 base pairs. The product size of primer was 70 to 200 base pairs. The GC content of primers was kept within 40 to 60%. Melting temperature (T_m) was kept as close as possible to 60 °C. The last 5 bases at the 3 prime ends should avoid 3 continuous nucleotides, especially for G and C. Finally, primer pairs were selected with a minimal number of potential primer dimers and primer hairpins as possible. Primer-BLAST was employed to check the specificity of the primers. Respective primer details are shown in Supplemental Data, Table S1. The amplification efficiencies of all primers were between 95 and 105%. The qPCR amplification was as follows: denaturation program 1 min at 95 °C, and 40 cycles of 95 °C (15 s) and 60 °C (60 s). To ensure specificity of the primers, a melting curve analysis (60–95 °C) was completed on each sample at the end of qPCRs. According to previous studies [6,11], ribosomal protein L (RpL13α), elongation factor 1 alpha (EF1-α), and β-actin were selected as housekeeping genes for normalization. The average threshold cycle value of the 3 reference genes was employed as reference to normalize the expression of messenger RNA (mRNA) for the target genes. Melt curves were used to validate the specificity of PCR products. Relative mRNA expression was determined by the ΔΔCT method [28].

Histology

After anesthesia, 1 male and 1 female fish per replicate tank (total of $n = 4$/treatment) were randomly taken for histological examination. Histological examination was performed for gonad morphology after the exposure. Histological preparation was performed as described by Silva et al. [29]. Briefly, gonad samples were dehydrated through a graded series of ethanol solutions, and after dehydration the samples were embedded in paraffin. Three longitudinal sections (4 μm) from different regions of the ovary and testis were examined in each individual. In total, 12 sections were stained by the hematoxylin–eosin protocol and examined using a Nikon Eclipse 50i light microscope (Nikon) to check the histological alteration in the gonad. The maturation stages of gonads were evaluated according to the guideline [30]. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals of the National Institute for Food and Drug Control of China.

Data analysis and statistics

GeneSpring software Ver 13 (Agilent) was used to analyze the microarray data for summarization and normalization. Differentially expressed genes (DEGs) were selected on the basis of their threshold values (≥2) compared with the solvent control group [26,27]. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to aid interpretation of the DEGs and determine significant gene ontology (GO) categories and biological pathways [31]. The significantly changed biological pathways and GO categories were identified based on the Benjamini-Hochberg–corrected p value calculated by the false discovery rate (= 0.05). A scatterplot was used to display the upregulated and downregulated genes after normalization.

Hierarchical clustering maps were constructed using Cluster Ver 3.0. Statistical analyses of the qPCR data and ovary and testis stages (histology) were performed in SPSS, Ver 13.0. The Kolmogorov-Smirnov and Levene tests were used to test for normality and homogeneity of variances, respectively. If necessary, raw data were transformed to meet the parametric tests. The significance of differences between the solvent control and NGT exposure groups in transcript levels, condition factors, and ovary and testis stages (histology) were analyzed by one-way analysis of variance, followed by Tukey's multiple comparison tests. Data were considered significantly different at $p < 0.05$.

RESULTS

Measured NGT exposure concentrations

The measured concentrations of NGT at the beginning of exposure (T_0) and prior to water renewal (T_{24}) are given in Table 1. The measured concentrations of NGT were close to the nominal concentration at T_0, whereas they were decreased at T_{24}, with measured concentrations being 25 to 57% lower than nominal concentrations (Table 1). The mean measured concentrations of NGT for the 3 nominal concentrations (10, 100, and 1000 ng/L) were 6.75, 83.1, and 912 ng L^{-1}, respectively. In the present study, the exposure solutions were renewed every day, with 24 h as a cycle. The

Table 1. Nominal and measured concentrations of norgestrel in adult zebrafish exposure experiments

	Measured concentration ($ng\,L^{-1}$)		
Nominal concentration	T_0 [a]	T_{24} [a]	Average
$10\,ng\,L^{-1}$	9.25[b]	4.20	6.75
$100\,ng\,L^{-1}$	95.1	71.1	83.1
$1000\,ng\,L^{-1}$	1030	793	912
$1000\,ng\,L^{-1}$	988[c]	755[c]	871[c]

[a]Exposure time (0 and 24 h).
[b]Measured concentrations are given as mean ($n = 2$ replicates).
[c]Measured concentrations in the microarray experiment.

experimental process and conditions were the same during exposure. Therefore, the concentration detected at 9 d postfertilization could represent other days. The measured concentrations of NGT from 2 samples also could represent the mean exposure concentration. In addition, previous studies have shown similar decreases in nominal concentrations for NGT, dydrogesterone, medroxyprogesterone acetate, P4, and drospirenone in adult exposure [6–8], while the concentrations of NGT, dydrogesterone, and medroxyprogesterone acetate did not decrease in embryo exposure [7,11]. The present results demonstrated that adult fish could accelerate the chemical decrease. A previous study further showed that the apparatus, sorption to tubing and fish debris, could also affect the exposure concentration [7].

