Characterization of a bystander effect induced by the endocrine-disrupting chemical 6-propyl-2-thiouracil in zebrafish embryos

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ABSTRACT

This study was conducted to evaluate possible bystander effects induced by the model chemical 6-propyl-2-thiouracil (PTU) on melanin synthesis. Zebrafish (*Danio rerio*) embryos were treated with PTU by either microinjection exposure, via waterborne exposure or indirectly through bystander exposure. Melanin content, related mRNA and protein expression were examined at the end of exposure (36 h post-fertilization). Direct exposure to PTU decreased the melanin content, up-regulated mRNA expressions of oculocutaneous albinism type 2 (*OCA2*), tyrosinase (*TYR*), dopachrometautomerase (*DCT*), tyrosinase-related protein 1 (*TYRP1*) and silver (*SILV*), and increased the protein expressions of TYR and SILV. Bystander exposure also up-regulated mRNA and protein expressions of TYR and SILV but increased melanin contents. Correlation analysis demonstrated that mRNA expressions of *OCA2*, *TYR*, *DCT*, *TYRP1*, *SILV* and protein expressions of TYR and SILV in bystander exposure groups were positively correlated with corresponding expressions in microinjection exposure groups. The results might have environmental implications and highlight the need to consider the bystander effects when assessing potential risks of endocrine-disrupting chemicals.

1. Introduction

Some chemicals can modulate the endocrine system either directly as hormone mimics or indirectly by affecting various signal transduction pathways or enzyme systems (Gracia et al., 2006; Solomon et al., 2008; Zhang et al., 2005, 2008a,b). Such chemicals are now commonly referred to as endocrine-disrupting compounds (EDCs). There have been reports of abnormalities in the endocrine systems of wildlife and humans that have been attributed to exposure to EDCs (Guillette and Gunderson, 2001; Rasier et al., 2006), which made US unveil new law that required Environmental Protection Agency (EPA) to develop and validate test systems to identify the EDCs in food and water (EDSP; U.S. EPA, 2006).

In fish, the endocrine system contains a number of hormones and signal transduction pathways as well as hundreds of pheromones. Intra-specific communication by means of pheromones, behavior and some metabolic products is well accepted in fish (Bergman and Moore, 2003; Giaquinto and Volpato, 1997; Liley, 1982; Rowland, 1999). Pheromones, behavior or related metabolites could cause altered physiology, metabolism or behaviors to maintain a constant degree of coordination between organisms (Bergman and Moore, 2003; Giaquinto and Volpato, 1997; Liley, 1982; Rowland, 1999). Therefore, an EDC that can disrupt endocrine function in one individual might cause a corresponding effect in individuals with which it comes into contact that were not originally exposed to the EDC. This is defined as the "bystander effect" in unexposed organisms. This effect is not caused by chemical(s) released from exposed animals, but is a result of exposed animals experiencing some behavioral/physiological (e.g., pheromones) alteration due to exposure, which is then having an impact on the bystanders, resulting in bystander effect.

Different in vivo and in vitro testing systems are currently used to assess the risks of exposure to EDCs already in the environment and of new chemicals that might be released in the

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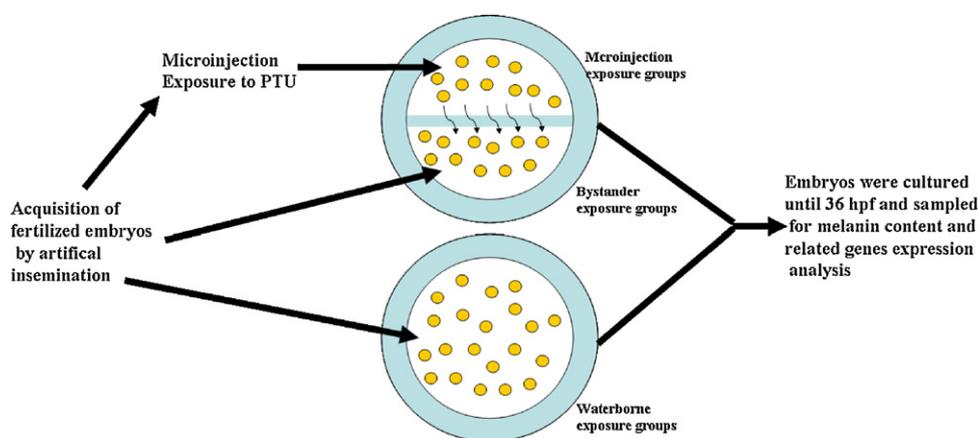E-mail addresses: ligy2001@163.com (G. Li), zhangxw@nju.edu.cn (X. Zhang).

