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Adipogenesis of Perfluorooctanesulfonate (PFOS) on Japanese Medaka (*Oryzias Latipes*) Embryo Using *Ovo*–nanoinjection–mRNA Seq Analysis

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Perfluorooctanesulfonate (PFOS) is a worldwide persistent organic pollutant and suspected to have effects on animals. The present study used *in ovo*–nanoinjection to simulate the maternal bioaccumulation and biotransformation of PFOS from parental Japanese medaka (*Oryzias latipes*) to embryos, investigated and elucidated the obesogenic activity *in vivo* and the gene expression changes. Results showed that no significant differences on the survival of medaka embryos and larvae between control, solvent control, and three PFOS exposure groups (0.05, 0.5 and 5 ng per embryo). Hatching time of medaka embryo was significantly delayed in the 0.05 ng PFOS exposure group and significantly accelerated in the 5 ng exposure group compare with those in control. The adipocyte areas were significantly increased in 0.05 and 0.5 ng per embryo PFOS exposure groups comparing to that of the control. The mRNA sequencing analysis on the medaka larvae chromosome gene expressions showed that 24 genes had significantly changed expressions ($p < 0.05$, $FDR < 0.05$) including genes involved in the pathways of metabolism and adipogenesis, like *g6pca.2* in the 'Insulin signaling pathway' and 'Adipocytokine signaling pathway'. These results suggested that *ovo*–nanoinjection–mRNA Seq analysis is a reliable method to evaluate and understand the maternal effect of chemicals.

Key words: Perfluorooctanesulfonate (PFOS), Adipogenesis, mRNA–Sequencing, Pathway analysis

INTRODUCTION

Perfluorooctanesulfonate (PFOS) is an anthropogenic fluorosurfactant and a worldwide persistent organic pollutant, even in the polar area (Rigét *et al*., 2016). PFOS has been used in a lot of industrial materials for our daily life. It has low toxicity but is bioaccumulative along the food chain of the environment (Quinete *et al*., 2009). It has been detected in various species (Taniyasu *et al*., 2003), even in the human being (Saikat *et al*., 2013). PFOS has been confirmed to be bioaccumulative and have toxic effects on human being, and possibly involved in increasing the risk of chronic kidney diseases in general US population (Shankar *et al*., 2011).

In the aquatic environment, many studies reported the appearances of PFOS in most waters (Liu *et al*., 2017; Loos *et al*., 2010; Wei *et al*., 2007), sediments (Mourier *et al*., 2019; Sammut *et al*., 2019), fish body and organs and other aquatic living organisms (Llorca *et al*., 2017). It has also been detected in multiple wildlife animal organs and plasma (Houde *et al*., 2006). PFOS showed developmental and other adverse effects in fish and its embryos (Fang *et al*., 2013; Huang *et al*., 2012; Huang *et al*., 2011).

Recently, PFOS was reported as a potential obesogen that may affect the adipogenesis (Qiu *et al*., 2018; Xu *et al*., 2016) and adipocyte differentiation (Watkins *et al*., 2015) of wildlife. It was also suggested that PFOS interacted the expression of genes encoding key proteins of peroxisome proliferator–activated receptors (PPARs) signaling pathway (Fang *et al*., 2012; Yamamoto *et al*., 2015). The PPARs are nuclear receptor proteins that work as transcription factors and regulating expression of genes (Berger and Moller, 2002; Kersten *et al*., 2000), which involved in development and metabolism. In the PPARs, pparα, pparβ/δ and pparγ are three key proteins involved in the lipid metabolism and adipocyte differentiation. The alteration of PFOS on the expression of PPARs genes has been reported in multiple animals, including hamster (Jacquet *et al*., 2012) and fish (Fang *et al*., 2012; Krøvel *et al*., 2008). The effects of PFOS on PPARs system may affect not only lipid metabolism and genesis, but also developmental processes and some diseases. It is necessary to study the mode of action of the PFOS toxicity in the developmental stages of animals.

Japanese medaka (*Oryzias latipes*) is a developing model in aquatic toxicity research (Wittbrodt *et al*., 2002). Due to its clarified life cycle, developmental stages (Iwamatsu, 2004), and draft genome information (Kasahara *et al*., 2007), medaka becomes a suitable model for aquatic toxicology and developmental

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research at both *in vivo* and molecular level (Owens and Baer, 2000). Generally, embryos are more sensitive to adult fish to the environmental pollution. Some of the developmental effects on fish can be discovered during embryonic developing stages. Maternal influence caused by chemicals accumulated *in vivo* may have more effect on embryos than adult fish. Together with the *in vivo* nano–injection, embryo exposed to PFOS can be used to stimulate maternal biotransformation.