Global gene transcription analysis

There were a large number of DEGs after exposure to NGT (Figure 1A,B). Statistical analysis revealed that exposure to NGT caused the differential expression of 11 545 genes (6332 upregulated and 5213 down-regulated; Figure 1B). A complete list of DEGs can be found in Supplemental Data, Table S1.

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis identified 1 predicted signaling pathway (notch signaling pathway) as particularly enriched among the selection of DEGs (Table 2; Supplemental Data, Table S2A). The GO functional enrichment analysis showed that many predicted biological processes and molecular functions were identified based on the DEGs. A total of 14 GOs were identified as significant (Table 3; Supplemental Data, Table S2B). The majority of the GOs were associated with general development processes (Table 3). General development processes affected in this group included organ development, system development, single-organism developmental process, and developmental process ($p < 0.05$). A number of DEGs associated with general development processes were clearly enriched by episodic exposure to $871\,ng\,L^{-1}$ NGT. As shown in the Venn diagram (Figure 1C,D), the overlapping regions indicate that 434 DEGs were common in all 5 development-related processes (Supplemental Data, Table S1C).

We used KEGG to identify predicted signaling pathways by using the overlapping genes (434 genes). Six signaling pathways associated with development were identified as being enriched, including the notch signaling pathway, the wnt signaling pathway, the TGF-beta signaling pathway, melanogenesis, the VEGF signaling pathway, and the ErbB signaling pathway (Figure 2A). It is interesting that the top toxicity pathway was the notch signaling pathway (Figure 2A). Therefore, the notch signaling pathway was selected as the target pathway for further study based on the GO and KEGG analyses. The mode of the notch signaling pathway is depicted

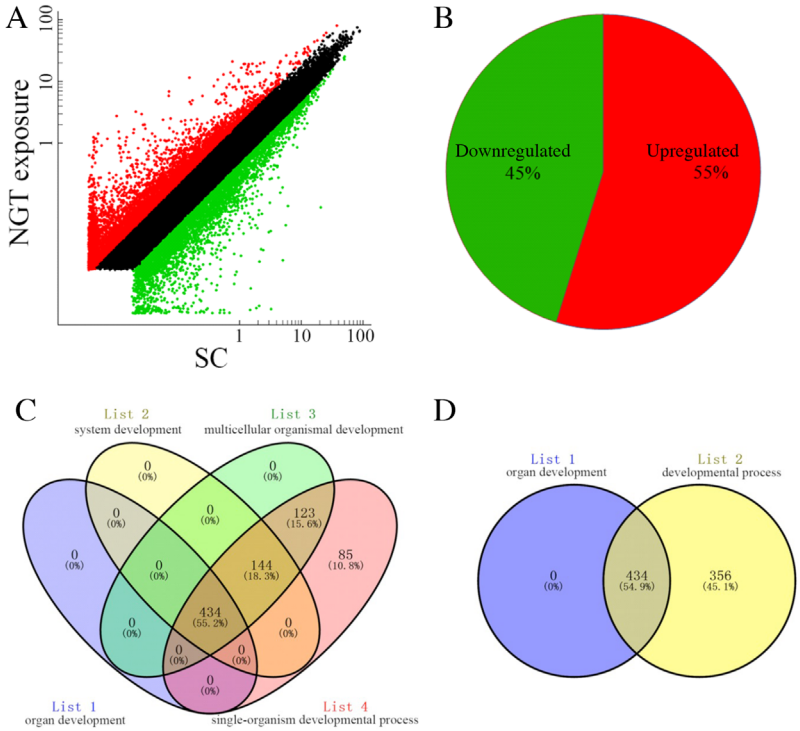

Figure 1. Gene transcription profiling and functional genomic analysis. (**A**,**B**) Global expression profile of zebrafish exposed to norgestrel (NGT) at $871\,ng\,L^{-1}$. Transcriptions of 11 545 genes were altered, and 55% of genes were up-regulated compared with the solvent control. Fold-change cutoff was set at ≥2. Red = upregulated; green = downregulated. (**C**,**D**) Venn diagrams representing the overlap of gene sets in zebrafish following a 14-d exposure to $871\,ng\,L^{-1}$ NGT. There were 434 enriched genes that were altered in the 5 general development processes after NGT exposure. Fold-change cutoff was also set at ≥2. List: cluster of differentially expressed genes involved in gene ontology biological processes. SC = solvent control.