Fig. 1. Flow diagram of the embryos exposure experiment.

future. One of the in vivo testing methods is the fish toxicity bioassay, in which individuals are exposed to constant concentrations of chemicals in static renew or continuous flow exposure systems. However, in the real world, concentrations of chemicals fluctuate, and avoidance and migration behaviors of fish result in that animals have different exposure histories. It is common that exposed and unexposed individuals spend at least some time in each other's proximity. Therefore, in the present study, a new exposure approach, "bystander exposure", was developed to investigate potential interactions between organisms exposed to different concentrations of chemicals. Specifically, in the present study, the model chemical 6-propyl-2-thiouracil (PTU) was chosen as a reference agent to study possible bystander effects on the melanin synthesis pathways of zebrafish embryos. This pathway was chosen because the results of previous study had demonstrated that color patterns in fish are multicomponent signals, which can be used to communicate between and among individual fish by physiological regulation or behavioral change (Price et al., 2008). In addition, this pathway allows simple observation without complicated experimental procedures. Zebrafish embryos have been suggested as an appropriate model for screening of melanogenic regulatory compounds (Choi et al., 2007). PTU is an inhibitor of tyrosinase that is used routinely to inhibit synthesis of melanin in zebrafish (Choi et al., 2007; Dryja et al., 1978; Peterson et al., 2000). It was hypothesized that PTU exposure could cause some behavioral/physiological (e.g., pheromones) alteration, which is then having an impact on the bystanders, subsequently causing the induction of bystander effects on melanin synthesis pathways.

2. Materials and methods

2.1. Materials and reagents

TRIzol regent and M-MLV reverse transcription kits were obtained from Invitrogen (New Jersey, NJ, USA) and Promega (Madison, WI, USA), respectively. SYBR Green kits and PTU were purchased from Toyobo (Tokyo, Japan) and Sigma (St. Louis, MO, USA), respectively. Primary antibodies against tyrosinase (TYR) and silver (SILV) were obtained from Epitomics (Burlingame, CA, USA) and Everest Biotech Ltd (Oxfordshire, United Kingdom), respectively.

2.2. PTU exposure protocol

Adult zebrafish (25-week-old) were maintained according to previously described protocols (Liu et al., 2009, 2010a). Fertilized

eggs were obtained by artificial insemination as described previously (Wu et al., 2006). PTU was purchased from Sigma (St. Louis, MO, USA) and was dissolved in sterile phosphate buffered saline (PBS) as stock solution. The experimental design for PTU exposure included a microjection exposure, bystander exposure and waterborne exposure (Fig. 1). Twelve custom-made dishes were fabricated, and each dish contained 2 shallow regions to accommodate embryos from microinjection and bystander groups, respectively. The custom-made dishes separated the two groups of embryos but allowed them to share the same medium. In the microinjection exposure group, approximately 3 nL of various concentrations of PTU solution was microinjected into fertilized embryo (final exposure concentrations: 0, 0.03, 0.3 or 3 ng/egg) at the one-cell stage under a dissecting microscope (Wu et al., 2006). Embryos that developed normally were cultured in the corresponding microinjection regions of custom-made dishes until sampled at 36 h post-fertilization (hpf). Simultaneously, bystander embryos, which were sham injected, were cultured in corresponding bystander regions. Each dish contained 40 mL embryo culture medium with 100 microinjection and bystander embryos in the corresponding regions. Each concentration was tested in triplicate. Exposure concentrations were chosen based on a previous range-finding study, in which the melanin contents were significantly decreased by PTU. The duration of exposure used in this study was chosen based on a previous study and a range-finding study, where measurement of contents using the method described below could be easily performed (Camp and Lardelli, 2001).

Two waterborne PTU exposure experiments were conducted in parallel to: (1) verify the effects of PTU on melanin synthesis pathway from the microinjection exposure; and (2) confirm that bystander effects were not affected by possible PTU release from embryos that had been exposed via microinjection. Briefly, fertilized embryos that developed normally were randomly distributed into dishes containing different concentrations of PTU exposure solution (0, 0.018, 0.18, 1.8 or 18 mg/L for experiment 1, and 0, 0.075, 0.75 or 7.5 µg/L for experiment 2) and were cultured until collected at 36 hpf. There were three dishes for each concentration with 200 embryos in each dish. Exposure concentrations of the first experiment were chosen based on a previous study (Choi et al., 2007) where PTU could significantly decrease melanin contents. Concentrations in the second experiment were chosen based on the hypothesis that all the PTU in the microinjected embryos was released into the medium, which would make the concentration of PTU in the medium reach 0.075–7.5 µg/L. The calculation formula was as follows: PTU concentration in the medium = (PTU content in each embryo × 100 (number of embryos))/40 mL (volume of medium).

Table 1
Sequences of primers for the genes tested.

Gene name	Sequence of the primers (5′–3′)	Accession number
OCA2	Forward: gctctgtttgtgtgctcatgga	XM_690715
	Reverse: ggcagtgaagggaatgttgt	
TYR	Forward: gacagacgaggagacggaag	NM_131013
	Reverse: ccttgggtttacggatgaga	
DCT	Forward: acgagcgcacttacacacac	AF280090
	Reverse: ttagacacgatcccctccac	
TYRP1	Forward: ctaatgtgcgtggtggtgac	NM_001002749
	Reverse: cgcctgtcagcagtaattga	
SILV	Forward: cagacatggtgatggtggag	AY864065
	Reverse: ggacgatctgcacactctca	
rpl8	Forward: ttgttggtgttgttgctggt	NM_200713
	Reverse: ggatgctcaacaggggttcat	

2.3. Melanin measurement

Concentrations of melanin were measured according to the method developed by Jin and Thibaudeau (Jin and Thibaudeau, 1999). Briefly, 30 embryos were digested in 0.25 mL of 1 M NaOH. The OD values were determined at 414 nm in the following day. Embryos exposed to each concentration were measured in triplicate. Concentrations of melanin in treated embryos was compared to appropriate controls (plotted as 100%) and expressed as percentage of control.

