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Authors

Lin, Jiebo
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Liu, Jinfeng
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Research paper

Up-stream mechanisms for up-regulation of miR-125b from triclosan exposure to zebrafish (*Danio rerio*)

Jiebo Lin^a, Caihong Wang^a, Jinfeng Liu^a, Randy A. Dahlgren^b, Weiming Ai^a, Aibing Zeng^a, Xuedong Wang^{a,*}, Huili Wang^{a,*}

^a School of Life Sciences, Wenzhou Medical University, Wenzhou 325035, PR China

^b Department of Land, Air and Water Resources, University of California-Davis, CA 95616, USA

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ABSTRACT

Triclosan (TCS) exposure has widely adverse biological effects such as influencing biological reproduction and endocrine disorders. While some studies have addressed TCS-induced expression changes of miRNAs and their related down-stream target genes, no data are available concerning how TCS impairs miRNA expression leading us to study up-stream regulating mechanisms. Four miRNAs (miR-125b, miR-205, miR-142a and miR-203a) showed differential expression between TCS-exposure treatments and the control group; their functions mainly involved fatty acid synthesis and metabolism. TCS exposure led to the up-regulation of mature miR-125b that was concomitant with consistent changes in pri-mir-125b-1 and pri-mir-125b-3 among its 3 pri-mir-125bs. Up-regulation of miR-125b originated from direct shear processes involving the two up-regulated precursors, but not pri-mir-125b2. Increased expression of pri-mir-125b-1 and pri-mir-125b-3 resulted from *nfe2l2*- and *c/ebpα*-integration with positive control elements of promoters for the two precursors. The overexpression of transcriptional factors, *nfe2l2* and *c/ebpα*, initiated the promoter activity for the miR-125b precursor. CpG islands and *Nfe2l2* were involved in constitutive expression of *mir-125b-1* and *mir-125b-3*. The activities of two promoter regions, −487 to −1 bp for pri-mir-125b1 and −1327 to +14 bp for pri-mir-125b-3 having binding sites for NFE2 and Nfe2l2/MAF:NFE2, were higher than other regions, further demonstrating that the transcriptional factor Nfe2l2 was involved in the regulation of pri-mir-125b1 and pri-mir-125b-3. TCS's estrogen activity resulted from its effects on GPER, a novel membrane receptor, rather than the classical ERα and ERβ. These results explain, to some extent, the up-stream mechanism for miR-125b up-regulation, and also provide a guidance to future mechanistic study on TCS-exposure.

1. Introduction

Triclosan (TCS, 5-chloro-2-[2,4-dichlorophenoxy]phenol) is an antimicrobial agent that has been broadly used in many products such as liquid hand soaps, household cleansers, and dental products for more than 30 years. In recent years, TCS is universally detected in the environment at ng/L to μg/L levels, leading to accumulation in biota and humans (Wang et al., 2015). TCS has adverse effects on biological growth and development such as influencing reproduction and endocrine disorders. Many previous studies document the acute or long-term exposure toxicity of TCS to various organisms, such as fish, invertebrates and amphibians (Huang et al., 2014). For example, Yueh and Tukey (2016) found that long-term TCS exposure led to liver tumors and produced oxidative stress in mice. Because TCS is a stable and lipophilic compound, it preferentially bioaccumulates in fatty tissue

and liver bile, and as a consequence is often detectable in these tissues or organs (Young et al., 2008). TCS was extensively metabolized in HepG2 cells overexpressing CYP1A2, CYP2B6, CYP2C19, CYP2D6, CYP2C18, lucuronosyltransferases (UGTs) and sulfotransferases (SULTs) (Wu et al., 2017). In zebrafish (*Danio rerio*), TCS impairs the mRNA expression level of beta-oxidation transcripts and lipid beta-oxidation genes, which included *ppara*, *cpt1*, *lpbe*, *cyp4a10* and *aco* (Ho et al., 2016). TCS at non-cytotoxic concentrations can induce human mesenchymal stem cells lipid accumulation by decreasing adipocyte protein 2, lipoprotein lipase, and adiponectin gene expression (Guo et al., 2012). However, there is a paucity of information regarding the specific microRNAs (miRNAs) induced by TCS exposure and the responsible transcription factors. miRNAs play an important role in lipid synthesis, oxidation and related diseases (Baldán and Tq, 2016). miRNA levels in cells are controlled by several mechanisms (e.g., transcription,

* Corresponding authors.

E-mail addresses: zjuwxd@163.com (X. Wang), whuili@163.com (H. Wang).

processing and stabilization) and an increase of pri-miRNA is a major indicator for quantitation of changes in miRNAs (Chang et al., 2011).