Table 2. Enrichment of the top 10 signaling pathways in females exposed to norgestrel compared with the solvent control

Signaling pathway	*p*	DEG number	%
Notch signaling pathway	0.021276272*	34	58.6
Purine metabolism	0.086932401	82	44.3
Torpedoed backbone biosynthesis	0.098396104	13	61.9
Lysine degradation	0.110921998	31	49.2
Fatty acid metabolism	0.112300677	28	50
Fatty acid degradation	0.112696676	22	52.4
RNA polymerase	0.125934124	16	55.2
Fatty acid biosynthesis	0.133256806	10	62.5
Pyrimidine metabolism	0.136090034	47	45.2
Spliceosome	0.153284143	58	43.6

*Significant enrichment ($p < 0.05$).
DEG = differentially expressed gene.

in Figure 2B. A total of 20 key genes involved in the notch signaling pathway were detected at different concentrations of NGT.

Validation of target genes by qPCR in the ovary

Real-time qPCR analysis was used to validate the data obtained from microarray analysis. Besides the target genes involved in notch signaling, several DEGs altered at a different threshold were also corrected (Figure 3; Supplemental Data, Figure S2). The upregulation of DEGs includes apolipoprotein (*apoa1a*, *apoa1b*, and *apoa4b*); uncoupling protein 1 (*ucp1*); vitellogenin 1 (*vtg1*); cytochrome P450, family 2, subfamily K; polypeptide 22 (*cyp2k22*); prostate transmembrane protein, androgen-induced 1 (*pmepa1*); MCF.2 cell line derived transforming sequence a (*mcf2a*); LIM domain binding 3a (*ldb3a*); and disheveled-binding antagonist of beta-catenin 2 (*dact2*). The downregulation of DEGs includes sulfotransferase family 1, cytosolic sulfotransferase 1 (*sult1st1*); insulin-like 3 (*insl3*); translocase of inner mitochondrial membrane 17 homolog B (yeast, *timm17b*); synovial sarcoma translocation gene on chromosome 18-like 2 (*ss18l2*); notch-regulated ankyrin repeat protein b (*nrarpb*); tetraspanin 15 (*tspan15*); adaptor-related protein complex 1, sigma 3, subunit a (*ap1s3a*); cathepsin Ba (*ctsba*); and major histocompatibility complex class I UFA (*mhc1ufa*). The differences among microarray and qPCR data occurred always in the same direction except for *dll4* and *notch3*. The fold-changes of 21 target genes measured by qPCR and microarray were very close (Figure 3; Supplemental Data, Figure S2A,B). However, there are some differences between the data of microarray and qPCR. Transcriptional alterations of *kat2a*, *kat2b*, *rbpjb*, *dvl2*, *psen1*, *ucp1*, and *insl3* (Supplemental Data, Figure S2C) were much stronger as determined by qPCR than by microarray, while the transcriptional changes of *dtx1*, *vtg1*, *timm17b*, *sult1st*, *apoa4b*, *dact2*, *ctsba*, and *mhc1ufa* were much weaker (Supplemental Data, Figure S2D).

Transcriptional alterations in notch signaling pathway

Heatmap analysis revealed a significant distinction of transcriptional response between female and male fish, whereas a similar level occurred between the high and intermediate doses and between the low dose and control in both ovary and testis. This is indicated by the distance and the linkage between the clusters in the hierarchical clustering analysis (Figure 4A). The qPCR analysis showed that NGT exposure also significantly affected the transcription of target genes involved in the notch signaling pathway at environmentally relevant concentrations in female and male zebrafish. As shown in Figure 4 and Supplemental Data, Figure S3, NGT exposure upregulated the expression of *notch1*a, *dvl2*, *dtx1*, *dtx2*, *ncor2*, *kat2a*, *kat2b*, *rbpjb*, and *ctbp1* mRNA with a dose-related increase in females and males. The gene transcription of *notch1a*, *dtx1*, *ncstn*, and *ncor2* was significantly upregulated at all concentrations in both females and males. Moreover, transcriptional variations of several other target genes (*kat2a*, *kat2b*, *her6*, and *notch3*) were observed at $6.7\,ng\,L^{-1}$ in females and males. Significant changes of gene transcription of *psen1*, *kat2b*, *tle3a*, and *her6* were observed in females at most exposure concentrations, whereas the transcription of these 4 target genes was slightly altered in males.