There were three main methods used in the present study, the *in vivo*–nanoinjection, Nile red staining and mRNA–Seq. The *in ovo*–nanoinjection is a unique method for early life stage toxicity studies in fish (Walker *et al*., 1996). It can inject an accurate amount of toxicant into embryos. It is a mature and efficient technique to evaluate active compound toxicity in the laboratory (Chen *et al*., 2017; Hano *et al*., 2007; Nassef *et al*., 2010). The Nile red staining assay can measure the lipid tissue areas. It has been used for staining intracellular lipid and measuring adipocyte areas *in vivo* of animals (Imrie and Sadler, 2010; Stone *et al*., 2004; Tingaud–Sequeira *et al*., 2011). In order to analyze the toxicity and adipogenesis effects from molecular level, the mRNA sequencing (mRNA–Seq), a powerful and sensitive method widely used in toxicology, was performed. The mRNA–Seq, which is one of the next generation sequencing analysis (Shi *et al*., 2008), has been developed as a highly sensitive and accurate method to quantify gene expression of whole transcriptomes with the ability to identify both known and novel transcript isoforms, gene fusions, and other changed gene expressions.

In this study, survival and hatching condition of medaka embryos were measured to evaluate the lethal toxicity of PFOS. An altered adipocyte area of PFOS on medaka early life stages has been measured by *in vivo* Nile red staining. The mRNA–Seq result provided evidence of the obesogen characteristic of PFOS at gene expression level. Altogether, the toxicity and adipogenesis effect of PFOS (0.05, 0.5 and 5 ng per embryo) stimulated from maternal fish were profiled from both physiological and molecular levels.

MATERIALS AND METHODS

Medaka fish and embryo culture

The adult fish that provided embryos and the embryos were cultured and pre–treated in the same way described in our previously published research in detail (Chen *et al*., 2017). Embryos were taken from 10 females and 5 male medaka mating pairs that were cultured in a tank (60 cm × 30 cm × 30 cm) with artificial sea water (1‰ salinity, 45 L, 27 ± 1˚C, light: dark = 14:10). The fish were fed two times every day with 24 h incubation *Artemia nauplii* juveniles. Healthy fertilized embryos (average embryo volume 0.52 μl, average embryo weight 1.04 mg) were taken within 2 hours after spawning. After embryos were sterilized in 0.9% H_2O_2 for 10 min to minimize the infection that may cause by fungal and bacterial (Marking *et al*., 1994), they were washed with and cultured in the embryo culture medium (ECM) solution (0.1% NaCl, 0.008% $MgSO_4$, 0.004% $CaCl_2$–H_2O, 0.003% KCl, 0.0002% Methylene Blue, pH = 7.0). Afterwards, embryos were cultured in six–well–culture plates (Becton, Dickinson and Company, USA), and no more than 10 embryos in each well which contained 12 ml of ECM solution. Plates were kept on a shaker (30 rpm) at 27 ± 1˚C.

Chemicals

Perfluorooctanesulfonate (PFOS) was dissolved in methanol with the concentration of PFOS 10 g l^{-1} methanol. Afterwards, triolein (95%, SIGMA–ALDRICH, USA) was used to dilute the PFOS–methanol solution. Input nitrogen to remove methanol from triolein diluted PFOS solution. Check and adjust PFOS concentration to 0.1 ng nl^{-1}, 1 ng nl^{-1} and 10 ng nl^{-1}. These triolein diluted PFOS solutions were used in the exposure group for *in ovo*–nanoinjection to medaka embryos, while triolein without PFOS were *in ovo*–nanoinjected to medaka embryos as solvent control group.

Nanoinjection

The method was previously described in the published papers of our laboratory (Chen *et al*., 2017; Hano *et al*., 2007), and the micro–capillary syringes (30 μm diameter pinhead with a 30˚ angle pinpoint) were prepared based on the methods introduced by Walker *et al*. (1996) for *in ovo*–nanoinjection of 0.5 nl per embryo of a liquid (about 0.1% embryo volume) into medaka embryos. The microcapillaries were coating with Sigmacote (Sigma–Aldrich) to increase the strength and cleaned with distilled water.

According to the medaka *in ovo*–nanoinjcetion description reported by Nassef *et al*. (2010), the embryos were stabilized on a 2% agarose plate and the oil droplets side were set on top. The syringes were filled with either triolein (solvent control) or PFOS solution (dosages mentioned above). The micromanipulator (SZX12, Olympus) and microinjector (FemtoJet, Eppendorf, Germany) were set up to inject 0.5 nl liquid into the oil droplets in the embryo yolk.