2.4. Quantitative real-time PCR assay

After exposure, embryos were collected and preserved in TRIzol regent (Invitrogen, New Jersey, NJ, USA) at $-20\,^{\circ}$C. Each concentration was measured in triplicate with a composite of 20 embryos. Isolation, purification and quantification of total RNA, first-strand cDNA synthesis, and quantitative real-time PCR were performed according to methods described previously (Liu et al., 2011). Briefly, total RNA was isolated by use of TRIzol regent, and digested by RNase-free DNase I (Promega, WI, USA) following the manufacturer's instructions. Concentrations of total RNA were estimated by use of 260 nm reading value and the quality was verified by measuring the 260/280 nm ratios. The 1% agarose-formal dehyde gel electrophoresis with ethidium bromide staining was used to further verify the quality of total RNA. First-strand cDNA synthesis was performed using commercial kits (Promega, WI, USA) following the manufacturer's instructions. Quantitative real-time PCR was analyzed on the ABI PRISM 7300 Sequence Detector system (PerkinElmer Applied Biosystems, CA, USA). The housekeeping gene ribosomal protein l8 (rpl8) did not vary under experimental conditions in the present study (data not shown) and was used as an internal control. Sequences of primers used in the study were designed with the Primer 3 software (http://frodo.wi.mit.edu/) (Table 1). The thermal cycle was set at $95\,^{\circ}$C for 10 min, followed by 35–45 cycles of $95\,^{\circ}$C for 30 s, $60\,^{\circ}$C for 20 s and $72\,^{\circ}$C for 1 min. The fold-change of the genes tested was obtained by the $2^{-\Delta\Delta Ct}$ method. Transcript expression was measured in triplicate.

2.5. Western blotting

Western blotting was performed as previously described (Li et al., 2011; Liu et al., 2010b) with some modifications. Each concentration was measured in triplicate with a composite of 30 embryos. Briefly, zebrafish embryos were homogenized in protein extraction buffer (Wuhan Boster Biological Technology, Wuhan, China) and centrifuged at $12,000 \times g$ for 10 min. Supernatants were collected for protein analysis. About 20 μg protein was denatured, electrophoresed and transferred onto a polyvinylidenedifluoride (PVDF) membrane. The membrane was blocked and incubated in
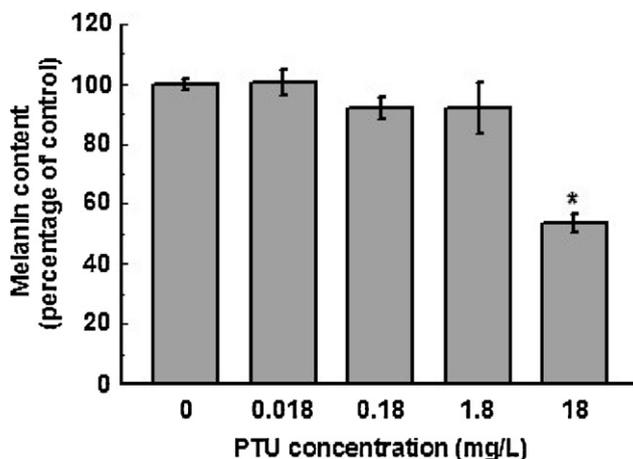
Fig. 2. Effects of waterborne exposure to different concentrations of PTU on melanin contents in zebrafish embryos. Values represent mean ± SEM ($n = 3$ samples; each sample included 30 embryos). Significant difference from the control group is indicated by $*P < 0.05$.

primary antibody against TYR (Epitomics, Burlingame, CA, USA) and SILV (Everest Biotech Ltd, Oxfordshire, United Kingdom), and then secondary antibody (KPL, Gaithersburg, MD, USA) following the manufacture's instructions. The immunoblot analysis was performed by using NBT/BCIP system. The quantification of the relative expression of TYR and SILV was performed by using Gene Snap software (Syngene, America). Each concentration contained 3 replicates.

2.6. Statistical analyses

All data were expressed as mean ± SEM. The statistical analysis was performed using Kyplot 5.0 Demo software (Tokyo, Japan). Data normality and homogeneity were evaluated by use of the Kolmogorov–Smirnow test and Levene's test, respectively. If the data did not meet the assumptions of the parametric tests, values were base-10 logarithmically transformed. Treatments were examined for differences by use of the one-way analysis of variance followed by Tukey's multiple range tests. Spearman correlation analysis with Bonferroni correction was used to examine relationships between mRNA and protein expressions in microinjection exposure and in bystander exposure. P-value < 0.05 was considered as statistically significant.