Herein, based on high throughput sequencing, we identified the dysregulated miRNAs among TCS-exposed groups and the control group. One of these dysregulated miRNAs, miR-125b-5p, was differentially expressed in three TCS-exposure groups when compared to the control group. In cancer research, miR-125b is associated with tumor suppressor by down-regulating target oncogene (Yueh et al., 2014). Additionally, several studies have examined miR-125b in relationship to metabolic disorders in mice and humans cell lines (Zhang et al., 2015; Tili et al., 2012). miR-125a/b was found to suppress Cd-induced apoptosis by target Bak and caspase-3 directly in LLC-PK1 cells (Chen et al., 2016a,b). Moreover, several previous studies demonstrate that expression changes of microRNAs in response to pollutant exposure result from the corresponding differential expression of down-stream genes (Pogribny et al., 2015; Harrill et al., 2016; Woeller et al., 2017). These previous reports mainly focused on down-stream regulation by miR-125b, but no studies have addressed related up-stream regulation mechanisms. Therefore, the specific objective of this study was to probe up-stream mechanisms regulating miR-125b expression changes resulting from TCS exposure to zebrafish (*Danio rerio*). Although previous researches identified several factors that positively regulate miR-125b expression in mammal cell lines, such as Nrf2, c/EBP α and ER α , whether or not miR-125b is also involved in glucose and lipid metabolism in zebrafish embryo remain uncertain (Tili et al., 2012; Joo et al., 2013; Zhang et al., 2015; Romero et al., 2015). Furthermore, the molecular mechanisms for abnormal expression of miR-125b from TCS exposure and the conservation of miR-125b in vertebrates have not been investigated. As a result, the above uncertain problems are the focus of this investigation.

2. Material and methods

2.1. Ethics statement

This study strictly followed protocols for the care and use of laboratory animals established by the Institutional Animal Care and Use Committee (IACUC) at Wenzhou Medical University (Wenzhou, China). All zebrafish surgery was performed on ice to decrease suffering.

2.2. Chemical reagents

Triclosan (5-chloro-2-[2,4-dichlorophenoxy]phenol) was purchased from Sigma-Aldrich (St. Louis, USA; CAS No. 3380-34-5, 99.9% purity). Formamide (CAS No.75-12-7, purity $\geq 99\%$) and xylene (CAS No. 1330-20-7, 98.5%) were purchased from Aladdin (Shanghai, China).

2.3. Zebrafish maintenance and exposure protocols

Wild-type (AB strain) zebrafish (*Danio rerio*) were raised in dechlorinated and filtered water at 28 °C with a 14-h light:10-h dark photoperiod (light on at 8 am). Zebrafish maintenance followed Westerfield (2000). A series of TCS-exposure concentrations was chosen according to its LC₅₀ and EC₅₀ values for embryonic and adult zebrafish (Oliveira et al., 2009), environmentally relevant concentrations, and preliminary experimental results. Embryos were exposed to a series of TCS concentrations (0, 62.5 $\mu\text{g/L}$ (0.22 $\mu\text{mol/L}$), 125 $\mu\text{g/L}$ (0.43 $\mu\text{mol/L}$) and 250 $\mu\text{g/L}$ (0.86 $\mu\text{mol/L}$)). Adult zebrafish were continuously exposed to low-dose TCS concentrations (0, 25 $\mu\text{g/L}$ (0.09 $\mu\text{mol/L}$), 50 $\mu\text{g/L}$ (0.17 $\mu\text{mol/L}$) and 100 $\mu\text{g/L}$ (0.35 $\mu\text{mol/L}$)) from embryos (4 hpf) to adults (90 dpf). Control embryos were treated with 0.0025% acetone (as reference to the highest 250 $\mu\text{g/L}$ TCS-exposure treatment) (Ho et al., 2016).

2.4. cDNA synthesis and qRT-PCR

Embryos were dissolved in TransZol Up[®] solution (Transgene, China) for total RNA extraction. Total RNA with an A₂₆₀/A₂₈₀ ratio of 1.8–2.0 was used. Briefly, 1 μg total cellular RNA was reversely transcribed using a PrimeScript RT[®] Reagent Kit with gDNA Eraser (Takara, Japan) and cDNA was transcribed with TransScript miRNA First-Strand cDNA Synthesis SuperMix for miRNA qRT-PCR (Transgene, China) with elfa and U6 as the endogenous reference (McCurley and Callard, 2008). All PCR reactions were performed in three biological replicates and each biological replicate included three technical replicates. Bio-Rad CFX Manager software was used to analyze results from qRT-PCR. Primers used for qRT-PCR are shown in Supplementary Table 1.

2.5. Promoter isolation, sequence analysis, construction, microinjection and luciferase assay

Zebrafish genomic DNA was extracted using the EasyPure Genome DNA kit (Transgene, China) according to the manufacturer's protocols. PCR was performed with 100 ng genomic DNA, a final concentration of 0.2 μM of primers; 2.5 units of TransStart FastPfu Fly DNA Polymerase, 1xTransStart FastPfu Fly DNA buffer (Transgene, China), and 0.2 mM dNTPS (Takara, Japan) in a total volume of 50 μL using a GeneAmp[®] PCR System 2700 thermocycler (Applied Biosystems, USA). PCR conditions were 35 cycles of 20 s at 95 °C, 20 s at 60 °C, and 30 s at 72 °C. Validated plasmid containing target fragments was gel-purified and ligated to pEASY-Blunt Simple Cloning Vector (Transgene, China). Several clones were sequenced by BGI (Beijing, China) and searched against the genome database using the ensemble BLAST/BLAT (http://www.ensembl.org/Danio_rerio/Tools/Blast?db=core;redirect=no).