Exposure test for survival and hatching observation

One control (with neither PFOS exposure nor *in ovo*–nanoinjection operation) group, one solvent control (without PFOS exposure, but with 0.5 nl per embryo triolein *in ovo*–nanoinjection) group, and three PFOS exposure (with 0.1 ng nl^{-1}, 1 ng nl^{-1}, and 10 ng nl^{-1} triolein diluted PFOS *in ovo*–nanoinjected to medaka embryo) groups were set in the present exposure test. In the three exposure groups, the dosages of PFOS in each embryo were 0.05, 0.5 and 5 ng. In each group, 50 fertilized and healthy medaka embryos were used. All the operations to embryos were done within 8 hours post fertilization (hpf). They were cultured in ECM solutions and the culture was renewed every day.

The survival and hatching condition of embryos were observed daily for 12 days. All 'dead' embryos were

removed from the culture well. On the 12th day post fertilization (dpf), the unhatched embryos were also identified and counted as 'dead', and the hatched larvae were taken as samples from each group.

Staining and measurement of adipocyte area

The adipocyte area of medaka larvae was measured with the method reported by Tingaud–Sequeira *et al.* (2011). In each test group, 15 individuals were randomly taken and stained with Nile Red solution (NR, 5 μg l^{-1}), which was diluted with ECM culture solution. The live larvae were stained with NR in the dark for 30 min at 28°C, so that the adipose tissue could be saturated. In order to remove the staining on the surface, larvae were rinsed twice in the ECM solution for 5 min (10 min in total). The adipocyte of medaka larvae were observed under a fluorescence microscope (SZX12, Olympus). Pictures of the fluorescence adipocyte were taken by Canon EOS Kiss X6i camera, and the area of adipose tissue was measured by Image J (National Institutes of Health, http://rsb.info.nih.gov/ij/).

The mRNA sequencing

On the 12th dpf, three larvae were randomly and separately taken and stored for mRNA sequencing as three replicates in each test group. Considering the result of adipocyte area measurement, samples from solvent control and 0.5 ng PFOS exposure group were sent for mRNA–Seq analysis.

In solvent control and 0.5 ng PFOS exposure group, larvae were separately transferred into three 1.5 ml tubes containing 1 ml trizol–1%SDS. The larvae were crushed and homogenized (vortex, 20 s). Total–RNA was extracted following the protocol developed by Chomczynski *et al.* (1987), and using RNeasy Mini Kit (Qiagen, USA).

The mRNA purification, quality check and mRNA sequencing procedures were the same as the published paper of our laboratory (Chen *et al.*, 2017). The mRNA was purified from total–RNA using the NEBNext® Poly (A) mRNA Magnetic Isolation Module (New England Biolabs Inc., USA). The mRNA quality was analyzed by the Agilent 2100 Bioanalyzer (Agilent Technologies, USA), and generated a parameter called the RNA integrity number (Rigét *et al.*, 2016). The mRNA samples were accepted and sent for sequencing when the RNA integrity number (RIN) was higher than 8.0. It was the threshold for high quality of mRNA. In the present study, the mRNA sequencing was operated by following the manufactures' instructions of the NEBNext® Ultra RNA Library Prep Kit for Illumina® (New England Biolabs Inc.), the NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1, New England Biolabs Inc.), and the Miseq Reagent Kit v3 (300 cycles) (Illumina, MS–102–3003, USA).

Statistical analysis

Survival rate, hatching time and adipocyte area data analysis

The significance between control, solvent control, and three PFOS exposure groups (0.05, 0.5 and 5 ng per embryo) on the survival rate, hatching time of medaka embryos and the adipocyte area of larvae were evaluated with Dunnett's Test by Microsoft Excel (2007). The significance threshold was $p < 0.05$, and $FDR < 0.05$.

The mRNA–Seq data analysis

The mRNA–Seq data analysis involved only samples from solvent control and 0.5 ng PFOS exposure group. The genome sequence and all information about genes (annotations, homologous, related protein functions, pathways, *etc.*) of Japanese medaka (*O. latipes*) were obtained from Ensembl (http://asia.ensembl.org/index.html), Gene Ontology Consortium (http://geneontology.org/), the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/), and Blast2Go (https://www.blast2go.com/).

For mRNA–Seq data analysis, FastQC (version 0.10.1), Trimmomatic (version 0.38), RiboPicker (version 0.4.3), Hisat2 (version 2.1.0), StringTie (version 1.3.5), R package edgeR (version 3.14.0), and Samtools (version 1.10) were used. Trimmomatic and RiboPicker were used to clean up the raw sequences and remove non–chromosomal sequences from raw data. Moreover, Integrative Genomics Viewer (IGV, version 2.3.37) was used to visualize the exon coverage of coding mRNA in each replicates. Gene expression difference was quantified as log counts per million (logCPM) and shown as log fold change (logFC). The values of the significance of differences (*p*–value and *FDR*–value) between solvent control and 0.5 ng PFOS exposure groups were automatically calculated by edgeR from all reliable experimental replicates.