3. Results

3.1. Melanin content

No significant effects on survival rates were observed after three different methods of exposure to PTU. Survival rates were >90% in all the exposure groups including controls. Waterborne exposure to 18 mg PTU/L resulted in significantly less melanin content (53.3%) in zebrafish embryos compared with the control, while exposure to lesser concentrations (0.018, 0.18 or 1.8 mg PTU/L) did not change melanin production (Fig. 2). Waterborne exposure to 0.075, 0.75 and 7.5 μg/L PTU did not change melanin content in zebrafish embryos (data not shown). Microinjection with 3 ng PTU/egg significantly decreased contents of melanin by 37.4% in zebrafish embryos, compared with the control while no significant changes were observed in melanin of embryos exposed to other concentrations of PTU (Fig. 3). Concentrations of melanin were significantly greater by 30.2% than that of the control in 3 ng PTU/egg bystander exposure group. Concentrations of melanin
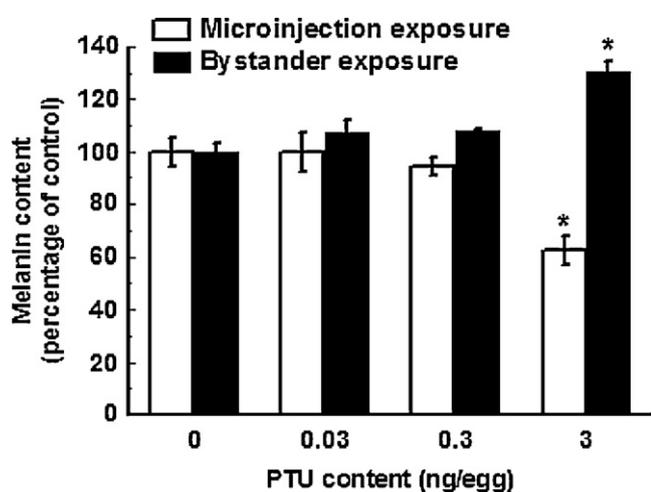
Fig. 3. Effects of two different types of exposure of zebrafish embryos to PTU (microinjection exposure and bystander exposure) on melanin contents. Values represent mean ± SEM (n = 3 samples; each sample included 30 embryos). Significant difference from the corresponding control group is indicated by *$P < 0.05$.

were not significantly different from those of the controls in 0.03 or 0.3 ng PTU/egg bystander exposure groups (Fig. 3).

3.2. mRNA expression

Waterborne exposure to PTU significantly up-regulated expression of mRNA of all genes that were studied. Exposure to 1.8 or 18 mg PTU/L significantly up-regulated expression of oculocutaneous albinism type 2 (OCA2), TYR and dopachrometautomerase (DCT) by 1.29- and 0.95-fold, 0.50- and 0.30-fold, and 0.62- and 0.91-fold, respectively (Table 2). Transcription of SILV was significantly up-regulated by 0.42-fold upon exposure to 18 mg PTU/L, while expression of mRNA for tyrosinase-related protein 1 (TYRP1) was not significantly different among treatments or the control (Table 2).

The abundances of mRNA of the studied genes in embryos exposed via both microinjection or bystander was significantly greater than that of the controls. Exposure to 0.3 or 3 ng PTU/egg via microinjection resulted in significantly more expression of DCT mRNA by 0.34- and 0.72-fold, respectively (Table 3). Expressions of mRNA of OCA2, TYR, TYRP1 and SILV were significantly up-regulated by 0.68-, 0.38-, 0.47- and 0.85-fold upon exposure to 3 ng PTU/egg, respectively (Table 3). Bystander exposure resulted in significant up-regulation of expression of TYR and SILV mRNA in 3 ng/egg exposure group by 0.77- and 0.57-fold, respectively (Table 3).

3.3. Protein abundance

Exposure to PTU via all three methods of exposure resulted in greater abundance of TYR and SILV proteins in a
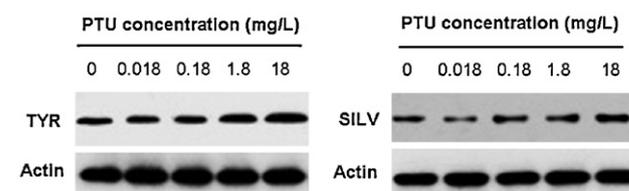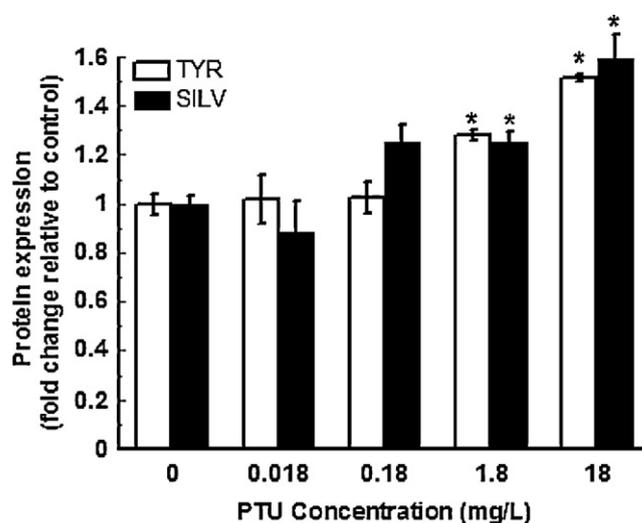
Table 2
mRNA expression of several melanin synthesis-associated genes after waterborne exposure to different concentrations of PTU. Values represent mean ± SEM (n = 3 samples; each sample included 20 embryos). *$P < 0.05$ indicates significant difference between exposure groups and the corresponding control group.

