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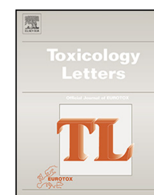

Circulating microRNA profiles altered in mice after 28 d exposure to perfluorooctanoic acid

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HIGHLIGHTS

- PFOA altered the profiles of circulating miRNAs in mice.
- PFOA increased level of two liver enriched miRNAs in serum, miR-122 and miR-192.
- miRNAs with level changed in serum altered in mice livers too after PFOA exposure.
- miR-28-5p, 32-5p, 122-5p, 192-5p, and 26b-5p may be biomarkers for PFOA exposure.

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ABSTRACT

Perfluorooctanoic acid (PFOA) is a stable man-made compound with many industrial and commercial uses. Recently, however, concern has been raised that it may induce various toxicological effects such as hepatotoxicity, immunotoxicity, and developmental toxicity. Because levels of circulating microRNAs (miRNAs) can be altered in several clinical diseases, they may serve as potential novel biomarkers. Here, we explored differences in the profiles of circulating miRNAs in mice after PFOA exposure. Using TaqMan miRNA arrays, we determined that the levels of 24 circulating miRNAs were altered in mice dosed with PFOA at 1.25 mg/kg/d and 73 were altered in mice dosed with 5 mg/kg/d. Eight miRNAs were further validated using TaqMan Real-Time PCR assays. Results were consistent with those obtained from the TaqMan miRNA arrays, except for miR-199a-3p. The most remarkable of the circulating miRNAs (miR-26b-5p and miR-199a-3p) were also up-regulated in the serum of occupational workers in our previous epidemiological study. We also found similar patterns in mice exposed to PFOS. These results demonstrated that circulating miRNA profiles were altered after exposure to high concentrations of PFOA and miR-28-5p, miR-32-5p, miR-122-5p, miR-192-5p, and miR-26b-5p in serum may be linked to effects of PFOA, especially in occupationally exposed people.

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1. Introduction

Perfluoroalkyl acids (PFAAs) are a family of perfluoroalkyl and polyfluoroalkyl substances (PFASs) with unique physical and chemical characteristics consisting of a carbon backbone and a charged functional moiety like carboxylate, sulfonate, or phosphonate (Lau et al., 2007). Perfluorooctanoic acid (PFOA) is one of the most widely known PFAAs and a useful anthropogenic compound, containing an eight-carbon backbone and used in products such as Gore-Tex and Teflon (Steenland et al., 2010). Toxicological PFOA and PFAS studies on laboratory animals such as rats and mice have shown that such compounds have hepatotoxic, immunotoxic, developmental, hormonal, and other toxicological effects (Lau et al., 2007). A great deal of information is available concerning the environmental distribution of PFOA and other PFASs, as well as their exposure to humans and wildlife (Lau et al., 2007). Previous studies have shown that PFOA, an agonist of peroxisome proliferator activated receptor alpha (PPARα) in both rodents and humans (Wolf et al., 2012), can disrupt lipid metabolism and even induce adenomas of the liver, testes (Leydig cell tumors) and pancreas (acinar cell tumors) of rodents (Klaunig et al., 2012; Olsen and Zobel, 2007). However, epidemiologic evidence on the effects of PFOA on human health is limited, and insufficient data makes it difficult to draw strong conclusions in relation to the association of PFOA and diseases of concern (Steenland et al., 2010).

Biomarkers reflect molecular and cellular alterations that occur along the temporal and mechanistic pathways, thereby connecting toxic chemical exposure to the presence or risk of clinical disease

Abbreviations: PFASs, perfluoroalkyl and polyfluoroalkyl substances; PFOA, perfluorooctanoic acid; PFOS, perfluorooctane sulfonate; miRNAs, microRNAs; PPAR, peroxisome proliferator activated receptor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

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(Decaprio, 1997). Since exposure to environmental pollutants does not usually result in rapid clinical symptoms, it is difficult to detect potential biomarkers associated with the effects of PFAS exposure for toxicity assessment and early clinical diagnosis (Tan et al., 2012). Proteome analysis demonstrated that proteins with various functions from different organs of laboratory animals were associated with PFASs exposure (Shi et al., 2009; Tan et al., 2012). Human epidemiological studies have also shown associations between PFOA and serum biochemical parameters like transaminase, γ-glutamyl transferase (GGT), high-density lipoprotein cholesterol (HDL-C), and adiponectin (Gallo et al., 2012; Lin et al., 2011; Wang et al., 2012b), although these associations have been inconsistent among different studies. Thus, finding stable biological molecules that can be reliably extracted and assayed for use as biomarkers of PFASs induced toxicological effects is challenging.

MicroRNAs (miRNAs) are endogenous ∼23 nt RNAs, which play crucial gene-regulatory roles in animals by pairing with mRNAs of protein-coding genes to direct their posttranscriptional repression (Bartel, 2009). To date, many studies have indicated that disruption of miRNA function contributes to diseases such as cancer, heart ailments, neurological dysfunctions, and metabolic disorders (Kerr et al., 2011; Pasquinelli, 2012; Rottiers and Naar, 2012). Several studies have examined the role of miRNAs in response to environmental agents and pollutants such as cigarette smoke, ethanol, benzo(a)pyrene, dioxin, and UV radiation (Sonkoly and Pivarcsi, 2011) and their possible involvement in chemical hepatocarcinogenesis (Koufaris et al., 2012). Certain evidence also indicates that changes in miRNA profiles are associated with perfluorooctane sulfonate (PFOS) exposure (Wang et al., 2012a; Zhang et al., 2011a), although, to our knowledge, no other laboratory results have shown correlations between PFASs and miRNAs. However, the association between miRNAs and PFOS suggests that miRNAs may be used as potential biomarkers of the toxicological effects induced by PFASs exposure.