RESULTS

Survival and hatching results

Survival of medaka embryos and larvae had been observed every day since fertilization. The result is shown in Table 1. There was no significant difference between all tested groups.

Figure 1 shows the average hatching time of medaka embryos in each tested group. There was no significant difference between the control and solvent control groups. Therefore the data of these two groups were combined and compared with the PFOS exposure groups. The Dunnett's test showed that the hatching time of medaka embryos in the 0.05 ng PFOS exposure group was significantly prolonged, while it was shortened in the 5 ng PFOS exposure group.

Adipose area analysis

Because the control and solvent control groups did not show any significant difference in the adipose tissue area in medaka larvae, the results of these two groups were combined and then compared with the PFOS exposure groups (Fig. 2). The Dunnett's test results showed that the 0.05 and 0.5 ng per embryo PFOS exposure medaka larvae had significantly increased adipocyte area compare to that of the control group.

Table 1. Survival number and rate of the medaka embryos and larvae in all tested groups since fertilization

Survival number of medaka embryos and larvae		Observation time (dpf)													Survival rate
		0	1	2	3	4	5	6	7	8	9	10	11	12	
PFOS dosage (ng/embryo)	Control	50	50	50	50	50	49	48	43	42	42	42	42	42	0.84
	Solvent control	50	43	43	43	43	43	43	43	42	42	41	41	41	0.82
	0.05	50	49	48	48	48	48	48	48	48	48	48	48	48	0.96
	0.50	50	41	41	41	41	41	41	40	39	39	39	35	35	0.70
	5.00	50	40	40	40	40	40	39	39	34	34	33	33	33	0.66

Embryos in the Control group were not in ovo–nanoinjected, embryos in the Solvent control group were in ovo–nanoinjected with triolein, and embryos in the exposure group were in ovo–nanoinjected with triolein dissolved PFOS at three concentrations. dpf: day post fertilization.

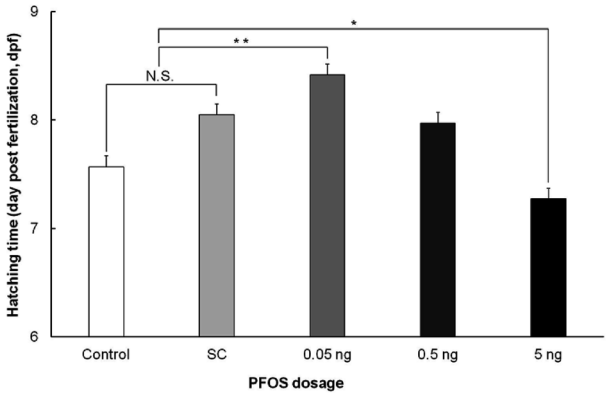

Fig. 1. The average hatching time of medaka embryos in different tested groups: control, solvent control, 0.05, 0.5 and 5 ng per embryo PFOS exposure groups.
SC: solvent control. N.S.: no significance, *: $p < 0.05$, **: $p < 0.01$ for difference in hatching time between tested groups.

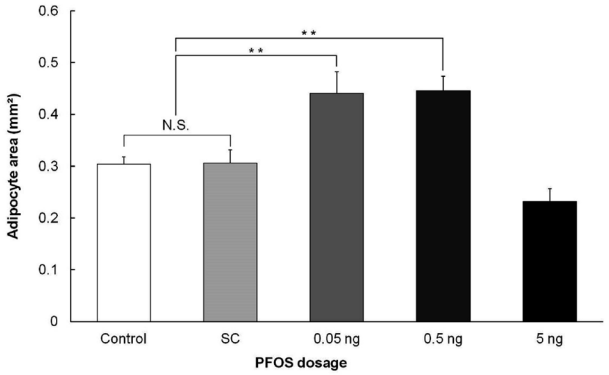

Fig. 2. The adipocyte area of hatched medaka embryos on the 12th day post fertilization in different tested groups.
The difference between the control and solvent control groups is not significant, therefore the data from the two groups were pooled and compared to the PFOS exposure groups. SC: solvent control, N.S.: no significance, **: $p < 0.01$ for differences in the adipocyte area between control and PFOS exposure groups.

The mRNA sequencing analysis

The mRNA RIN values of all tested groups were over 8.0, which means the quality of the mRNA samples taken from these replicates was acceptable for mRNA–Seq data analysis. Considering the results of hatching time and adipocyte area, hatched medaka larvae samples of 0.5 ng PFOS exposure group and the solvent control group were sent for mRNA–Seq analysis, because this dosage shows the least PFOS toxicity on medaka embryo hatching time and the highest potential on adipogenesis.