Gene	PTU concentrations (mg/L)				
	0	0.018	0.18	1.8	18
OCA2	1.00 ± 0.13	0.91 ± 0.15	1.23 ± 0.13	2.29 ± 0.13*	1.95 ± 0.30*
TYR	1.00 ± 0.11	1.03 ± 0.05	1.13 ± 0.21	1.62 ± 0.03*	1.91 ± 0.07*
DCT	1.00 ± 0.09	0.87 ± 0.09	0.86 ± 0.19	1.50 ± 0.10*	1.30 ± 0.04*
TYRP1	1.00 ± 0.09	1.00 ± 0.13	1.04 ± 0.21	1.26 ± 0.20	1.07 ± 0.15
SILV	1.00 ± 0.07	0.89 ± 0.16	1.04 ± 0.10	0.93 ± 0.07	1.42 ± 0.09*

Fig. 4. Effects of waterborne exposure to PTU on expression of TYR and SILV protein expression in zebrafish embryos. Values represent mean ± SEM (n = 3 samples; each sample included 30 embryos). Significant difference from the control group is indicated by *$P < 0.05$.

concentration-dependent manner. Waterborne exposure to 1.8 or 18 mg PTU/L resulted in significant up-regulation of TYR and SILV protein expression by 0.28- and 0.52-fold, and 0.25- and 0.59-fold, respectively (Fig. 4). Expression of TYR and SILV proteins were significantly up-regulated by 0.97- and 1.25-fold, and 0.35- and 0.56-fold after microinjection exposure to 0.3 and 3 ng PTU/egg, respectively (Fig. 5). Bystander exposure significantly up-regulated TYR and SILV proteins expression in 3 ng/egg exposure group by 0.49- and 0.35-fold, respectively (Fig. 6).

3.4. Correlation analysis

Expressions of mRNA of OCA2, TYR, DCT, TYRP1 and SILV in embryos exposed via microinjection were positively correlated with expression of the corresponding genes in embryos exposed as bystanders, and correlation probabilities (CP) were 0.037, 0.002, 0.024, 0.016 and 0.011, respectively (Fig. 7). Expressions of TYR and SILV proteins in embryos exposed via microinjections were also positively correlated with their protein expressions in bystander exposure groups, and the CP were 0.014 and 0.029, respectively (Fig. 7).

4. Discussion

The model chemical PTU is a TYR inhibitor and exposure to PTU could significantly decrease melanin production in zebrafish (Choi et al., 2007). The results reported here were consistent with previous observations. Both waterborne and microinjection exposure of embryos to PTU resulted in significantly less production of melanin. Furthermore, mRNA and protein expression analysis demonstrated that PTU exposure significantly up-regulated OCA2, TYR, DCT, TYRP1 and SILV mRNA expression and increased protein TYR and SILV abundance in waterborne and microinjection exposure groups. These genes tested are involved in melanin synthesis pathway

Table 3
mRNA expression of several melanin synthesis-associated genes after two different forms of PTU exposure. Values represent mean ± SEM ($n = 3$ samples; each sample included 20 embryos). *$P < 0.05$ indicates significant difference between exposure groups and the corresponding control group.

Exposure forms	Gene	PTU contents (ng/egg)			
		0	0.03	0.3	3
Microinjection exposure	OCA2	1.00 ± 0.15	1.06 ± 0.15	0.97 ± 0.23	1.68 ± 0.22*
	TYR	1.00 ± 0.27	1.05 ± 0.09	1.38 ± 0.08	2.42 ± 0.13*
	DCT	1.00 ± 0.08	1.16 ± 0.03	1.34 ± 0.14*	1.72 ± 0.28*
	TYRP1	1.00 ± 0.09	1.08 ± 0.04	1.11 ± 0.08	1.47 ± 0.07*
	SILV	1.00 ± 0.15	1.24 ± 0.07	1.39 ± 0.16	1.85 ± 0.34*
Bystander exposure	OCA2	1.00 ± 0.16	0.96 ± 0.15	0.97 ± 0.22	1.11 ± 0.12
	TYR	1.00 ± 0.41	0.91 ± 0.06	1.45 ± 0.19	1.77 ± 0.09*
	DCT	1.00 ± 0.02	0.97 ± 0.19	1.09 ± 0.08	1.15 ± 0.26
	TYRP1	1.00 ± 0.05	1.00 ± 0.04	0.98 ± 0.13	1.13 ± 0.10
	SILV	1.00 ± 0.16	0.99 ± 0.16	0.99 ± 0.19	1.57 ± 0.27*

(Fig. 8). The gene *OCA2* encodes for transporter protein, which resides in the melanosomal membrane and is essential for melanin synthesis (Braasch et al., 2007). The protein TYR catalyzes the first two rate-limiting steps of melanin synthesis in conversion of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and DOPAquinone as well as two later steps (Braasch et al., 2007). DCT is responsible for conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TYRP1 is involved in synthesis of indole-5,6-quinone carboxylic acid from DHICA (Braasch et al., 2007). The protein encoded by the *SILV* gene catalyzes the final step of melanin synthesis in the conversion of indole-5,6-quinone carboxylic acid to eumelanin (Braasch et al., 2007). Therefore, the up-regulation of the expression of these genes was considered as a compensatory mechanism for lesser production of melanin. Negative feed-back structure in endocrine system is one of the key mechanisms that allow the living organism to maintain a relatively stable physiological status (Nichols et al., 2011). Previous studies also demonstrated that exposure to prochloraz could significantly decrease plasma testosterone and 17β-estradiol concentrations but up-regulate several steroidogenic genes expression as a compensatory mechanism in fish (Ankley et al., 2009; Liu et al., 2011; Zhang et al., 2008c).