Histological alterations in gonads

To study the histopathological changes in fish gonad after 14-d exposure to NGT, the percentages of ovaries and testis at each stage were determined. Typical histological sections of

Table 3. Significant enrichment of gene ontology biological processes in females exposed to norgestrel compared with the solvent control

GO category	*p*	DEG number	%
Organ development	0.02470951*	434	42.9
Movement of cell or subcellular component	0.027853422*	161	46.1
System development	0.033083243*	582	61.2
Retinal ganglion cell axon guidance	0.039607791*	18	76.2
Smoothened signaling pathway	0.039708203*	20	41.9
Cell motility	0.040505211*	112	54.3
Localization of cell	0.040505211*	112	69.2
Multicellular organismal development	0.040538262*	706	66.7
Single-organism developmental process	0.041885689*	794	47.1
Cell migration	0.04304192*	107	47.1
Developmental process	0.043976934*	798	41.4
Renal system development	0.044464059*	44	41.1
Inner ear development	0.046172077*	47	49.1
Anatomical structure formation involved in morphogenesis	0.047394185*	195	47.1

*Significant enrichment ($p < 0.05$).
GO = gene ontology; DEG = differentially expressed gene.

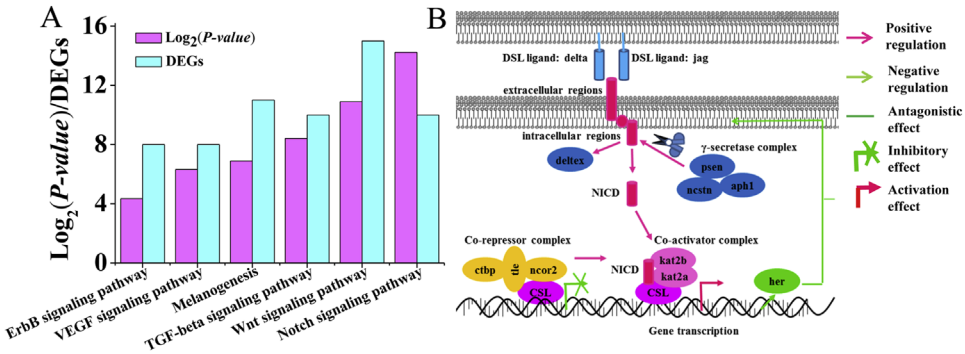

Figure 2. Toxicity pathways associated with the overlapping genes. (**A**) Significantly enriched signaling pathways by Kyoto Encyclopedia of Genes and Genomes analysis. The notch signaling pathway was enriched as the top toxicity pathway. (**B**) Schematic illustration of the notch signaling pathway supported by the current knowledge. The notch signaling pathway is an evolutionarily conserved, intercellular signaling mechanism essential for development of invertebrate and vertebrate species. Notwithstanding the complexity of the developmental action of notch, some general principles underlying the action of this fundamental cell-interaction mechanism have emerged. Canonical notch signaling involves activation of the notch receptor (*notch1a*, *notch 1b*, and *notch3*) at the cell surface by ligands of the DSL family, including delta (*dlb*) and serrate/jagged (*jag2b*). The γ-secretase complex (*ncstn* and *psen1*) cleaves the intracellular regions of notch to release the notch intracellular domain (NICD). After binding to the NICD, the CSL (*rbpjb*) regulates gene transcription by combining with a coactivator (*kat2a* and *kat 2b*), resulting in modulating expression of downstream genes. Regulatory factors (*numb* and *dvl*) also play an important role in controlling the steady-state levels of the notch receptors at the cell surface [15,42]. DEG = differentially expressed gene; TGF = transforming growth factor; VEGF = vascular endothelial growth factor.

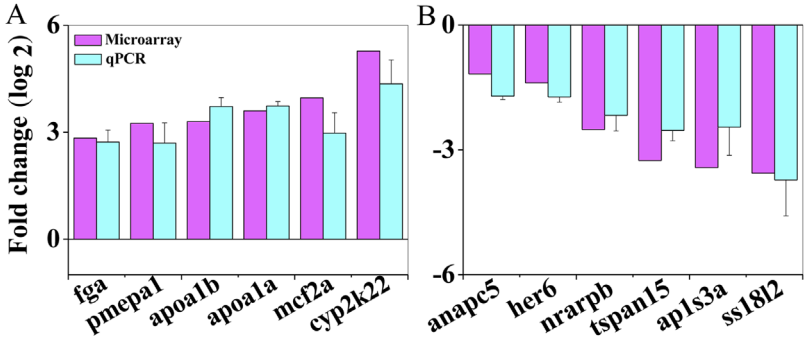

Figure 3. Confirmation of the microarray data with quantitative polymerase chain reaction (qPCR). (**A**,**B**) Confirmation of the differentially expressed genes at a different threshold by qPCR analysis. Fold-changes ($\log_2$) of qPCR and microarray compared to solvent controls was determined by the $2^{-\Delta\Delta CT}$ method and ratio of array spot intensity, respectively.