Although most miRNAs are intracellular, a significant number have been observed in various body fluids (Etheridge et al., 2011). MiRNAs are stable in circulation, and thus may be potential non-invasive biomarkers (Bala et al., 2012). Recent studies have shown that the pattern of circulating miRNAs is altered in several clinical diseases. For example, circulating miR-29a appears to be correlated with colorectal liver metastasis (Wang and Gu, 2012) and changes in circulating miR-122 levels may be an indication of liver disease (Starkey Lewis et al., 2011; Trebicka et al., 2013; Tryndyak et al., 2012; Wang et al., 2009). Previous research has shown that levels of circulating miRNAs in fluorochemical plant workers and nearby residents were associated with serum PFOA concentrations (Wang et al., 2012b). However, whether PFASs alter circulating miRNA profiles is still unknown.

In this study, we analyzed the impact of PFASs (PFOA and PFOS) on circulating miRNA levels in mice and determined which specific circulating miRNAs may be used as potential biomarkers of the toxicological effects induced by PFASs exposure.

2. Materials and methods

2.1. Chemicals and reagents

Perfluorooctanoic acid (PFOA, CAS number 335-67-1, 96% purity) and perfluorooctane sulfonate (PFOS, CAS number 2795-39-3, 98% purity) were purchased from Sigma–Aldrich (St. Louis, MO). The PFOA and PFOS solutions were prepared by Milli-Q water. All other chemicals used were of the highest grade commercially available.

2.2. Animal treatment

Male BALB/c mice (6–8 weeks old) were obtained from Weitong Lihua Experimental Animal Center (Beijing, China). Mice were housed in an environmentally controlled (12:12 h light:dark cycle, 20–26 °C and 40–60% relative humidity) mass air displacement room. Food and water were provided *ad libitum* throughout the study. After one week of adaptation, 96 mice were randomly divided into six groups of equal size and dosed by gavage with either Milli-Q water or PFOA at concentrations of 0.08, 0.31, 1.25, 5 or 20 mg/kg/day (gavage volume of 10 mL/kg) for 28 d. The PFOA doses were chosen according to earlier toxicological studies and our previous experiments (Son et al., 2008). For practical reasons, another experiment was conducted in which 48 mice were randomly assigned into three groups of equal size and dosed by gavage with either Milli-Q water or PFOS at 1.25 or 5 mg/kg/day (gavage volume of 10 mL/kg) for 28 d. Body weight (BW) was measured every 7 d. At the end of treatment, 16 mice in each group were weighed, bled by retro-orbital puncture and subsequently sacrificed by cervical dislocation after a night of fasting. Following sacrifice, the livers were removed, weighed, immediately frozen in liquid nitrogen, and stored at −80 °C until further use. Blood was clotted at room temperature for 1 h and was then centrifuged for 5 min at 5000 rpm to collect serum. Serum was stored at −80 °C until further use. All animal care and treatment protocols were approved by the Committee on the Ethics of Animal Experiments from the Institute of Zoology, Chinese Academy of Sciences (Permit Number: EET-015-08-2012).

2.3. Serum PFOA determination

Serum PFOA concentrations ($n = 3$) of each mouse group were analyzed using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). Serum samples were mixed with nine different volumes of Milli-Q water for PFOA analysis. With some modification, PFOA was extracted as per previous reports (Wang et al., 2012b; Zhang et al., 2012). Briefly, samples were extracted with acetonitrile (ACN) twice, with both extractants then combined and concentrated to approximately 0.5 mL under a nitrogen gas flow at 40 °C. We added 0.5 mL of methanol (MeOH) to each concentrated solution, which were diluted using 10 mL of Milli-Q water for SPE cleanup. Each solution was then passed through pre-equilibrated Oasis WAX cartridges (Oasis HLB; 150 mg, 6 cm^3; Waters). After loading all samples, the cartridges were first rinsed with Milli-Q water and then with 25 mM of acetate buffer solution (pH 4). Any water remaining in the cartridges was removed by vacuum filtration. PFOA was eluted by 4 mL of MeOH and 4 mL of 0.1% NH_4OH in MeOH, and finally concentrated to approximately 1 mL under nitrogen gas.

The instrumental chromatographic setup consisted of a P680 binary gradient pump, an UltiMate 3000 autosampler, and a Chromeleon 6.70 chromatography workstation (Dionex, USA). Mass spectra were collected using an API 3200 triple quadrupole tandem mass spectrometer fitted with an electro-spray ionization source and operated in negative ionization mode. Quantification was performed using Analyst 1.4.1 software. Chromatographic separations were carried out on an Acclaim 120 C18 column (4.6 mm × 150 mm, 3 μm) (Dionex, USA) with a binary gradient. Methanol (solution A) and 50 mM of ammonium acetate (NH_4Ac) (solution B) were employed as mobile phases. A calibration curve was prepared from a series of concentrations (0, 10, 50, 100, 500, 1000, 5000, 20 000, and 50 000 pg/mL) for PFOA concentration calculation, with standard deviations determined to be less than 20%. All samples were properly diluted before PFOA determination to ensure the concentration fit the calibration curve.