In the present study, the increases in mRNA expression were relative small, and the possible reasons might be attributed to weak feedback mechanisms in embryos. In addition, the consistency of effects after two different forms of PTU exposure confirmed that exposure to PTU through microinjection is a feasible and effective method.

When assessing the risk of EDCs the most frequently used in vivo testing system is fish toxicity test where traditional exposure forms (static renew or continuous flow exposure) were employed, few studies have been conducted to evaluate interactions between organisms with different exposure levels although this occurs commonly in the real world and might have implications for assessing the risk of exposure to EDCs (Partridge et al., 2010; Saaristo et al., 2009a,b, 2010). Therefore, zebrafish embryos were employed to study possible bystander effects of model EDC PTU in the present study. Previous studies of bystander effects have focused on radiation and only a few studies have evaluated several genotoxic chemicals in vitro (Asur et al., 2009, 2010; Choi et al., 2010; Cogan et al., 2010; Mothersill et al., 2006, 2007, 2009; Preston, 2005; Smith et al., 2007). Major effects include micronuclei, sister
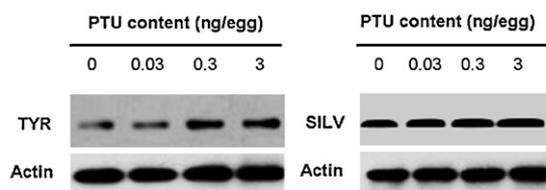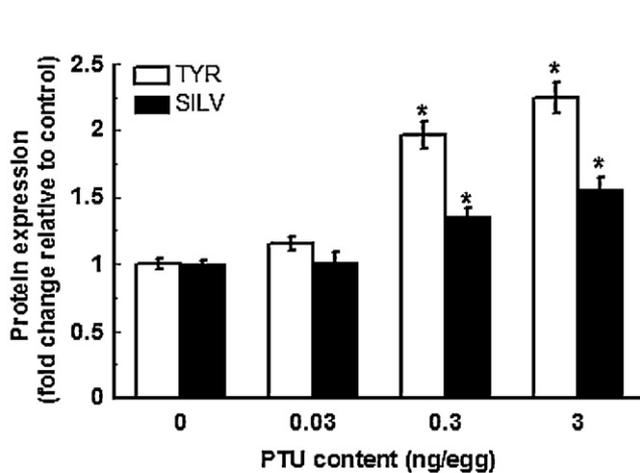

Fig. 5. Effects of exposure to PTU via microinjection on TYR and SILV protein expression in zebrafish embryos. Values represent mean ± SEM ($n = 3$ samples; each sample included 30 embryos). Significant difference from the control group is indicated by *$P < 0.05$.
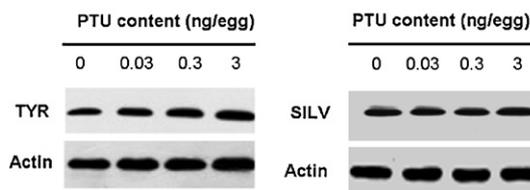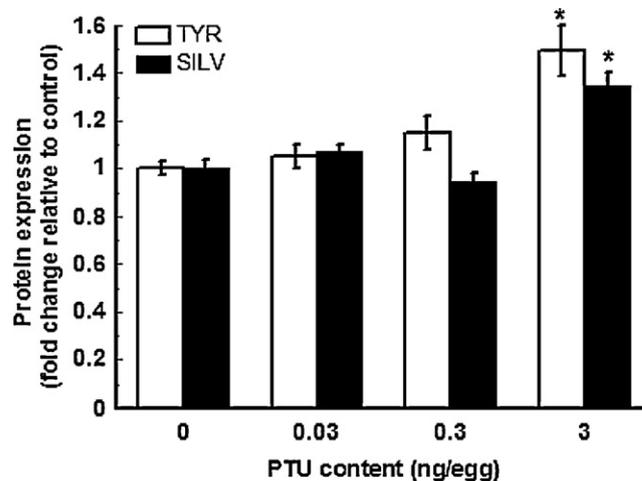