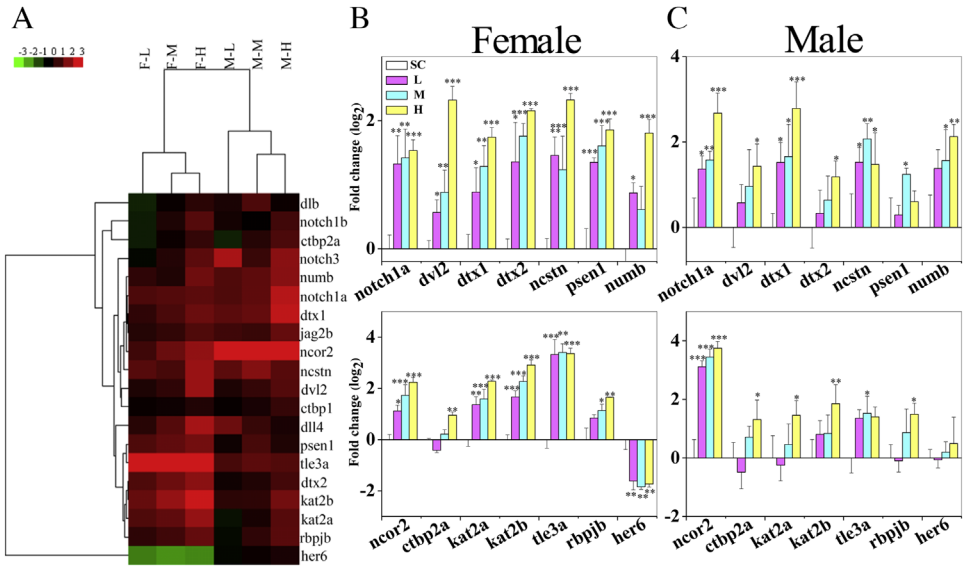

Figure 4. Transcriptional responses of target genes in the notch signaling pathway in the gonad. (**A**) Hierarchical clustering analysis of quantitative polymerase chain reaction (qPCR) results in females and males. (**B**) Transcriptional alteration of target genes involved in the notch signaling pathway at environmentally relevant concentrations in the gonads. Data are shown as mean ± standard deviation of 4 biological replicates. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. F- = female; M- = male; H = high dose (912 ng L^{-1}); L = low dose (6.7 ng L^{-1}); M = medium dose (83 ng L^{-1}); SC = solvent control.

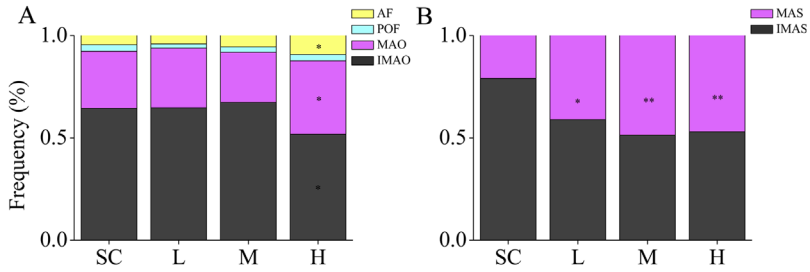

Figure 5. Effects of norgestrel (NGT) on gonadal development in zebrafish. (**A**) Histological change of ovaries induced by NGT. Each bar from top to bottom indicates atretic follicles, postovulatory follicles, mature vitellogenic oocytes, immature vitellogenic oocytes. Data are shown as mean values of 4 replicates. Asterisks indicate statistically significant differences between the solvent control and the exposure groups: $^{*}p < 0.05$ and $^{**}p < 0.01$. (**B**) Histological change of testis induced by NGT. Each bar from top to bottom indicates mature sperm and immature sperm. Data are shown as mean values of 4 replicates. Asterisks indicate statistically significant differences between the solvent control and the exposure groups: $^{*}p < 0.05$ and $^{**}p < 0.01$. AF = atretic follicle; IMAO = immature vitellogenic oocyte; IMAS = immature sperm; MAO = mature vitellogenic oocyte; MAS = mature sperm; POF = postovulatory follicle; H = high dose (912 ng L^{-1}); L = low dose (6.75 ng L^{-1}); M = medium dose (83.1 ng L^{-1}); SC = solvent control.

gonads are shown in the Supplemental Data (Figures S4 and S5). In the ovaries, no significant difference was observed at the lower concentrations (6.7 and 83 ng L^{-1} NGT). However, the percentages of immature vitellogenic oocytes were significantly lower after exposure to 912 ng L^{-1} NGT, whereas significantly increased frequencies of mature vitellogenic oocytes and atretic follicles were observed at 912 ng L^{-1}. In the testis, a higher percentage of mature sperm and a lower percentage of immature sperm compared with the solvent control group were observed at all NGT exposure concentrations (Figure 5). For fish growth, NGT exposure significantly increased the total body length at the high concentration in females, whereas no significant differences in wet weight and condition factor (K factor) in adult fish were observed between the solvent control and treatment groups (Supplemental Data, Figure S1).