2.4. TaqMan miRNA array analysis

We chose the group dosed with 1.25 mg/kg/d of PFOA, which had no significant change in BW, and the 5 mg/kg/d group, which lost 12.6% BW, to compare with the control group for miRNA array analysis. Although the 5 mg/kg/d group showed 12.6% BW loss, exceeding the 10% criteria for overt toxicity (Buckley and Dorato, 2009), it was relatively close to the borderline. Thus, 200 μL of serum from three mice from each group was pooled to ensure sufficient serum for miRNA analysis as well as reduce individual differences within the same group and reduce the cost of analysis (Kendziorski et al., 2005; Zhang and Gant, 2005). Three serum samples from each dose group (0, 1.25 and 5 mg/kg), with each sample pooled from three animals, were used for miRNA array analysis. Total RNAs including small RNAs were prepared using the mirVana miRNA Isolation Kit according to the manufacturer's procedures. cDNAs were synthesized from total RNAs using Megaplex RT Primers and the TaqMan MicroRNA Reverse Transcription Kit according to the manufacturer's instructions. Preamplification was performed by adding TaqMan PreAmp Master Mix and Megaplex PreAmp Primers to the cDNA sample. MiRNA expression profiling was performed using TaqMan® Array Rodent MicroRNA A + B Cards Set v3.0 containing a total of 384 TaqMan MicroRNA Assays per card and enabling quantitation of 641 and 373 unique microRNAs for mouse and rat, respectively, consistent with Sanger miRBase v15 (rodent). All kits and reagents in the TaqMan miRNA array analyzes were obtained from Life Technologies-Applied Biosystems, CA, USA. Quantitative real-time PCR was performed using an Applied BioSystems 7900HT Fast Real-Time PCR system and data were analyzed with SDS Relative Quantification software v.2.4.

We selected U6 snRNA as the endogenous control because its expression level was the most stable and there were no significant differences between U6 snRNA levels in the control and PFOA exposed samples.

2.5. Validation experiment of circulating miRNAs with altered levels

Four mouse serum samples per group, with each sample being a pool of three animals, were used for miRNA assay analysis. The samples used for the

verification experiment were different from the ones used for the TaqMan miRNA arrays. Total RNAs including small RNAs from the pooled mouse serum were isolated and the preamplification products were prepared following the same procedures as described above.

Preamplification products of serum total RNA or cDNA samples of liver total RNA were added with TaqMan Universal Master Mix II, no UNG and the specific primer/probe combination provided with each TaqMan assay in accordance with manufacturer's recommendations. The miRNAs selected for miRNA assay were miR-28-5p (assay ID. 000411), miR-32-5p (assay ID. 002109), miR-122-5p (assay ID. 002245), miR-192-5p (assay ID. 000491), miR-26b-5p (assay ID. 000407) and miR-199a-3p (assay ID. 002304). All kits and reagents used in TaqMan miRNA analysis were obtained from Life Technologies-Applied Biosystems, CA, USA. Real-time PCR was performed using a MP3000P q-PCR system (Stratagene, La Jolla, CA) with MxPro QPCR software. We selected U6 snRNA (assay ID. 001973) as the endogenous control, and relative quantification of each miRNA was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.6. TaqMan miRNA assay in livers

Total RNAs from mouse livers ($n = 6$) were isolated by TRIzol (Life Technologies-Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and measured by a NanoDrop 2000 spectrophotometer (Thermo Scientific). The cDNAs were synthesized from about 50 ng of RNA using a TaqMan MicroRNA Reverse Transcription Kit and specific reverse transcription primer provided with each TaqMan assay. We then used the cDNA samples of liver total RNA for analysis, as mentioned in Section 2.5.

2.7. Serum biochemical assay

Thawed individual serum samples ($n = 6$) were used for detection of serum lipids and enzymes using a HITAC7170A automatic analyzer (Hitachi, Japan) following standard spectrophotometric methods.

2.8. Statistical analysis

Differentially expressed miRNAs in the control and PFOA exposed mice were identified using Random Variance Model (RVM) *t*-tests (Clarke et al., 2008; Wright and Simon, 2003) in the TaqMan miRNA array analysis. Multiple testing was corrected using false discovery rate (FDR) analysis. The differentially expressed miRNAs were then statistical filtered by applying the RVM *t*-test with $p < 0.05$. The data were presented as fold changes calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Body and organ weight, serum biochemical levels and TaqMan miRNA assay analyses were performed using SPSS for Windows 17.0 Software (SPSS, Inc., Chicago, IL). The results were expressed as means with standard errors (mean ± SE). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test. A *p*-value of < 0.05 was used to determine significant differences between the treatment groups and the control.

3. Results

3.1. Toxicological effects of PFOA

After PFOA exposure, the BW of mice in the 5 and 20 mg/kg/d dose groups significantly decreased compared with the control group. In addition, a significant increase in absolute liver weight as well as relative liver weight was observed in the 0.31 mg/kg/d and higher dosed groups in a dose-dependent manner (Table 1).

Serum biochemical levels of each group were analyzed (Table 1). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) levels significantly increased at the highest dose compared with the control group, indicating that PFOA induced liver damage in mice particularly at higher doses. Total cholesterol (T-CHO) and high density lipoprotein cholesterol (HDL-C) levels decreased after PFOA treatment, but triglyceride (TG) levels increased in the middle dose groups (0.31 and 1.25 mg/kg/d) and decreased in the higher dose groups (5 and 20 mg/kg/d).

Serum concentrations of PFOA were analyzed (Fig. 1), which showed an increase depending on the dose of PFOA. The highest serum level of PFOA was 105.29 μg/mL in mice dosed with PFOA at 20 mg/kg/d.