Fig. 6. Effects of bystander exposure to PTU on TYR and SILV protein expression in zebrafish embryos. Values represent mean ± SEM ($n = 3$ samples; each sample included 30 embryos). Significant difference from the control group is indicated by *$P < 0.05$.
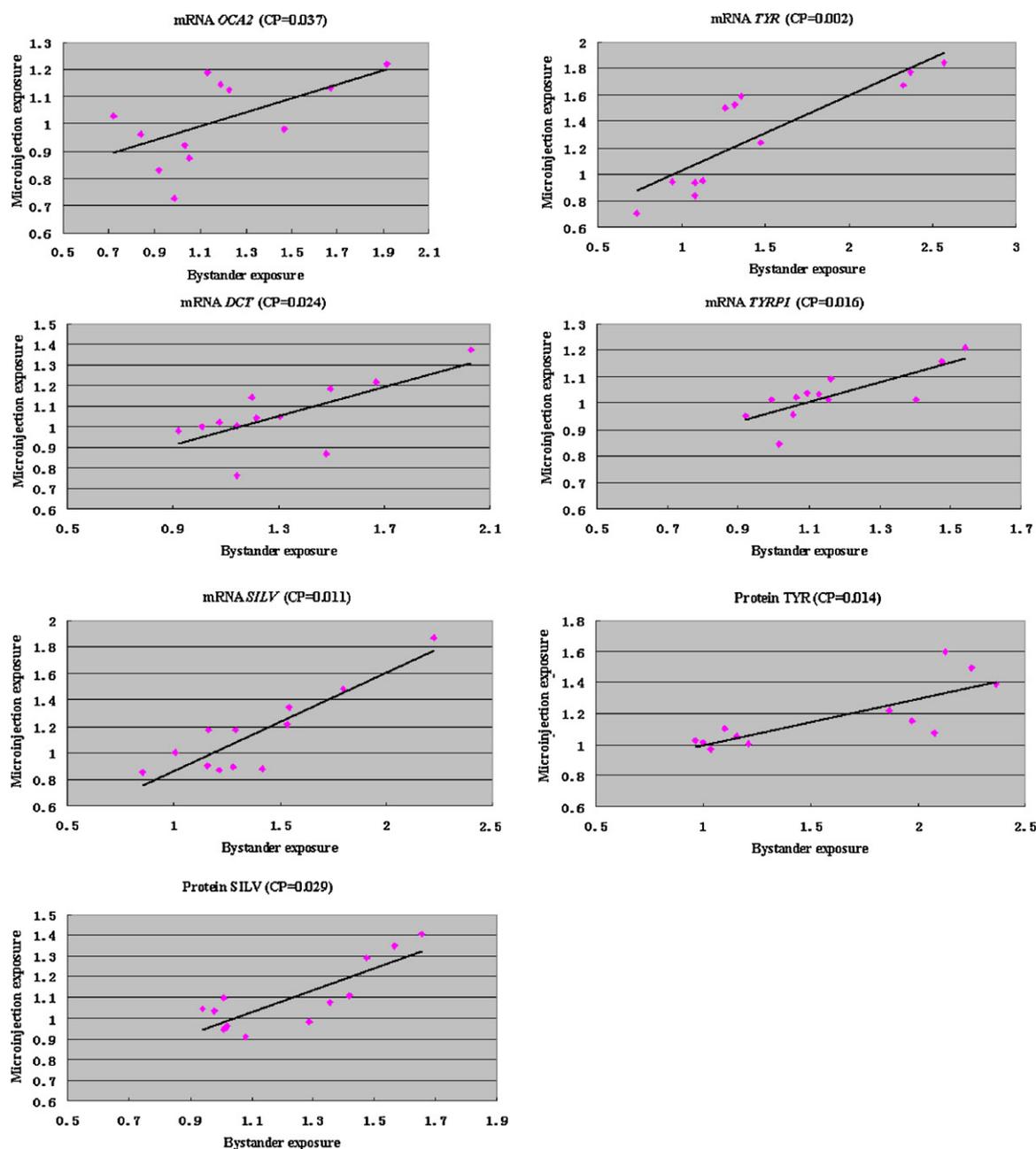
Fig. 7. Spearman rank correlation probabilities (CP) between the same parameters after two different forms of PTU exposure in zebrafish embryos (n = 24).

chromatid exchange, oxidative DNA damage and DNA double strand breaks, chromosome aberrations, mutations, apoptosis and neoplastic transformation. To our knowledge, this is the first report on bystander effects induced by EDC in living organisms. In the present study, bystander exposure up-regulated all tested genes (*OCA2*, *TYR*, *DCT*, *TYRP1* and *SILV*) although only the up-regulation of *TYR* and *SILV* was statistically significant. We further analyzed the proteins expression coded by genes *TYR* and *SILV*, and the results demonstrated that bystander exposure significantly increased the abundances of these two proteins. Proteins TYR and SILV play key roles in melanin synthesis (Braasch et al., 2007). Results of a previous study demonstrated that the knockdown of gene TYR resulted in less pigment in the retina and skin of rainbow trout embryos (Boonanuntanasarn et al., 2004). A mutation in the *SILV* gene leaded to defects in melanin biogenesis in zebrafish (Schonthaler et al., 2005). Therefore, up-regulation of these mRNA and protein expressions would result in more synthesis of melanin. In this study,