DISCUSSION

In the present study, we evaluated the transcriptomic effects of NGT in the ovary and found that NGT exposure could affect the transcription of target genes involved in development pathways and cause histological alteration at environmentally relevant concentrations. Microarray analysis clearly identified a number of DEGs associated with general development processes, which are involved in particular in organ development, system development, multicellular organismal development, and single-organism developmental processes (Figure 1). The microarray results from the present study were consistent with the reported transcriptomic changes in zebrafish brain and ovary after P4 and drospirenone exposure in a previous study [8,22], which identified single-organism developmental processes and cellular processes in the ovary and brain. Therefore, the present microarray results predicted the potential effects of NGT on normal development in zebrafish at the transcriptomic level. Interestingly, HPG axis–related pathways were not significantly identified after P4, drospirenone, and NGT exposure in adult zebrafish by microarray analysis [8,22]. However, exposure to P4 and NGT altered transcription of HPG axis–related genes in juvenile fish and embryos/larvae [6,7,11]. These results showed that the transcription of HPG axis–related genes was more sensitive in juvenile fish than in adult fish for P4, drospirenone, and NGT exposure.

Further data analysis indicated that 434 DEGs were common in all general development processes (Figure 1). The overlapping DEGs involved in several development processes are important for the development of zebrafish. The present study showed that overlapping DEGs were all from organ development, which was identified as the top GO (Table 3). Several previous studies showed the effect of progestins on different organs in zebrafish, especially the gonad [5,7,8,32]. For instance, progestins could cause gonadal change at physiological, histological, and biochemical levels at environmentally relevant concentrations [5,7,8,32]. Furthermore, development-related processes, the circadian rhythm network, and the phototransduction cascade were significantly enriched in brain of adult zebrafish after progestin exposure; and transcriptional alterations of the circadian rhythm network were observed in the eyes of adult zebrafish [22,33]. Taken together, progestins had extensive effects on different organs of adult zebrafish. However, different progestins had diverse effects on the organs of zebrafish. Mainly P4 affected ion homeostasis and regulation of insulin secretion–related biological processes in the ovary [8]. In the present study, similar pathways were also enriched, although this was not significant. The present microarray results showed that NGT primarily influenced development-related processes in the ovary. The degree of effect on sex differentiation and histological alteration in the gonad induced by P4 and NGT could explain the major difference between NGT and P4. Exposure to NGT could alter sex differentiation and gonadal development in juvenile zebrafish, whereas P4 had a slight effect on sex differentiation [5,6,32]. These studies presented important insights for us into the effect of NGT exposure on gonadal development in zebrafish. Thus, although we did not detect an effect on testis at the transcriptomic level, the results from the present study did suggest that NGT exposure could affect gonadal development in adult zebrafish.

The KEGG analysis showed that 6 specific pathways associated with development were enriched by using these 434 overlapping DEGs (Figure 2). This analysis was conducted to evaluate the type of developmental toxicity pathway that was affected by NGT exposure. It is interesting that the notch signaling pathway was identified as the top developmental toxicity pathway, which was consistent with the KEGG analysis using all the DEGs (Table 2 and Figure 2).

The notch signaling pathway is a vitally important pathway for fish because it modulates many cell processes, including cell differentiation, cell fate, apoptosis, and cell growth [15]. Dysregulated notch signaling pathway would cause a variety of disorders, including overdifferentiation and tissue damage [16–19,34]. Previous studies reported that alteration of the notch signaling pathway could affect organ development and embryonic development [16–18]. Moreover, the notch signaling pathway was enriched after embryonic exposure to

road salt in Atlantic salmon at the eyed egg stage [24]. These results suggest that the notch signaling pathway is a potential target pathway for response to developmental toxicity.