Primers used for the promoter activity assay are shown in Supplementary Table 1 and were synthesized by Shanghai Sangon Biotechnological Corporation (Shanghai, China). The PCR product containing *mir-125b-1* and *mir-125b-3* promoters was independently digested with SacI and HindIII or NcoI restriction endonucleases (NEB, USA), and fragments containing the promoter region were collected by EasyPure[®] Quick Gel Extraction Kit (Transgene, China). Promoter fragments of *mir-125b-3* were inserted into pGL3-basic (Promega, USA) using SacI and NcoI sites. The *mir-125b-1* promoter fragments were inserted into pGL3-basic (Promega, USA) using SacI and HindIII sites.

Plasmids, which included various lengths of *mir-125b-1* or *mir-125b-3* promoter regions and the pRL-TK vector in a ratio of 2:1, were microinjected (ca. 2 nL of solution) into the one-cell-stage of zebrafish eggs, and the pRL-TK vector was used as an internal control reporter. After injection, eggs were incubated at 28 °C for 48 h for luciferase assay. Protein extracts of 48 hpf embryos in passive lysis buffer were placed in an ice bath, and aliquots of the extracts (20 μL) were used for luciferase assays using a Dual Luciferase Reporter Assay System (Promega, USA) following manufacturer instructions (Varioskan Flash, Thermo Fisher Scientific, USA).

2.6. RNA preparation, sequencing and data mapping

Total RNA was extracted using Trizol Reagent (Transgene, China) according to the manufacturer's instructions. RNA sequencing libraries were prepared for each RNA-seq sample using a TruSeq[®] Stranded Total RNA Sample Preparation kit (Illumina, USA) following manufacturer's protocols. For miRNA sequencing, the RNA samples from 96-hpf zebrafish for each group were pooled together based on an equal RNA quantity. After quality control (OD₂₆₀/OD₂₈₀ > 1.8 and OD₂₆₀/OD₂₃₀ > 1.5), sequencing of all libraries was performed by Illumina HiSeq2000/2500 according to manufacturer's instructions (LC Sciences, USA). Small RNA library sequencing was completed by Hangzhou LC-Bio Co., Ltd. (Hangzhou, China). Analyses of RNA-Seq data were conducted according to the protocols previously reported by our group (Zheng et al., 2016; Li et al., 2015).

2.7. Histopathological observation for liver tissue

After TCS exposure from 4 hpf to 90 dpf, zebrafish liver was dissected for histopathological observation. Haematoxylin and eosin (H & E) staining of the isolated liver tissue was conducted following manufacturer's instructions for the Hematoxylin-Eosin/HE[®] Staining Kit (Solarbio, China). Microscopic observation of liver damage was performed and photographed using a transmission electron microscope (TEM7500, Hitachi, Japan).

2.8. Whole-mount in situ hybridization (WISH) and immunohistochemistry

WISH was carried out using Dig-labeled mRNA antisense probes as described by [Thisse and Thisse \(2008\)](#) and [He et al. \(2011\)](#). Embryos, exposed to TCS from 6 to 72 or 96 hpf, were fixed in 4% paraformaldehyde (PFA)/phosphate buffer saline (PBS) overnight at 4 °C, washed twice in PBS at room temperature, and then maintained in absolute methanol at −20 °C until use. WISH results were recorded using a microscope camera (SZX16, Olympus, Japan).

Immunofluorescence (IF) was performed according to [Doganli et al. \(2016\)](#). The primary antibody was anti-GPER (rabbit; Alomone Labs, Israel) and the secondary antibody was FITC-labeled goat anti-rabbit (GeneTex, USA).

2.9. Statistical analysis

Each treatment and control group was performed using three

biological replicates and three technical replicates, and all data were recorded as mean ± SD (standard deviation, n = 3). Each biological replicate included 20 zebrafish (female:male = 1:1) and thus 60 zebrafish (3 × 20) were used in each group for RNA-seq, qRT-PCR, IF, WISH, luciferase analysis and histopathological observations. One-way analysis of variance (ANOVA) was used to analyze TCS-exposure effects, followed by Dunnett's tests to independently compare TCS-exposed treatments with the control group, and also used for bioinformatics analyses of sequencing data. All statistical analyses were conducted with SPSS 18.0 (SPSS, USA) using a $p < 0.05$, $p < 0.01$ or $p < 0.001$ significance level.

3. Results

3.1. Screening of differentially expressed miRNAs after TCS exposure

To identify differentially expressed miRNAs, we performed miRNA Illumina sequencing analysis on total miRNAs of zebrafish exposed to TCS from 4 to 96 hpf, in 62.5, 125 and 250 µg/L treatment groups as compared to the control group (0 µg/L). Inter-group comparisons revealed an altered miRNA biosignature. In total, 20 miRNAs demonstrated differential expression between treatment and control groups (p -value < 0.05 and Log_2 (|fold-change|) ≥ 0.5, per million fragments mapped (FPKM) ≥ 100), among which four miRNAs (miR-125b, miR-205, miR-142a and miR-203a) showed a consistent differential expression between each treatment group and control group ([Fig. 1A](#)). To better understand the biological functions of the four miRNAs, a

Fig. 1. Venn diagram and clustering analyses of four miRNAs along with their target gene-concerned pathways.