bystander exposure significantly increased melanin production in zebrafish embryos. Therefore, the increase of melanin production was considered as a result of the up-regulation of corresponding gene expression. Because PTU is an inhibitor of TYR and it only decreased melanin production and the results of previous studies have demonstrated that PTU only decreased production of melanin (Choi et al., 2007; Dryja et al., 1978; Peterson et al., 2000). It is not possible that the up-regulation of mRNA and protein expression and increase of melanin production in embryos of bystander exposure is directly resulted from waterborne exposure to PTU due to the possible PTU release from microinjection embryos. In addition, it was hypothesized that all the PTU in the microinjected embryos were released into the medium, which would make the concentration of PTU in the medium reach 0.075–7.5 µg/L. The results of the waterborne exposure demonstrated that exposure to 0.075, 0.75 and 7.5 µg/L PTU did not change melanin content in zebrafish embryos. It is also not possible that the increase in melanin
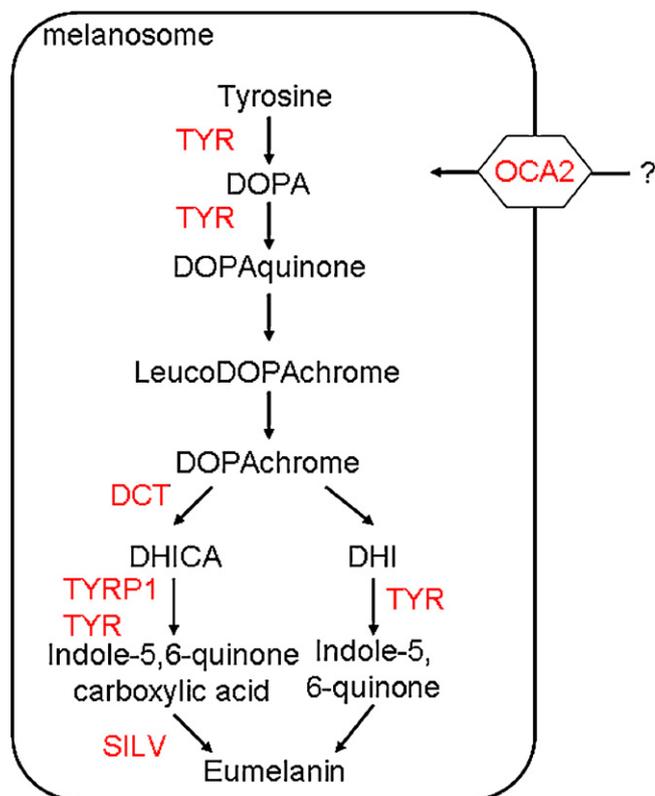
Fig. 8. Eumelanin synthesis pathway in vertebrates, adopted from Braasch et al. (2007). TYR: tyrosinase; DCT: dopachrometautomerase; TYRP1: tyrosinase-related protein 1; SILV: silver; OCA2: oculocutaneous albinism type 2.

synthesis resulted from waterborne exposure to PTU metabolites due to the possible release from microinjection embryos, since elimination of chemical from fish body is finished mainly by excretory system, which consists of gill, kidney and bile. By the end of exposure (36 hpf), these organs have not completed development (Drummond, 2005; Lorent et al., 2010; Jonz and Nurse, 2005). Therefore, the release of PTU metabolites from the embryos is not possible at this stage of development. Furthermore, correlation analysis demonstrated that mRNA expressions of *OCA2*, *TYR*, *DCT*, *TYRP1*, *SILV* and protein expressions of TYR and SILV in microinjection exposure groups were positively correlated with these mRNAs and proteins expression in bystander exposure groups, respectively, which suggested that the up-regulation of the expression of these might be caused by the same factor(s). It was speculated that factor(s) might be released by microinjection embryos in response to PTU exposure. It is also possible that PTU exposure caused some behavioral alterations, which then had an impact on the bystanders, resulting in bystander effects. Similarly, a previous study reported a radioadaptive response induced by alpha-particles in zebrafish embryos and the authors stated that when an individual living organism is subjected to a radiation exposure the adaptive response will be induced in the entire population (Choi et al., 2010). The main objective of this study was to character bystander effects after PTU exposure, therefore, further study is needed to explore the mechanisms of bystander effects.

The data presented in this study provide evidence of a bystander effect induced by the EDC PTU in zebrafish embryos. The results suggest that PTU exposure through microinjection could alter the physiology or behavior of zebrafish embryos, which affected bystander embryos and caused bystander effects in the population-level responses. Characterizing this type of effect is important to understand the impact of EDCs on unexposed animals in the environment. It also may need to be incorporated into a testing strategy

to support reliable evaluations of the risk of EDCs. The results of studies conducted in the laboratory have demonstrated that concentrations of chemicals in a given fish species depend on many factors, such as exposure concentration, exposure duration, pre-exposure history, fish age and body conditions (Dabrowska et al., 1999; Playle et al., 1993; Taylor et al., 2000). Therefore, these factors might result in individual fish carrying different concentrations of chemical. Furthermore, almost all biomornitoring data have demonstrated that fish might have different exposure histories. Specifically, coefficients of variation of concentrations of residues among individual fish can be equal to or greater than 100%, which is indicative of a skewed distribution (Giesy and Allred, 1985). In addition, migration behavior occurs commonly for kinds of fish (e.g., Sakhalin taimen (*Hucho perryi*), pink salmon (*Oncorhynchus gorbuscha*)) to maximize their reproduction success and fitness, where animals with different exposures might reside and spawn together, resulting in bystander exposure. Therefore, the results of this study might have implications for assessing the effects of EDCs in the environment.

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