Results of mRNA–Seq showed that 24 genes have significantly changed expressions in the 0.5 ng PFOS exposure group comparing to that of the solvent control group (Table 2, Fig. 3). Expressions of 18 genes were up–regulated by PFOS exposure, while it was down–regulated that of the other 6 genes. Annotation of all 24 genes showed that they were mainly related to the regulation of transcription (*c–fos, cebpb, cebpd, si:ch211–220e11.3, safb*, and *rbm6*), stress response (*c–fos, hsp90aa1.2, hspa8b, ddit4, hpx*, and *ENSORLG00000003753*), metabolism and enzymatic activity (*dpp7, seripinh1b, upp2, arrdc2, g6pca.2, nfrkb*, and *ENSORLG00000003753*), metal ion binding (*zgc: 162730, dsg2, hpx*, and *ENSORLG00000003753*), and some cellular components (*dsg2, stx18, tm2d2, fbrs, arl2*, and *ENSORLG00000003753*). Some proteins encoded by the same gene have multiple functions due to different basis of grouping, for example, protein encoded by the gene *ENSORLG00000003753*. It is related to heme oxidation and catabolic process, iron ion homeostasis, and response to oxidative stress. It also has a molecular function of metal ion binding. Regarding to its functions, this protein usually appears across membrane, which is an integral component of membrane.

Genes related to adipogenesis and metabolism

In the present study, the genes encoded the key proteins, *ppar and rxr, in adipogenesis, pparaa, pparab, ppard, pparg, ppargc1a, rxraa, rxrba, rxrbb*, and *rxrgb*, did not show significant expression changes between solvent control and 0.5 ng PFOS exposure groups. Regarding to results of KEGG pathway analysis, genes *g6pca.2, upp2* and *ENSORLG00000003753* were involved in metabolic pathways. Gene *g6pca.2* and *upp2* were up–regulated, but *ENSORLG00000003753* was down–regulated. Gene *g6pca.2* encodes a subunit

Table 2. The mRNA–seq result of genes that have significant expression changes ($p < 0.05$, $FDR < 0.05$) between the solvent control and 0.5 ng PFOS exposure groups

Gene_shortname	GeneID	logFC	logCPM	FDR	Description
dpp7	ENSORLG00000017735	5.133	4.762	4.15E–02	dipeptidyl–peptidase 7
c–fos	ENSORLG00000024739	3.808	5.193	1.58E–02	c–fos protein
cebpb	ENSORLG00000015032	3.557	6.004	1.13E–05	CCAAT enhancer binding protein beta
zgc:162730	ENSORLG00000027149	3.307	6.122	9.80E–06	zgc:162730
seripinh1b	ENSORLG00000014312	2.181	6.653	5.34E–05	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1b
upp2	ENSORLG00000016737	2.120	6.169	7.04E–03	uridine phosphorylase 2
hsp90aa1.2	ENSORLG00000025267	2.059	8.026	3.91E–11	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2
hspa8b	ENSORLG00000005316	1.899	6.781	3.17E–04	heat shock cognate 70 kDa protein
rsrp1	ENSORLG00000017529	1.786	8.543	3.91E–11	arginine/serine–rich protein 1
ddit4	ENSORLG00000009171	1.711	7.455	1.43E–05	DNA–damage–inducible transcript 4
arrdc2	ENSORLG00000008572	1.697	7.073	3.17E–04	arrestin domain containing 2
cebpd	ENSORLG00000022254	1.683	6.302	4.19E–02	CCAAT enhancer binding protein delta
si:ch211–220e11.3	ENSORLG00000025379	1.635	6.361	4.19E–02	TSC22 domain family member 3
DSG2	ENSORLG00000017110	1.279	7.271	1.68E–02	desmoglein–2
g6pca.2	ENSORLG00000018711	1.170	7.852	5.50E–03	glucose–6–phosphatase a, catalytic subunit, tandem duplicate 2
safb	ENSORLG00000016989	1.164	8.358	4.24E–04	scaffold attachment factor B
rbm6	ENSORLG00000027833	1.162	7.386	3.88E–02	RNA binding motif protein 6
hpx	ENSORLG00000010251	1.114	7.824	1.33E–02	hemopexin
fbrs	ENSORLG00000024419	–1.770	6.996	6.10E–05	fibrosin; autism susceptibility gene 2 protein homolog
nfrkb	ENSORLG00000006270	–2.400	5.602	2.15E–02	nuclear factor related to kappaB binding protein
stx18	ENSORLG00000016755	–2.423	5.431	4.44E–02	syntaxin 18
tm2d2	ENSORLG00000016282	–3.701	4.878	4.15E–02	TM2 domain containing 2
arl2	ENSORLG00000005571	–5.174	4.656	1.98E–02	ADP–ribosylation factor–like 2
novel gene	ENSORLG00000003753	–5.181	4.658	1.98E–02	heme oxygenase