Note: (1) A, Venn diagram for the dysregulated miRNAs in comparisons of control vs. 62.5 µg/L treatment, control vs. 125 µg/L treatment and control vs. 250 µg/L treatment; (2) B, clustering analyses of four conserved and differentially expressed miRNAs; (3) C, heat map and clustering patterns of the differentially expressed miRNAs and their target gene-related pathways; (4) C, prediction using human miRNAs (p -value threshold < 0.05 and MicroT threshold < 0.08); and (5) D, prediction using zebrafish miRNAs (p -value threshold < 0.05 and MicroT threshold < 0.08).

clustering analysis on their expression pattern changes was conducted under different TCS-exposure conditions using Multi-Experiment Viewer (V4.9) Software (Fig. 1B). The expression pattern for the four miRNAs showed different trends in different TCS-exposure groups (Fig. 1B). For example, miR-125b was slightly and prominently up-regulated in the 62.5 and 250 µg/L treatments, respectively, but slightly down-regulated in the 125 µg/L treatment. In contrast, expressions for miR-205, miR-142a and miR-203a showed a concentration-dependent up-regulation. Their target pathways in humans and zebrafish are shown in Fig. 1C and D, respectively, based on analysis with DIANA miRPath V3 software (<http://snf-515788.vm.okeanos.grnet.gr/>). Except for has-miR-203a, miR-125b is mainly associated with fatty acid synthesis and metabolism, O-glycan biosynthesis, Hippo signaling pathway and so on; hsa-miR-205 mainly takes part in nucleotide excision repair, cell cycle, ErbB signaling pathway and so on; hsa-miR-142 mainly participates in the p53 signaling pathway, prostate cancer, glioma and etc. (Fig. 1C). In zebrafish, dre-miR-205, dre-miR-203a and dre-miR-142a mainly participate in fatty acid biosynthesis and metabolism pathways, but there is a lack of information on dre-miR-125b

function (Fig. 1D). Three of the four miRNAs were highly conserved across vertebrate species, only dre-miR-203a exhibited two different bases (Fig. 2B).

The miRNA expression patterns are remarkably specific and diverse, suggesting that expression sites and distribution of miRNAs in different tissues and organs are strongly related to their functions (Kloosterman et al., 2006). To further illustrate the functions of differentially expressed miRNAs, we analyzed the main expression site of the four miRNAs (miR-125b, miR-205, miR-142a and miR-203a) in three species (zebrafish, human and mice) based on results from previous studies (<http://www.microna.org/microna/getExprData.do>; Wienholds et al., 2005; Landgraf et al., 2007). The expression system and distribution of the four miRNAs in organs demonstrated overlapping occurrence in the three species (Fig. 2A). This was especially evident for miR-125b, the main expression system was observed in the central nervous system, digestive system and cardiac system, and the main distribution was found in the brain, liver, heart, adipose tissue and kidney of zebrafish, humans and mice. The miR-142a and miR-205 entities were highly conserved in terms of their sequences in zebrafish, humans and mice (Fig. 2B). In contrast, two bases in miR-203a

Fig. 2. The regulation network, conservative analyses and expression site of four miRNAs and the 3'UTR binding site of dre-miR-125b target genes.
Note: (1) A, miRNA expression patterns in zebrafish, human and mice; (2) B, evolutionary conservation of miR-125b, miR-142a, miR-203a and miR-205 sequences; (3) C, the network diagram plotted by Cytoscape (v3.5.1) software; and (4) The binding site of the potential target genes of dre-miR-125b in 3'UTR sequence.

were different among the three species.

In zebrafish, the regulation network of the four miRNAs and their related target genes were involved in lipid metabolism (Fig. 2C; Moon and Horton, 2003; Wang et al., 2003; Broeder et al., 2015). The related target genes of dre-miR-125b were *pparda* and *ppargcla*, as a function of fatty acid oxidation, and *acsl3b* and *acsl14a*, as a function of fatty acid synthesis (Fig. 2C). The target genes of miR-125b binding sites are shown in Fig. 2D. Moreover, the target genes of dre-miR-125b, dre-miR-203, dre-miR-142a and dre-miR-205 overlapped in the fatty acid synthesis pathway, suggesting that all four miRNAs participated in fat metabolism regulation. Because few previous studies have focused on the metabolism function of miR-125b in zebrafish, we chose this miRNA for further functional analysis.

3.2. Effect of miR-125b abnormal expression on lipid metabolism

TCS can affect key enzymes in fatty acid and cholesterol metabolism in cell lines (Tili et al., 2012). The previous section's analyses demonstrated that the dysregulated miR-125b, dre-miR-203, dre-miR-142a and dre-miR-205 were involved in fatty acid metabolism in humans. Therefore, we examined lipid metabolism using Oil Red O (ORO) staining after TCS exposure to zebrafish. The regulatory role of miR-125b in lipid metabolism was also characterized by qRT-PCR analysis and ORO staining in zebrafish embryos, which were injected with miR-125b agomir for overexpression, and antagomir for suppression.

Preliminary embryo-injected experiments showed that when the injected miR-125b concentration was 200 ng/ μ L and 2 nL/zebrafish, no obvious toxicological effects or phenotypic malformation of zebrafish were observed (data not shown). qRT-PCR results showed that agomir significantly increased the expression of miRNA-125b by ca. 2.7-fold ($p < 0.001$; Fig. 3A). In contrast, antagomir significantly decreased the expression of miR-125b when compared with the control group (no injection of agomir or antagomir; $p < 0.01$; Fig. 3A). These results showed that miR-125b agomir and antagomir had prominent over-expression and suppression functions, respectively. Interestingly, we found accumulation of lipid droplets in zebrafish yolk, brain and liver regions in miR-125b agomir-injected embryos (Fig. 3B and 3C). The increase of TCS-exposure concentrations was concomitant with increasing lipid synthesis and up-regulation of miR-125b (Fig. 3D–F), and this result was the same as the miR-125b agomir-injected experiments. These results imply that up-regulation of miR-125b due to TCS exposure was associated with an increase of lipid synthesis. Consequently, we conclude that abnormal expression of miR-125b affected zebrafish normal lipid metabolism.