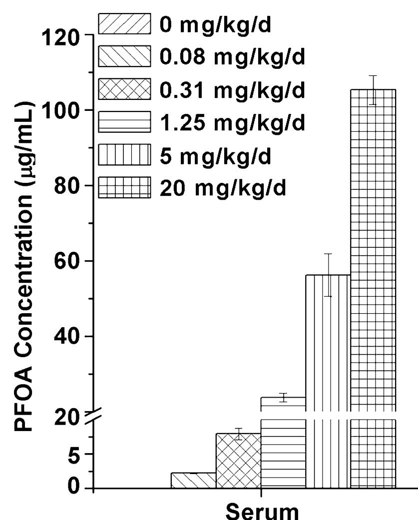

Fig. 1. PFOA concentration in mice serum after 28 d PFOA exposure. PFOA was extracted from the serum of mice exposed to PFOA at concentrations of 0, 0.08, 0.31, 1.25, 5 and 20 mg/kg/d using SPE-Oasis-WAX-method and analyzed with LC–MS/MS. *Error bars* indicate standard error (SE, $n = 3$).

3.2. Circulating miRNA profiles altered after PFOA exposure

Among the 641 circulating miRNAs checked, we found that the levels of 24 miRNAs were altered in mice dosed with 1.25 mg/kg/d PFOA and the levels of 73 miRNAs were altered in the 5 mg/kg/d dose group (Table 2 and Table S1 in Supporting information, Ct values and miRBase Accession Numbers of miRNAs in Supplementary data). Out of these two groups, 22 miRNAs were altered in both. Among these miRNAs, the levels of 21 miRNAs increased in both dose groups, and the level of one miRNA (miR-96-5p) decreased in the 1.25 mg/kg/d dose group and increased in the 5 mg/kg/d dose group (Table 2). Levels of nine circulating miRNAs showed a 30-fold increase, two (miR-295-3p and miR-669h-5p) showed a fold change increase in both analyzed dose groups (Table 2), and seven (miR-200c-5p, miR-294-3p, miR-376a-5p, miR-379-5p, miR-669d-5p, miR-1194 and miR-1982.1-3p) increased in the 5 mg/kg/d dose group only (Table S1).

TaqMan miRNA assays were performed to validate those miRNAs with altered levels after PFOA exposure, as detected from miRNA arrays. Among the 22 miRNAs with levels altered in both PFOA treated groups, 14 had the same sequences between mice and humans (http://www.mirbase.org/), indicating that they were conserved. Four conserved miRNAs with dose-dependent altered levels (miR-28-5p, miR-32-5p, miR-122-5p, and miR-192-5p) were selected from the 14 miRNAs for validation experiments and further analysis. To explore the potential relationship between circulating miRNAs and PFOA hepatotoxic effects, two other conserved miRNAs (miR-34a-5p and miR-200c-3p) were also selected for analysis since they may be associated with liver tumors in rats and humans and their levels were altered in mice dosed with 5 mg/kg/d of PFOA (Ladeiro et al., 2008; Sukata et al., 2011). Our results showed that serum levels of the selected miRNAs increased in the 1.25 mg/kg/d and higher dose groups (Fig. 2). This confirmed the initial array data, indicating that the miRNA array results were credible. In particular, the level of miR-122-5p showed a significant 391.8-fold change increase in mice dosed with 20 mg/kg/d of PFOA.

3.3. Effects of PFOA on circulating miR-26b-5p and miR-199a-3p in mice

Our previous study found that levels of circulating miR-26b-5p and miR-199a-3p were elevated in accordance with serum

Table 1
Alteration of body weight (BW, $n = 16$), relative liver weight ($n = 16$), and serum biochemical levels ($n = 6$) after PFOA exposure, data are mean ± SE, significantly different from control group.

	PFOA (mg/kg/d)					
	0	0.08	0.31	1.25	5	20
BW before treatment (g)	19.4 ± 0.3	19.5 ± 0.4	19.3 ± 0.3	19.4 ± 0.3	19.6 ± 0.2	19.5 ± 0.3
BW after treatment (g)	21.4 ± 0.3	21.3 ± 0.5	20.9 ± 0.4	22.0 ± 0.4	18.7 ± 0.2**	15.5 ± 0.3**
Absolute liver weight (g)	0.92 ± 0.02	1.00 ± 0.03	1.17 ± 0.02**	1.89 ± 0.03**	2.25 ± 0.05**	2.37 ± 0.07**
Relative liver weight (%)	4.3 ± 0.0	4.7 ± 0.0	5.6 ± 0.1**	8.6 ± 0.1**	12.0 ± 0.2**	15.3 ± 0.2**
ALT (IU/L)	25 ± 3	29 ± 2	47 ± 4	127 ± 21	322 ± 16	1738 ± 255**
AST (IU/L)	75 ± 5	71 ± 4	75 ± 4	86 ± 8	148 ± 10	552 ± 79**
ALB (g/L)	21.6 ± 0.4	22.6 ± 0.3	23.0 ± 0.2	24.0 ± 0.2*	24.8 ± 0.6**	23.7 ± 1.3*
ALP (IU/L)	88 ± 3	93 ± 3	99 ± 17	221 ± 8	549 ± 9**	1179 ± 129**
TBA (μmol/L)	0.9 ± 0.1	1.5 ± 0.3	0.6 ± 0.0	1.6 ± 0.2	7.5 ± 1.7	41.3 ± 8.9**
T-CHO (mmol/L)	3.1 ± 0.1	3.1 ± 0.1	3.1 ± 0.1	2.5 ± 0.1**	2.7 ± 0.1**	2.1 ± 0.1**
TG (mmol/L)	1.23 ± 0.08	1.27 ± 0.08	1.95 ± 0.11**	1.77 ± 0.10**	0.83 ± 0.15*	0.68 ± 0.13**
HDL (mmol/L)	3.24 ± 0.08	3.10 ± 0.06	3.04 ± 0.10	2.38 ± 0.08**	2.42 ± 0.10**	2.15 ± 0.13**
LDL (mmol/L)	0.18 ± 0.02	0.24 ± 0.08	0.22 ± 0.03	0.19 ± 0.01	0.21 ± 0.03	0.17 ± 0.05
LDH (U/L)	512 ± 39	429 ± 10	376 ± 18	586 ± 45	651 ± 17	991 ± 136**