Besides the target genes involved in the notch signaling pathway, microarray analysis also identified several important DEGs in other pathways (Figure 3; Supplemental Data, Figure S2). For instance, target genes belonging to steroid metabolic processes were upregulated by microarray analysis, such as *apoa1a*, *apoa1b*, *apoa4b*, *cyp2k22*, and *pmepa1*, while these genes were not found in microarray data after P4 and drospirenone exposure. The expression of *pmepa1* was regulated by androgen [35]. Overexpression of *pampa1* implied that NGT might increase the level of androgen in the ovary, which is consistent with the androgenic effect of NGT. These results predicted that NGT exposure might have a strong effect on steroid metabolism in adult zebrafish. Furthermore, the transcription of several target genes involved in negative regulation was suppressed after NGT exposure, such as *nrarpb* and *insl3*. The *nrarpb* gene participates in negative regulation of the notch signaling pathway [36]. Low expression of *nrarpb* indicated activated notch signaling. Similarly, *insl3* displays antiapoptotic effects on meiotic germ cells in mammals [37]. Thus, low expression of *insl3* might induce apoptosis. These results predicted that NGT exposure might activate the notch signaling pathway and induce apoptosis, which is consistent with the present study.

However, differences among microarray and qPCR data occurred (Supplemental Data, Figure S2C,D). The main difference was centered on the fold-change of target genes. There could be several reasons for the difference between the microarray and qPCR data. Firstly, the analytical methods fundamentally differ between microarray analysis and qPCR [38]. Global normalization is required for microarray analysis, while several housekeeping genes are applied to qPCR for normalization. Analytical methods could influence the correlation between microarrays and qPCR [38]. Moreover, array spot intensity and the cycle threshold value are used to calculate the fold-change for microarray and qPCR compared with solvent control, respectively. A previous study showed the effects of array spot intensity on correlation and found that low-intensity spots had considerably lower correlations with qPCR data than high-intensity spots [39]. In the present study, several genes with a high fold-change displayed low intensity (such as *bact2* and *mhc1ufa*). Secondly, enough replicates were not conducted on the microarray experiment in the present study. Though pooling could obtain sufficient mRNA amounts for microarray, individual differences in the zebrafish would exist in microarray results. Regardless, the aim of the microarray analysis was to predict the effect at the transcriptome level and assist studying the target pathways in further investigation.

We further detected some key genes involved in the notch signaling pathway at environmentally relevant concentrations based on the microarray analysis results. Significant upregulation of target genes (*notch1a*, *dlv2*, *dtx1*, *dtx2*, *ncstn*, *psen1*, *ncor2*, *kat2a*, *kat2b*, and *tle3a*) was observed in females and males (Figure 4). These target genes play vital roles in the notch signaling pathway [19]. Firstly, *notch* encoded a key receptor containing extracellular regions and intracellular regions. Canonical notch signaling involves activation of the notch receptor at the cell surface by ligands of the DSL family, which includes Delta (*dlb*) and Serrate/Jagged (*jag2b*). The γ-secretase complex (*ncstn* and *psen1*) cleaves the intracellular regions of notch to release the notch intracellular domain, which is translocated to the nucleus [12,15]. After binding to the notch intracellular domain, the recombining binding protein suppressor of hairless (CSL, *rbpjb*) regulates gene transcription by combining with a coactivator (*kat2a* and *kat2b*), resulting in modulation of cell processes [12,15]. Regulatory factors encoded by *numb* and *dvl* also play an important role to control the steady-state levels of the notch receptors at the cell surface (Figure 2). Overexpression of the target genes could be explained by the notch signaling cascade (Figure 2B). These results revealed that increased transcription of these target genes (*notch1a*, *notcha1b*, *dlb*, *jag2b*, *kat2a*, *kat2b*, and *rbpjb*) indicated that notch signaling was activated and translocated to the nucleus.

The aim of the present study was not focused on the specific mechanism of these activations. However, the transcriptional alterations of several target genes are interesting after NGT exposure. Firstly, in the absence of notch activation, CSL proteins typically act as transcriptional repressors of transcription factors in concert with corepressor complexes (*tle3a*, *ncor2*, *ctbp1*, and *ctbp2a*). But, in the present study, the increased transcription of *notch*, *tle3a*, *ncor2*, and *ctbp1* was observed in females and males after NGT exposure. These results could be explained by the fact that CSL overexpression strongly enhances the corepressor complexes (*tle3a*, *ncor2*, *ctbp1*, and *ctbp2a*) [40,41]. In addition, the hairy-related proteins encoded by *her* are antagonists of notch [12,42]. Notch was responsive to changes in hairy-related proteins [12,42]. Similarly, *nrarpb* participates in negative regulation of the notch signaling pathway [36]. In the present study, low expression of *her* and *nrarpb* was consistent with overexpression of *notch*. These results further indicated that the transcriptional changes of target genes were consistent with the notch signaling cascade [12,42]. It is known that stable notch signaling is very important to control cell development and maintain homeostasis of mature tissues in the adult organism [15]. Based on this, the upregulation of these key genes involved in the notch signaling pathway may disturb normal cell development in gonads. Previous studies reported that imbalance of notch signaling leads to an erroneous cell cycle [15,19]. Moreover, growth hormone promoted oocyte maturation via activation of the notch signaling pathway in ovarian tissue [34]. Thus, we hypothesize that increased notch signaling alters gonadal maturity stages.