Histopathological observations following HE staining of the control group showed that the liver tissue was clear in texture, the hepatic plate was arranged in an orderly fashion, the structure of the hepatic sinusoid was normal, and the nucleus, located in the center of cell, was large and round as well as displaying clear staining (Fig. 4A). In contrast, TCS exposure led to a series of pathological phenomena: (a) the disse space

Fig. 3. Oil red O staining, and expression of dre-miR-125b by miRNA-seq or qRT-PCR.

Note: (1) A, overexpression or inhibition of miR-125b by qRT-PCR after microinjection of agomir-125b or antagomir-125b; (2) B, Red O staining in zebrafish embryos injected with agomir-125b or antagomir-125b; (3) C, IOD analysis of Oil Red O staining; (4) D, the differential expression of miR-125b by miRNA-seq or qRT-PCR; (5) E, Red O staining in zebrafish embryos exposed to TCS; (6) The capital letters in Fig. 3B and E: B, brain; L, liver; and Y, yolk; (7) F, The IOD analysis of Oil Red O staining in control and TCS-exposure groups; and (8) **, ***, and **** indicate significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Fig. 4. Histopathological observations on adult zebrafish liver.

Note: (1) A–D, HE dyeing of adult zebrafish liver; (2) E–H, TEM images of hepatocyte in zebrafish; (3) I–L, TEM images of hepatin in zebrafish; (4) Nu, nucleus; Nm, nuclear membrane; Ch, chromatin; Mi, mitochondria; ER, endoplasmic reticulum; He, hepatin; Ld, lipid droplet; (5) “a arrow” in Figs. 4B, TCS-exposure leading to the decrease in nucleus number; and (6) “b arrow” in Fig. 4C and D, steatosis.

of hepatocytes was narrow and vague in the 62.5 µg/L treatment (Fig. 4B); and (b) steatosis occurred in the 125 and 250 µg/L treatments (cavities formed when xylene was dissolved by fat in the process of xylene-dewaxing) (Fig. 4C and D). These abnormal phenomena implied that long-term TCS exposure could result in an increase of fat particles and further formation of fatty liver in adult zebrafish liver. TEM observation showed no obvious differences for hepatocytes between the control and 62.5 µg/L treatment (Fig. 4E and F). However, a cavity occurred in the 125 and 250 µg/L treatments resulting from the injury of organelle (Fig. 4G and H). Similarly, no significant differences were observed in hepatin between the control and 62.5 µg/L treatment (Fig. 4I and L). However, the 125 and 250 µg/L treatments produced accumulation of large lipid droplets (Fig. 4K and L). Although no prominent inflammatory symptoms occurred, the effects were very similar to nonalcoholic fatty liver disease (NAFLD) indicating that long-term TCS exposure might contribute to development of NAFLD (Iancu and Manov, 2011).

3.3. Origin for up-regulation of miR-125b and screening for transcriptional factors

The miR-125b unit has three pri-miR-125bs (pri-miR-125b1, pri-miR-125b2 and pri-miR-125b3) in the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>). To assess the role of pri-miR-125bs in dysregulated miR-125b, we performed qRT-PCR analyses for pri-miR-125bs. Upon TCS exposure, pri-miR-125b1 and pri-miR-125b3 were prominently up-regulated, which was consistent with that of mature miR-125b. An extremely significant up-regulation ($p < 0.01$) was observed

for pri-miR-125b1 in the 250 µg/L treatment and pri-miR-125b3 in the 62.5 µg/L treatment (Fig. 5A). However, up-regulation was not as strong for pri-miR-125b2. As a result, the up-regulation of mature miR-125b mainly resulted from pri-miR-125b1 and pri-miR-125b3 rather than from miR-125b2.

To further confirm the distribution and expression changes of pri-miR-125b1 and pri-miR-125b3 upon exposure to TCS, two antisense oligonucleotide probes (248 and 386 bp) were synthesized, using in vitro transcription, for WISH analysis of pri-miR-125bs. The primers prepared for the above probes are listed in Supplementary Table 1. Sequencing results showed that the correct probes were synthesized for pri-miR-125b1 and pri-miR-125b3 (Supplementary Fig. 1). WISH showed that pri-miR-125b1 had similar expression sites to that of pri-miR-125b3, i.e., high expression in head and slight expression in visceral mass, liver and vertebrate (Fig. 5C and 5E). Upon TCS exposure to 72-hpf zebrafish, the expression of pri-miR-125b1 increased with the increase of TCS concentration, especially for the 250 µg/L treatment ($p < 0.01$). It was similar for 96-hpf zebrafish where 62.5, 125 and 250 µg/L treatments all significantly increased ($p < 0.05$) the expression of pri-miR-125b1 compared to the control (Fig. 5D). For 72- and 96-hpf larvae, significant up-regulation of pri-miR-125b1 occurred in vertebrate of the 62.5 µg/L treatment, but down-regulation occurred in the 125 and 250 µg/L treatments (Fig. 5C). The expression of pri-miR-125b3 was higher in 72-hpf larvae than in 96-hpf larvae of the control group; however, it increased in the brain and liver upon TCS exposure (Fig. 5E and F). In summary, the changing trends for the precursors of pri-miR-125b1 and pri-miR-125b3 were in general agreement with that of mature miR-125b, implying that up-regulation of miR-125b originated

Fig. 5. WISH and qRT-PCR of transcriptional factors and pri-miR-125bs.