This logFC value represents the gene expression increase of the two groups. logFC: log2 (fold change), logCPM: log2 (counts per million), FDR: FDR value is a *p*–value adjusted for multiple tests (by the Benjamini–Hochberg procedure), 'Description' shows gene annotations matched to NCBI database.

of protein glucose–6–phosphatase (*g6pc*), which is an important enzyme in the 'Adipocytokine signaling pathway' and 'Insulin signaling pathway', and directly affects the inhibition of gluconeogenesis. The other 2 genes encoded proteins related to base metabolism processes. The annotation results of GO analysis showed other genes encoded proteins related to protein metabolism like *dpp7, serpinh1b* and *nfrkb* also had abnormal expressions after 0.5 ng PFOS exposure. Proteins encoded by the former 2 genes have dipeptidyl–peptidase and endopeptidase inhibitor activities respectively. Their expressions were up–regulated by the exposure of PFOS. Protein encoded by gene *nfrkb* is a protease binding protein has a decreasing expression after exposing to PFOS. Gene *upp2* encoding protein uridine phosphorylase 2 had an increasing expression after exposure test. It has nucleotide catalytic activity and transferase activity. Generally, most of medaka embryonic genes related to protein and adipogenetic metabolism were up–regulated after being exposed to 0.5 ng PFOS.

Genes related to other biological processes and molecular function

Genes *c–fos, cebpb, cebpd, si:ch211–220e11.3, safb*, and *rbm6* encoding proteins related to regulation of transcription. Expressions of these were all up–regulated by PFOS exposure. Proteins encoded by the upper 4 genes have the molecular function of DNA binding, while the latter 2 genes have that of RNA–binding. Protein c–fos, encoded by gene *c–fos*, also involved in the biological process of multicellular organismal response to stress. Its expression was up–regulated. Other expression changed genes related to stress–response processes were *hsp90aa1.2, hspa8b, ddit4, hpx* and *ENSORLG00000003753*. Among them, only *ENSORLG00000003753* has down–regulated expression. The heat shock proteins have been well known as the first responder to environmental stress. The GO annotation showed that proteins encoded by *hsp90aa1.2* and *hpx* involved in a biological process of response to estrogen stimulus. Proteins encoded by genes *ddit4* and *ENSORLG00000003753* have response to hypoxia and oxidative stress. Hemopexin and heme oxygenase encoded by gene *hpx* and *ENSORLG00000003753* respectively have the molecular function of iron/heme ion binding. Gene *dsg2* encoding protein desmoglein–2 has the molecular function of calcium ion binding. It is also functioning in cell adhe-

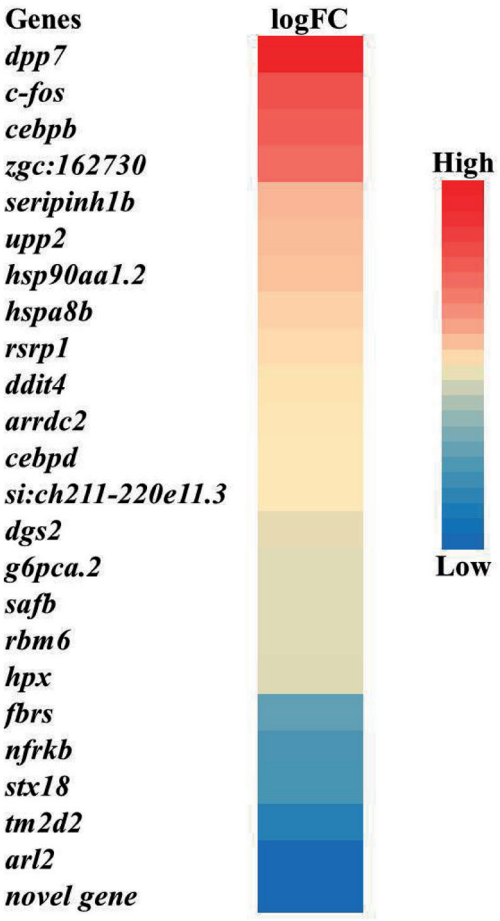

Fig. 3. Heatmap of the gene expressions (mRNA–Seq result, logFC) in different biological functions and processes. The genes have significantly changed expressions ($p < 0.05$, $FDR < 0.05$) between Solvent control and 0.5 ng PFOS exposure groups. logFC: logarithm value of fold change value of each gene.

sion and cell–cell adhesion, and an integral component of membrane. Other genes (*stx18, tm2d2, fbrs* and *arl2*) encoded proteins that were cellular components related to membrane, fibrosin or microtubule–cytoskeleton were down–regulated after 0.5 ng PFOS exposure.