*$p < 0.05$, **$p < 0.01$

concentrations of PFOA in workers and nearby residents of a fluorochemical plant (Wang et al., 2012b). The miRNAs array results in the present study demonstrated that circulating miR-26b-5p had a significant 3.2-fold change increase in mice dosed with 5 mg/kg/d of PFOA (Table S1 in Supporting information), but the level of miR-199a-3p remained unchanged. To explore whether levels of circulating miR-26b-5p and miR-199a-3p changed in mice after PFOA exposure, we also analyzed the levels of these two miRNAs in serum using TaqMan RT-PCR miRNA assays.

The results showed that levels of circulating miR-26b-5p significantly increased in mice dosed with 1.25 mg/kg/d of PFOA and showed a 4.4-fold increase in the 20 mg/kg/d PFOA exposed mice group (Fig. 3). Although circulating miR-199a-3p was unchanged in TaqMan miRNA array analysis, its level increased in the 1.25 mg/kg/d and higher dosed groups after PFOA exposure using TaqMan RT-PCR miRNA assays.

3.4. Effects of PFOA on levels of eight selected miRNAs in liver

It has been reported that miR-122-5p and miR-192-5p are enriched in mice livers (Chang et al., 2004; Wang et al., 2009). Several studies have demonstrated that levels of miR-122-5p and miR-192-5p increased in circulation but decreased in the liver following liver injury (Trebicka et al., 2013; Wang et al., 2009). Previous research has also indicated that miR-34a-5p and miR-200c-3p are associated with liver tumors in rats and humans (Ladeiro et al., 2008; Sukata et al., 2011) and animal studies have suggested that the liver is an important target organ for PFOA physiological activity (Lau et al., 2007). Using TaqMan miRNA assay, we analyzed the levels of the above-mentioned miRNAs in the liver after PFOA exposure (Fig. 4).

The data indicated that levels of miR-28-5p, miR-34a-5p, and miR-200c-3p significantly increased in mice after PFOA exposure. Interestingly, the expression levels of miR-122-5p and miR-192-5p in mice livers increased in the 0.08 and 0.31 mg/kg/d dose groups and then decreased to the same or lower levels as the control group. The fold change of miR-32-5p expression was increased in the 0.31 and 20 mg/kg/d dose groups but did not significantly change in the other dose groups after PFOA exposure. The level of miR-199a-3p significantly increased in the 0.31 and 20 mg/kg/d dose groups after PFOA exposure, and the level of miR-26b-5p decreased in the 5 and

Table 2
Circulating microRNAs with significantly altered expression levels ($p < 0.05$) in the 1.25 and 5 mg/kg/d PFOA exposure groups.

miRNAs	PFOA (1.25 mg/kg/d)		PFOA (5 mg/kg/d)	
	Fold change (*p*-value)	FDR	Fold change (*p*-value)	FDR
# let-7i-5p	1.83 (0.0412)	0.4990	2.40 (0.0076)	0.0633
# miR-28-5p	2.32 (0.0421)	0.4990	5.31 (0.0010)	0.0240
# miR-32-5p	2.35 (0.0301)	0.4990	6.57 (0.0028)	0.0358
# miR-96-5p	0.03 (0.0040)	0.3720	8.02 (0.0311)	0.1440
# miR-122-5p	2.88 (0.0199)	0.4000	15.45 (0.0017)	0.0306
# miR-130a-3p	1.83 (0.0075)	0.4000	1.92 (0.0031)	0.0378
# miR-191-5p	1.92 (0.0486)	0.5420	1.73 (0.0281)	0.1340
# miR-192-5p	1.67 (0.0334)	0.4990	5.25 (0.0019)	0.0306
# miR-194-5p	2.82 (0.0098)	0.4000	11.80 (0.0004)	0.0145
# miR-328-3p	1.62 (0.0346)	0.4990	1.64 (0.0321)	0.1450
# miR-339-5p	2.15 (0.0215)	0.4000	2.64 (0.0014)	0.0297
# miR-363-3p	2.60 (0.0155)	0.4000	2.69 (0.0085)	0.0675
# miR-532-3p	1.73 (0.0185)	0.4000	2.88 (0.0021)	0.0306
# miR-652-3p	1.98 (0.0309)	0.4990	2.70 (0.0022)	0.0306
miR-295-3p	10.73 (0.0018)	0.3720	172.95(0.0002)	0.0114
miR-322-5p	2.79 (0.0360)	0.4990	5.25 (0.0040)	0.0432
miR-466j	4.17 (0.0030)	0.3720	7.44 (0.0006)	0.0180
miR-503-5p	2.28 (0.0429)	0.4990	2.85 (0.0051)	0.0473
miR-598-3p	2.51 (0.0154)	0.4000	2.91 (0.0320)	0.1450
miR-669h-5p	30.02 (0.0064)	0.4000	39.97 (0.0048)	0.0461
miR-704	3.23 (0.0179)	0.4000	2.58 (0.0096)	0.0727
miR-879-5p	5.97 (0.0204)	0.4000	6.03 (0.0256)	0.1270

#: miRNA sequence was the same between mouse and human, as per the miRBase database (http://www.mirbase.org/).