Note: (1) A, pri-miR-125b gene expression in 96-hpf zebrafish larvae exposed to TCS from 6 to 96 hpf; (2) B, the expression of transcriptional factors in 96-hpf zebrafish larvae; (3) C, WISH of *pri-125b-1* expression in 72- and 96-hpf zebrafish larvae; B: brain; L: liver; Ve: vertebra; (4) E, WISH of *pri-125b-3* expression in 72- and 96-hpf larvae; (5) G, WISH of *nfe2l2* and *c/EBPα*; G, gill; Ob, olfactory bulb; Ss, swimming sac; (6) Fig. 5D and F indicate IOD of *pri-miR-125b-1* and *pri-miR-125b-3* genes, respectively; (7) Fig. 5H denotes the IOD of *nfe2l2* and *c/EBPα* genes; (8) “*”, “**” and “***” in Fig. 5D, F and H indicate the significance levels at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Fig. 6. Prediction for binding sites of transcriptional factors.

Note: (1) The binding sites for transcriptional factors are in box; (2) green font for CEBPA binding site, blue font for NFE2, red font for MAF:NFE2, purple font for Nfe2l2 and yellow font for overlap region.

from direct shear processes associated with the above two up-regulated precursors at the transcriptional level.

Previous research identified that miR-125b transcription was activated by ER α , C/EBP α and Nrf2 in mammal cell lines (Zhang et al., 2015; Romero et al., 2015). To explore which transcription factor regulated pri-miR-125bs expression in zebrafish embryos, we used the JASPAR web service (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl, Relative profile score threshold was 80%) and found several C/EBP α and Cap'n'collar (CNC) family bZip protein (NFE2, Nfe2l2/MAF:NFE2) putative binding sites at the 5' upstream promoter region (-2 to +1 kb) of mir-125b genes (Johnsen et al., 1998). Two NFE, two C/EBP α and one Nfe2l2/MAF:NFE2 binding sites were found between -2000 to -137 kb of *dre-mir-125b-1* (Fig. 6). Additionally, one NFE, two C/EBP α and one Nfe2l2/MAF:NFE2 binding sites were found between -2000 to

-707 kb of *dre-mir-125b-3*. The detailed information on binding sites of transcriptional factors in promoters is summarized in Supplementary Table 2. Moreover, NFE2, Nfe2l2/MAF:NFE2 distribution on the promoter of pri-mir-125b genes was similar in human as in zebrafish (*Danio rerio*), suggesting that these transcriptional factors played an important regulation role in expression activities of pri-miR-125b-1 and pri-miR-125b-3.

The expression levels of transcription factors in TCS-exposed 96-hpf zebrafish embryos were also characterized by qRT-PCR analysis. Expression of transcriptional factors showed a generally decreasing trend for ER α and ER β (Fig. 5B). In contrast, a generally increasing trend was observed for *nfe2l2*, *nfe2* and *c/ebp α* (Fig. 5B), which was consistent with pri-mir-125b1, pri-mir-125b-3 and mature miR-125b (Fig. 5A). These results imply that increased expression of pri-mir-125b-

Fig. 7. Promoter characteristics and activity analyses of *mir-125b-1* and *mir-125b-3*.

Note: (1) A, schematic of CpG island in the promoter region of *mir-125b-1* and *mi-125b-3*; (2) Green region: putative CpG island; (3) B, the activities of promoter fragments in *mir-125b-1* and *mir-125b-3*.

1 and pri-mir-125b-3 was possibly due to *nfe2l2*- and *c/ebpα*-integration with positive control element(s) of promoters for the above two precursors. Two antisense oligonucleotide probes (654 and 740 bp) were synthesized, using in vitro transcription, for WISH of *nfe2l2* and *c/ebpα*. The primers prepared for the above probes are listed in Supplementary Table 1. The sequencing results showed that the correct probes were synthesized for *nfe2l2* and *c/ebpα* (Supplementary Fig. 1). By means of WISH analysis, the expression of *c/ebpα* was mainly observed in the liver (Fig. 5G), which significantly increased ($p < 0.05$) with increasing TCS-exposure concentrations (Fig. 5H). As for *nfe2l2*, its expression was broadly found in the nasal cavity, gills, liver and swimming bladder. With increasing TCS-exposure concentrations, the expression of *nfe2l2* especially increased in the liver and swimming bladder ($p < 0.05$ or $p < 0.01$; Fig. 5G & 5H). From these results, we infer that TCS exposure leads to up-regulation of *nfe2l2* and *c/ebpα* and the overexpression of transcriptional factors initiated the promoter activity of the miR-125b precursor.