DISCUSSION

Survival rate and hatching time of medaka embryos

The insignificant survival rate of medaka embryos in all tested groups (Table 1) indicates the low lethal toxicity of PFOS on medaka embryos. Some previous studies by other researchers also showed low doses of PFOS have high survival rates on zebrafish and marine medaka embryos (Fang *et al*., 2012; Li *et al*., 2015; Shi *et al*., 2008). On the other hand, Shi *et al*. (2008) reported a delayed hatching of zebrafish embryo after exposed PFOS (1.0, 3.0, 5.0 mg/l waterborne exposure). In the *in ovo*–nanoinjected zebrafish embryo, LC_{50} at 72 and 96 hpf were about 5.93 ng PFOS per embryo, and the hatching delay was also observed in the 2.0 and 20.0 ng per embryo PFOS exposure groups at 72 hpf (Li *et al*., 2015). Besides, result in the study of Wu *et al*. (2012) showed significantly accelerated hatching on marine medaka embryo, *Oryzias melastigma*, after exposure to higher dosage of PFOS (4 and 16 mg/l waterborne exposure). Considering the results of these previous studies, especially the *in ovo*–nanoinjection of PFOS on zebrafish (Li *et al*., 2015), which used the same exposure method as in the present study, LC_{50} of microinjected PFOS to zebrafish embryo was around 5.93 ng, and the high dosage of PFOS might cause a delayed hatching on fish embryo. However, the low dosage of PFOS on medaka embryo hatching time through *in ovo*–nanoinjection was lack of investigation.

In the present study, the highest dosages of PFOS exposure (5.0 ng) were lower than the LC_{50} in the previous research. The survival rates in the present study were consistent with the results in the studies mentioned above. Similar to the result of Li *et al*. (2015), the low dosage (0.05 ng) of *in ovo*–nanoinjected PFOS on medaka *O. latipes* embryo showed a significantly delayed hatching (Fig. 1) in the present experiment. According to the research of Wu *et al*. (2012), which represented an opposite effect of PFOS on marine medaka *O. melastigma*, the increasing expressions of hatching related enzymes genes (*hce* and *lce*) were responsible for the acceleration of marine medaka embryo hatching. Unfortunately, we did not collect samples at hatching time, and the mRNA–Seq result in the present study showed insignificant expression changes of the hatching related genes between control and the 0.5 ng POFS exposure groups. The survival and hatching time results of medaka embryos suggested a low lethal but effective toxicity of the tested PFOS concentrations. PFOS can affect the development of Japanese medaka embryo.

Adipogenesis effect of PFOS

The adipose area analysis measured by Nile red staining assay confirmed the adipogenetic effect of PFOS (Fig. 2). Adipocyte area of medaka larvae from 0.05 and 0.5 ng per embryo PFOS exposure group significantly increased, suggesting lower dosage of PFOS can activate the adipogenesis progress in the development of medaka early life stages. There is one research (Fang *et al*., 2012) reported that the key genes, *ppar*α and *ppar*β, in the PPAR pathway related to adipogenesis had significantly changed gene expression after being exposed to PFOS. The abnormal expressions of these key lipid compound receptors are able to affect the metabolism and synthesis of adipocytes, and therefore lead to the size difference of adipocyte areas *in vivo*.

However, in the present experiment, there was no significant expression change on the key genes involved in the PPAR pathway. This result reflected that one–time exposure with low dosage (0.5 ng) of PFOS on medaka embryo was not able to affect the expressions of *ppar* and *rxr* genes. Meanwhile, the significantly increased adipocyte area of hatched medaka larvae in 0.05 and 0.5 ng per embryo PFOS exposure groups indi-

cate an effective influence of PFOS on other pathways or metabolism progress related to adipogenesis.