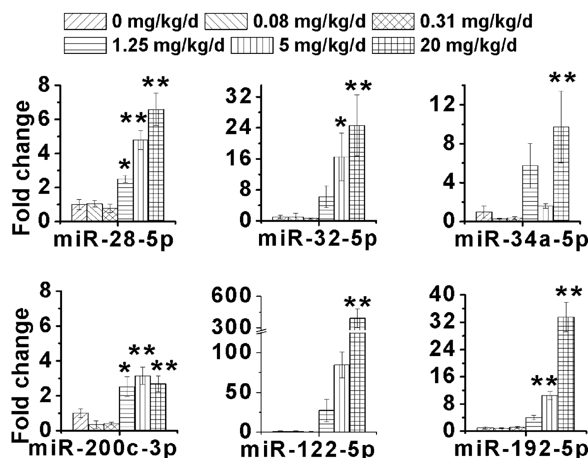

Fig. 2. Selected circulating miRNAs changed with a pattern similar to miRNA arrays after PFOA exposure. Each sample was pooled with the serum of three individual mice. Total RNAs were isolated using a mirVana miRNA Isolation Kit and each miRNA was quantitated using miRNA assay, respectively. Four independent serum samples were taken for analysis. U6 snRNA was used as the endogenous control and the miRNA levels represent relative value to the control group. Data are mean ± SE. Significantly different from control group (*$p < 0.05$, **$p < 0.01$).

20 mg/kg/d dose groups after PFOA exposure but without statistical significance (Fig. S1 in Supporting information).

3.5. Effects of PFOS on mice were similar to PFOA

After exposure to PFOS, a significant increase in absolute and relative liver weight was observed, but no significant effect was observed in relation to BW (Table S2 in Supporting information). The levels of ALT, AST, ALB and ALP increased after PFOS exposure. Results also demonstrated that PFOS exposure decreased HDL levels but increased LDL levels in mice (Table S2). The eight miRNAs mentioned above were also analyzed in the serum of PFOS exposed mice using TaqMan miRNA assays. We found that all levels of miRNAs, except miR-34a-5p and miR-199a-3p, increased in mice dosed with 5 mg/kg/d of PFOS (Figs. S2 and S4 in Supporting information).

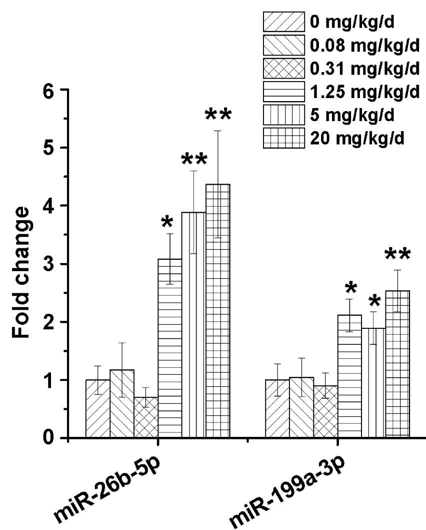

Fig. 3. Circulating miR-26b-5p and miR-199a-3p expression levels were altered after PFOA exposure. Each sample was pooled with the serum of three individual mice. Total RNAs were isolated using a mirVana miRNA Isolation Kit and each miRNA was quantitated using miRNA assay, respectively. Four independent serum samples were taken for analysis. U6 snRNA was used as the endogenous control and the miRNA levels represent relative value to control group. Data are mean ± SE. Significantly different from control group (*$p < 0.05$, **$p < 0.01$).

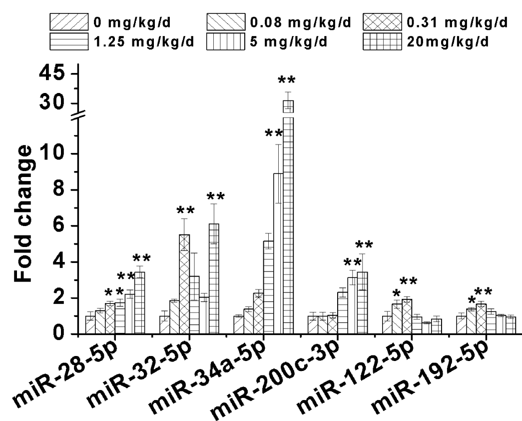

Fig. 4. Expression levels of selected circulating miRNAs in mouse livers after PFOA exposure. Total RNAs were isolated from liver tissues ($n = 6$) by TRIzol reagent and each miRNA was quantitated using miRNA assay respectively. U6 snRNA was used as the endogenous control and the miRNA levels represent relative value to control group. Data are mean ± SE. Significantly different from control group (*$p < 0.05$, **$p < 0.01$).

With the exception of miR-34a-5p, which increased at the dose of 5 mg/kg/d, the expression levels of miRNAs in the livers of mice after PFOS exposure did not significantly change (Figs. S3 and S5 in Supporting information).