3.4. Promoter characteristics and activity analysis of *mir-125b-1* and *mir-125b-3* genes

To further understand the promoter characteristics of miR-125b genes, the promoter regions of pri-miR-125b-1 and pri-miR-125b-3 were analyzed by constructing a series of reporter plasmids containing different regions of the promoter (Fig. 7A, Supplementary Fig. 2). The activities of promoter segments were detected at 48 hpi (hour post injection) by transferring mir-125bs promoter region constructs and pGL 3.0 Basic into zebrafish embryos. pRT-TK was co-injected as an internal reference plasmid with the above constructs and the promoter-less plasmid into zebrafish embryos consisting of three biological and three technological replicates. The promoter activities of mir-125b-3 in the

region from -615 to $+14$ bp and -1327 to $+14$ bp were approximately 4- to 5-fold higher compared to that from the promoter-less construct (pGL 3.0 Basic). In contrast, gene expression from -1872 to $+14$ bp was significantly lower than other regions, suggesting that there might be negative control element(s) between -1872 and -1327 bp (Yoon et al., 2004). Further, positive control element(s) might be present between -1327 and $+14$ bp. But the promoter activities of miR-125b-1 from -1969 to -1 bp and from -1490 to -1 bp were significantly lower than from -487 to -1 bp. Epigenetic alterations such as DNA methylation, histone modifications and non-coding RNA (including microRNA) associated gene silencing have been identified as a major characteristic in human cancers (Kuosmanen et al., 2007). The presence of multiple methylated CpG sites in CpG islands of promoters causes stable silencing of genes (Deaton and Bird, 2011) and occurs frequently in promoters for microRNAs. In addition, regions located within 2 kb of CpG islands are also involved in regulation of miRNAs (Rongione and Webster, 2009). Consequently, we used EMBOSS CpGpilot web service to identify CpG islands in pri-mir-125b-1 gene promoters (Observed/Expected ratio > 0.60 Percent C + Percent G > 50.00 Length > 200). The putative CpG islands were found between -1392 and -456 bp in the promoter of the pri-mir-125b-1 gene (Fig. 7A). These results suggested that methylation of CpG islands might be involved in suppression of the pri-mir-125b-1 gene in zebrafish embryos, which could explain why the promoter activity of pri-mir-125b-1 containing CpG islands was lower than that without CpG islands (Fig. 7A and B). Additionally, the pri-mir-125b-3 gene contained two relatively GC-rich regions (-1267 to -970 bp and -544 to -327 bp), which might be involved in self-regulation (Fig. 7C). Moreover, as shown in Fig. 7D, the promoter activity of pri-mir-125b-3 containing the above two GC-rich regions (-1267 to -970 bp and -544 to -327 bp) was lower than that containing just one GC-rich region. Both

Fig. 8. qRT-PCR and immunofluorescence of GPER.

Note: (1) A, differential expression of GPER gene by qRT-PCR; (2) B, Immunohistochemistry for GPER in 96-hpf zebrafish; (3) The capital letters in Fig. 8B: B, brain; L, liver; G, gill; Ob, olfactory bulb; and (4) “*”, “**” and “***” indicate significance levels at $p < 0.05$ and $p < 0.01$, respectively.

of the promoter regions, −487 to −1 bp for pri-mir-125b1 and −1327 to +14 bp for pri-mir-125b-3, had the binding sites of NFE2 and Nfe2l2/MAF:NFE2 (Fig. 6). The activities of the above regions were higher than other ones, which further demonstrate that the transcriptional factor Nfe2l2 is associated with the regulation of pri-mir-125b1 and pri-mir-125b-3.

3.5. Effect of TCS exposure on G protein-coupled estrogen receptor (GPER)

Since environmental estrogen-like pollutants such as bisphenol A, nonylphenol, dichlorodiphenyltrichloroethane and genistein can bind to GPER, they may promote or antagonize GPER signaling (Zimmerman et al., 2016). GPER is a novel estrogen receptor that can mediate multiple estrogen functions independent of classic estrogen receptors (Carmeci et al., 1997). Also, it can crosstalk with multiple signal transduction pathways, and induce estrogen-like diseases. As a result, GPER can be used as a potential new target for treatment of estrogen-like diseases, and also for monitoring direct targeting of estrogen effects.

As reported above, the classic estrogen receptors, ERα and ERβ, exhibited different expression trends (i.e., no positive dependence) compared with those of pri-mir-125b1 and pri-mir-125b3 at transcriptional levels, and thus they were not the direct factor up-regulating miR-125b. We infer that the estrogen-like TCS can affect GPER expression and may promote or antagonize GPER-signaling pathways. The potential estrogen effects of TCS exposure on GPER were investigated at transcriptional and protein expression levels by qRT-PCR and immunofluorescence (IF). The transcription levels of the GPER gene were significantly increased ($p < 0.05$ for 62.5 and 125 μg/L treatments; $p < 0.01$ for 250 μg/L treatment) after 96-h exposure, showing a concentration-dependent relationship (Fig. 8A). IF results showed that upon TCS exposure, GPER expression was significantly increased ($p < 0.05$ for 125 μg/L treatment; $p < 0.01$ for 62.5 and 250 μg/L treatments), especially in the zebrafish forebrain and trunk (Fig. 8B and C).