Analyzing the mRNA–Seq result, gene *g6pca.2* related to adipocytokine and insulin signaling pathway has been found to have significantly changed expressions in the 0.5 ng PFOS exposure group (Table 2, Fig. 3). Previous research (Yan *et al.*, 2015) reported the abnormal expression of gene *g6pc* in the liver of mice after being exposed to perfluorooctanoic acid (PFOA). Their results indicated that after long–term (28 days) exposure to low dosage of PFOA, the expression of *g6pc* has increased. In the present study, expression of *g6pca.2* significantly up–regulated by the exposure of 0.5 ng PFOS, which belongs to the same compound family with PFOA. There was another research showed a high fat diet (HFD) of mice would significantly up–regulate the expression of gene *g6pc* (Tan *et al.*, 2013). HFD has been a main reason for adipocyte. Considering both HFD and PFOS exposure can lead to the up–regulation of *g6pc* expression, and integrate the increasing adipocyte area in medaka fish into account, the 0.5 ng PFOS exposure may cause adipogenesis effect to medaka embryo. In the present study, PFOS was one–time injected to medaka embryo. The toxicity might be decreasing during embryonic development. Therefore, other genes related to the adipogenetic process did not show significant expression changes at the sampling time point.

Other gene expression changes in the mRNA–Seq result

After being exposed to PFOS, some other genes of medaka embryo during development had changed their expressions. The proteins encoded by these genes have functions in multiple biological progresses. The up–regulated expression of genes involved in transcriptional regulation reflected effects of 0.5 ng PFOS on DNA transcription. According to the gene ontology analysis, some of these genes have molecular function of DNA–binding, while others have that of RNA–binding. They have effect on the genetic information transportation from DNA to mRNA. These effects also indicated the toxicity effects of PFOS on the embryonic development of medaka fish. However, to clarify the effect of PFOS on metabolism pathways needs further studies.

There were some genes encoding stress response processes responded to the PFOS exposure on medaka embryo with increasing expressions, for example the *hsp* family. The heat shock protein family was well known as fast response proteins to some environmental stresses, such as heat shock and chemical exposure. They mainly have functions on remodeling and ensuring correct folding and refolding of damaged proteins under environmental stress (Kregel, 2002; Wegele *et al.*, 2004). T he abnormal expressions of *hsp* genes have been identified in many ecotoxicology studies (Glushkova *et al.*, 2010). The induced expressions of them represented the stressful effects caused by PFOS exposure during medaka embryo development. In the present study, the up–regulated expressions of gene *hspa8b* and *hsp90aa1.2* indicated a stress–responding process and toxic effects on functional proteins caused by PFOS exposure.

There was a gene *ENSORLG00000003753*, responding to oxidative stress. It encoded protein heme oxygenase (Kimberly and Herbert, 1999). The significantly decreasing expression of this gene may lead to the inhibition of heme oxygenase activity and heme catabolic process. Protein ddit4 also responses to hypoxia and relates to apoptotic process. However, it has a different mechanism in responding to oxidative stress. The increased expression of gene *ddit4* (DNA damage–inducible transcript 4) involved in signaling processes also suggested that PFOS may cause DNA damage during the development of medaka embryo. It is a regulator of mTOR signaling pathway that regulates various cellular functions, including growth and apoptosis, and is related to diseases like diabetes (Zoncu *et al.*, 2011). The GO analysis suggested protein ddit4 involved in the biological process of dorsal/ventral pattern formation of medaka fish. The up–regulation of gene *ddit4* indicated the toxicity effect during embryonic development of medaka caused by 0.5 ng PFOS.

The mRNA–Seq result showed that PFOS, even at low lethal effect dosage level can disturb various biological processes during the embryonic development of Japanese medaka.

CONCLUSION

This is a new study on the effects of low dosage of PFOS by using both *in ovo*–nanoinjection and mRNA–Seq on the early development stages of Japanese medaka (*Oryzias latipes*). The *in ovo*–nanoinjection is an effective and sufficient method to simulate the maternal bioaccumulation and biotransformation from to the next generation of an environmental pollutant. The transcriptomes analysis is a sensitive and efficient method to analyze the genome gene expression of medaka fish. The adipogenesis effect of PFOS may be related to functional pathways and biological processes other than the regulation of ppar family. Due to the exposure concentration and one–time injection, the toxicity effect and adipogenesis effect of PFOS has not been clarified. However, the combination use of mRNA–Seq and lipid area measurement was very helpful in studying the mode of PFOS action.

AUTHOR CONTRIBUTIONS

K. Chen did all the data analysis, including mRNA–Seq analysis, and wrote the paper. N. Iwasaki performed the exposure experiment, measured the endpoints during development, and run the test of adipocyte area. X. Qiu contributed to the mathematic analysis, mRNA–Seq analysis, adipocyte area test and paper writing. H. Xu helped with mRNA–Seq analysis and paper writing. Y. Takai helped with experiment design, supervised the exposure experiment in detail, and assisted the mRNA–Seq data analysis. K. Tashiro performed the HiSeq test of mRNA sequencing. Y. Shimasaki supervised the work.

Y. Oshima designed the experiment, provided fund for the study and supervised the work. All authors assisted with the editing and modification of the manuscript and approved the final version.

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