4. Discussion

Previous research has shown PFOA to be associated with liver enlargement in rodents and nonhuman primates and with hepatocellular adenomas in rats (Lau et al., 2007). Histopathological studies on the toxicological effects of PFOA on rats indicated that liver damage occurred after 28 d of exposure at PFOA concentrations of 20 mg/kg/d (Cui et al., 2009). Our results also indicated that PFOA exposure could induce liver injury and disrupt lipid metabolism in mice.

It is interesting to note that the PFOA level in mice serum in the 0.08 mg/kg/d group was 2.24 μg/mL, similar to the median serum level of PFOA (1.64 μg/mL) observed in occupational participants from a fluorochemical plant of Jiangsu Province, China (Wang et al., 2012b). Although no significant toxicological endpoints were observed in mice dosed with 0.08 mg/kg/d for 28 d in this study, we could not exclude that adverse health effects may appear after long-term PFOA exposure at low doses in mice as well as humans. Several medical surveillance studies of fluorochemical production workers have suggested that PFOA exposure may be negatively associated with serum HDL-C levels and positively associated with serum ALT levels (Gallo et al., 2012; Wang et al., 2012b). Triglycerides have been found positively associated with PFOA in humans, although the association does not appear to be consistent across locations (Olsen and Zobel, 2007; Steenland et al., 2010). The present results showed that triglycerides increased in the middle dose groups (0.31 and 1.25 mg/kg/d) and decreased in the higher dose groups (5 and 20 mg/kg/d). Further study is required to clarify whether the inconsistent findings between mice and humans in relation to PFOA exposure are due to differences in toxicokinetics, PPAR subtype expression and function, or some other factor (Lau et al., 2007).

Many studies on circulating miRNA profiles responding to hepatic diseases have been reported (Bala et al., 2012; Starkey Lewis et al., 2011; Sukata et al., 2011; Trebicka et al., 2013; Tryndyak et al., 2012; Wang et al., 2009). An earlier study using acetaminophen overdose-induced liver injury in a mouse model system observed highly significant differences in the plasma levels of miRNAs between the control and overdosed animals. Two miRNAs enriched

in the liver tissue, that is, miR-122-5p and miR-192-5p, were demonstrated to be potential biomarkers for drug-induced liver injury (Wang et al., 2009). Research on the association between nonalcoholic fatty liver disease (NAFLD) and altered circulating miRNA profiles in different strains of inbred male mice suggested that levels of circulating miR-34a-5p, miR-122-5p, miR-181a-5p, miR-192-5p, and miR-200b-3p were significantly correlated with the severity of NAFLD-specific liver pathomorphology (Tryndyak et al., 2012). In addition, circulating miR-122-5p has been reported to alter in patients with acute liver injury (Starkey Lewis et al., 2011). In the present study, levels of liver enriched miRNAs, miR-122-5p, and miR-192-5p showed significantly increased circulation after PFOA exposure. In addition, miR-200c-3p, which may be associated with liver tumors in humans (Ladeiro et al., 2008), showed increased serum levels in the 1.25 mg/kg/d and higher dosed groups after PFOA exposure. The increase in the levels of liver specific enzymes such as ALT and AST, as well as the increase in the levels of other serum biochemicals such as alkaline phosphatase (ALP) and total bile acid (TBA), implied that hepatic injury may occur after PFOA exposure, particularly at the highest doses. These results suggest that PFOA significantly induced liver injury in mice at the highest doses and also resulted in increased serum levels of liver-enriched miR-122-5p and miR-192-5p. Several studies on the relationship between circulating miRNAs and diseases have also mentioned miRNAs with levels altered, as observed in our miRNA array results. For example, let-7i-5p may be used as a potential serum biomarker for blast-induced traumatic brain injury (Balakathiresan et al., 2012), miR-130a-3p is apparently associated with atherosclerosis obliterans (Li et al., 2011) and miR-191-5p may be related to renal diseases (Hauser et al., 2012; Luo et al., 2013). However, whether the levels of circulating miRNAs changed in mice exposed to PFOA hint at extrahepatic systemic toxicity unrelated to peroxisome proliferation in rodents still needs further study.

Our results also indicated that levels of circulating miR-26b-5p and miR-199a-3p increased in mice after PFOA exposure. Taken together with our previous results showing that levels of circulating miR-26b-5p and miR-199a-3p were associated with PFOA concentrations in human serum (Wang et al., 2012b), our findings imply that these two miRNAs may be used as possible biomarkers of the toxicological effects induced by PFOA exposure. Because PFOS and PFOA share a similar toxicological profile in rodents (Lau et al., 2007), our present results demonstrate that levels of circulating miRNAs may also alter with a similar pattern after exposure to PFOA and PFOS.

Several studies on changes in the profiles of circulating miRNAs during liver injury examined the expression of miRNAs in the liver. Results demonstrated an inverse trend in the levels of certain circulating miRNAs compared with those miRNAs in the liver (Trebicka et al., 2013; Wang et al., 2009). For miR-122-5p, one explanation is that it is released from the liver into the bloodstream, causing circulating levels to sharply increase in response to hepatic injury (Trebicka et al., 2013). Contrary to previous studies, our results showed that levels of miR-122-5p and miR-192-5p in the liver did not decrease after PFOA exposure. This discrepancy might be due to the characteristics of PFOA toxicological effects. Many reports have demonstrated that PFOA can interact with or activate several nuclear receptors, especially PPARα in vivo and in vitro (Peters and Gonzalez, 2011). As far as we know, the regulation of miR-200c by PPARα has been verified (Zhang et al., 2011b) and the expression of several miRNAs may be regulated by PPARγ (John et al., 2012). Thus, in the present study, PPARs activity may relate to the higher expression levels of miRNAs in mice livers after PFOA exposure. However, the mechanism details for how PFOA-induced miRNA profiles are altered still require further study.