4. Discussion

Herein, we identified four up-regulated miRNAs (miR-125b, miR-203a, miR-205, miR-142a) that were associated with fatty acid synthesis pathways, and also documented that the expression sites for miR-125b were strongly related to fat metabolism, mainly in the brain and liver. Given the paucity of information regarding the fat metabolism pathway of miR-125b, this study provides new information to assess various regulating mechanisms. Bioinformatic analysis demonstrated

that there were many potential fat metabolism-related target genes such as *pparda*, *ppary*, *acsl3b* and *acsl4a*. Previous reports showed that these genes are involved in fat synthesis and oxidization, suggesting that miR-125b plays an important role in fat metabolism (Moon and Horton, 2003; Wang et al., 2003; Broeder et al., 2015).

An increase of pri-miRNA was a major indicator for documenting changes in miRNAs. TCS exposure led to up-regulation of pri-mir-125b-1 and pri-mir-125b-3. The miR-125b sequencing and qRT-PCR results following TCS exposure to zebrafish did not show concentration-dependent relationships. This may result from whole zebrafish analysis that does not reflect preferential accumulation and impact on specific target organs/tissues. In contrast, WISH analysis documented changes in specific tissues such as the liver and brain, where expression of pri-mir-125b-1 and pri-mir-125b-3 showed an obvious positive concentration-dependence.

Upon TCS exposure, the changing trends for the transcriptional factors, *nfe2l2* and *C/EBPα*, were similar as those of pri-mir-125b-1 and pri-mir-125b-3. There were also potential binding sites for *C/EBPα* and *Nfe2l2* in the promoter regions of *mir-125b-1* and *mir-125b-3*. The gene-encoding *Nfe2l2*, belonging to CNC-bZIP proteins, has been shown to be highly polymorphic and these functional risk alleles and haplotypes have been identified in various oxidative stress-related human disorders (Motohashi et al., 2004). *Nfe2l2* can heterodimerize with Maf proteins, and Maf:CNC heterodimers interact with anti-oxidant/electrophile responsive elements (ARE) on the gene promoters leading to active gene expression (Kuusmanen et al., 2007). The consensus sequence for the ARE is TGACnnnGC, and *C/EBPα* proteins interact with the CCAAT box motif. Because *C/EBPα* and *Nfe2l2* have binding sites in *mir-125b-1* and *mir-125b-3*, they can interact with the promoter motif to induce expression of the above-mentioned genes. As a consequence, TCS exposure can lead to increased expression of miR-125b indirectly by up-regulation of the transcriptional factors (*Nfe2l2* and *C/EBPα*).

Luciferase analysis of cells and fertilized eggs detected promoter activities for miRNA in zebrafish for the first time. *In vivo* detection is a more sensitive approach for detecting the activity of promoters. The luciferase analytical results showed that CpG islands were involved in constitutive expression of *mir-125b-1* and *mir-125b-3*. Additionally, according to the higher activity of promoter segments containing *Nfe2l2* binding sites, we infer that *Nfe2l2* was possibly the transcriptional factor inducing up-regulation of *mir-125b-1* and *mir-125b-3*.

TCS is a type of weak estrogen that affects classical estrogen nuclear receptors (ERα and ERβ) (Gee et al., 2010; Huang et al., 2014). However, the present study demonstrated that TCS's estrogen activity results from its effects on GPER, a novel membrane receptor, rather than its effects on the classical ERα and ERβ receptors. GPER can mediate

Fig. 9. Predicted up-stream mechanisms for up-regulation of miR-125b from TCS exposure.

multiple signal transduction pathways, such as PI3 K/Akt and MAPK (Revankar et al., 2005). Activation of GPER1 protects neonatal rat cardiomyocytes against H_2O_2 -induced cell injury by up-regulating and activating Nrf2 expression via the PI3 K/Akt signaling pathway (Li et al., 2014a,b; Chen et al., 2016b). Based on these findings, we inferred that interaction between TCS and GPER activated the down-stream signaling pathway PI3 K/Akt or others, further activating and increasing the expression of Nrf2 (Nfe2l2) in the nucleus. Nrf2 can form heterodimers with maf, and these heterodimers could interact with the promoter regions (ARE elements) of mir-125bs, which further promote the expression of *mir-125b-1* and *mir-125b-3*, and finally led to the up-regulation of miR-125b (Fig. 9). Our findings provide guidance for further investigations to verify the proposed TCS-induced up-stream mechanisms inferred from our results.

5. Conclusions

The characteristics of pri-miR-125b gene promoter sequences were analyzed in detail, and several transcription factors were proved to promote miR-125b expression, resulting in changes to lipid metabolism-related down-stream target genes and further lipid accumulation as well as fatty liver diseases. The up-regulation of miR-125b was demonstrated to be originated from the direct shear processes of two up-regulated precursors (pri-mir-125b-1 and pri-mir-125b-3), whose increased expression possibly resulted from *nfe2l2*- and *c/ebpa*-integration with positive control elements of promoters. Additionally, CpG islands were possibly concerned with constitutive expression of *mir-125b-1* and *mir-125b-3*. TCS's estrogen activity resulted from its effects on GPER, a novel membrane receptor, rather than the classical ER α and ER β . These new findings enhance our understanding of mechanisms inducing liver diseases from exposure to environmental pollutants and also provide a guidance to the future up-stream mechanistic study on TCS-exposure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2017.10.021>.

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