It is interesting that alterations of gonadal maturity stages were observed in females and males after NGT exposure (Figure 5). To better show the frequencies of gonadal development cells, the gonadal cells from mature and immature stages were counted. Generally, the mature vitellogenic oocyte is characterized by an increased accumulation of vitellogenic granules that displace the cortical alveolar material to the periphery of the cytoplasm [30]. It is easy to identify the mature vitellogenic oocyte compared to the immature vitellogenic oocyte. For instance, the phase of cortical alveolar oocytes belonging to immature stages is characterized by the appearance of cortical alveoli (yolk vesicles) within the ooplasm. In the testis, it is easier to distinguish between mature sperm and immature sperm. Mature sperm have dark, round nuclei and exist as scattered individual cells within the tubular lumen [30]. In the present study, there was a higher proportion of maturity stages in both ovaries and testis. Moreover, NGT decreased the frequency of immature vitellogenic oocytes at high concentrations. The alterations of ovary in adult zebrafish ovaries were consistent with a previous study with dydrogesterone exposure [7]. The

increased frequency of mature vitellogenic oocytes was also observed in fathead minnows after levonorgestrel exposure [43]. In the testis, the change of mature sperm was similar to that of previous reports on levonorgestrel, dydrogesterone, and drospirenone [7,8]. The increased frequency of mature sperm suggested a potential androgenic activity. Some recent studies have indicated that levonorgestrel and NGT had strong androgenic activity and induced a male phenotype in fish at environmentally relevant concentrations [5,6,32]. Thus, it is not surprising that NGT exposure increased a proportion of mature sperm at all concentrations in adult zebrafish. In addition, NGT treatment increased the frequency of atretic follicles at the high concentration in the present study. Ovarian follicle atresia is an apoptotic process leading to reabsorption of maturing oocytes rather than ovulation [44]. Various environmental stressors, such as progestins, antiprogestin, and bisphenol-A, have been shown to increase atresia [7,8,44,45]. These results revealed that the atretic follicles in fish gonad were sensitive to exposure to endocrine-disrupting chemicals, and the frequency of atretic follicles may be a potential histological marker of endocrine disruption in fish. In a word, these results revealed that NGT exposure could affect gonadal development at the histological level.

The gonadal maturity stages represent a cell differentiation process in gonads, whereas ovarian follicle atresia is an apoptotic process in ovaries [15,43]. It is known that the notch signaling pathway controls the cell fate, including cell differentiation and cell apoptosis [15]. Consequently, we proposed a hypothesis that an activated notch signaling pathway leads to a lower percentage of immature stages and a higher percentage of mature stages in the gonad. Moreover, inordinate notch signaling might cause cell apoptosis, resulting in a high frequency of atretic follicles. The low expression of *inl3* supported this hypothesis. Previous studies provided evidence for our hypothesis [15,16,34]. For instance, growth hormone promoted oocyte maturation via activation of the notch signaling pathway in ovarian tissue [34]. The notch pathway was activated during spermatogenesis in the mouse [16]. However, the notch signaling pathway is so complex and has broad biological functions by regulating downstream signaling pathways [20,46]; thus, the specific mechanism requires further investigation. In addition, microarray analysis predicted that NGT exposure would affect the transcription of a large number of DEGs. Because of space limitations, the present study mainly focused on the notch signaling pathway and did not investigate the other signaling pathways, such as the wnt signaling pathway and membrane protein–related pathways (*tspan15*, *ap1s3a*, *dact2*, and *timm17b*). However, microarray analysis supported important insights for further study.

CONCLUSION

Large transcriptional changes of genes associated with general development processes were observed in adult zebrafish after NGT exposure at the high dose according to microarray analysis. The KEGG analysis found that the notch signaling pathway was the top toxicity pathway. Exposure to NGT significantly induced histological changes in fish, which were accompanied by the transcriptional regulation of target genes involved in the notch signaling pathway. Activation of the notch signaling pathway was suggested to be the mechanism that led to increasing mature stages and decreasing immature stages in gonads of adult fish. Future studies are needed to understand the mechanisms behind various toxicological effects of NGT in fish.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3894.

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Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (guangguo.ying@gig.ac.cn).

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