Since their discovery, miRNAs have been recognized as crucial regulators of gene expression in animals (Bartel, 2009; Kerr et al., 2011; Pasquinelli, 2012; Rottiers and Naar, 2012). Recent research on the functions of the miRNAs selected in the present study have reported that miR-34a-5p is transactivated by p53 and its activation can broadly influence gene expression and recapitulate elements of p53 activity, including cell-cycle progression and apoptosis (He et al., 2007). Inactivation of miR-34a-5p strongly attenuates p53-mediated apoptosis and may contribute to tumorigenesis (Hermeking, 2010). Furthermore, miR-34a-5p may be involved in the regulation of neuronal development (Aranha et al., 2011; Liu et al., 2012), pathological cardiac remodeling (Bernardo et al., 2012), and hepatic lipid homeostasis (Rottiers and Naar, 2012). Our present study demonstrated that PFOA exposure could induce liver injury and disturb lipid metabolism in mice, which might be correlated with the level change of miR-34a-5p in the liver. In addition, miR-200c-3p has been reported to suppress tumor formation in vivo and loss of its expression may be involved in tumor progression (Ladeiro et al., 2008; Shimono et al., 2009). Although fewer studies on miR-28-5p have been reported in the literature compared with miR-34a-5p and 200c-3p, it may also be associated with cancer (Almeida et al., 2012). In the present study, the levels of miR-32-5p and miR-199a-3p changed independently of PFOA dose in the liver, and levels of miR-26b-5p decreased in the 5 and 20 mg/kg/d dose groups after PFOA exposure but without statistical significance. However, the levels of all three miRNAs were altered in serum after PFOA exposure. Various PFOA toxicological effects may contribute to this phenomenon. Earlier studies on miR-32-5p have reported that miRNAs may be involved in HIV-1 Tat C function and inhibit human myeloid leukemia cells apoptosis (Gocek et al., 2011; Mishra et al., 2012). MiR-26b-5p is down regulated in cancers and may suppress cell growth, induce apoptosis and regulate pituitary development (Lin et al., 2012; Rahbari et al., 2011; Wu et al., 2011). Levels of miR-199a-3p have been reported to decrease in malignancies such as hepatocellular carcinoma (Dettmer et al., 2013; Duan et al., 2011; Fischer et al., 2011; Hou et al., 2011; Iorio et al., 2007), and decrease in the plasma of osteosarcoma patients (Ouyang et al., 2013). Functional studies on miR-199a-3p indicate that it can induce cardiac regeneration (Eulalio et al., 2012), regulate human multipotent stromal cell differentiation by decreasing leukemia inhibitory factor secretion (Oskowitz et al., 2008), and modulate the cell cycle (Duan et al., 2011; Fornari et al., 2010). MiR-199a-3p may also be associated with the progression of liver fibrosis (Murakami et al., 2011). Because one miRNA can be identified in different tissues and many biological processes may affect miRNA expression and PFOA toxicological effects, the origins of circulating miRNAs with altered levels and the reasons why these miRNAs levels change after PFOA exposure still needs further investigation. Furthermore, although exposure to PFOA may have altered the profiles of circulating miRNA and affected liver miRNA expression in this study, it is difficult to determine whether the liver is an important source of altered circulating miRNAs after PFOA exposure due to the diverse origin of circulating miRNAs. The impact of miRNAs altered by PFOA in the liver needs further investigation.

To our knowledge, our study is the first report on altered circulating miRNA profiles in mice following PFOA exposure, which may also affect miRNAs expression in mice livers. Eight miRNAs were further validated using TaqMan miRNA assays, which showed that levels were altered similarly to the initial array results, except for miR-199a-3p. Additionally, we also found that PFOA exposure affected the expression of the selected miRNAs in the mouse livers. It is interesting to observe that higher levels of circulating miR-122-5p and miR-192-5p, which belong to liver enriched miRNAs and have been reported as potential biomarkers for liver injury, were induced by PFOA. Remarkably, of the miRNAs with altered levels, miR-26b-5p and miR-199a-3p were also up-regulated in the serum of occupational workers in our previous epidemiological results. Our present study demonstrated that miR-28-5p, miR-32-5p,

miR-122-5p, miR-192-5p, and miR-26b-5p in serum may be linked to effects of PFOA exposure. In addition, the effects of PFOS on miRNAs were similar to the effects of PFOA, and almost every miRNAs involved in the validation experiment showed altered levels after PFOS exposure, except for miR-34a-5p and miR-199a-3p. However, many questions remain unresolved in our present research. In particular, the origin and pathological meanings of the circulating miRNAs that altered after PFOA exposure, as well as the correlation between the altered circulating miRNAs and various PFOA toxicological effects, need further investigation. Because circulating miRNA profiles are also altered in several clinical diseases or following exposure to some substances, reduction in potential false positives when using circulating miRNAs as biomarkers for PFOA exposure needs further investigation. The cost of circulating miRNA analysis is high and the process is not simple, therefore it is also important to develop circulating miRNA analysis technology that is cheaper and easier to operate for the application and dissemination of circulating miRNA biomarkers.

Conflict of interest

The authors declare that there are no conflicts of interest.

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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.toxlet.2013.10.017.

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