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Perfluorooctanoic acid affects endocytosis involving clathrin light chain A and microRNA-133b-3p in mouse testes

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ABSTRACT

Perfluorooctanoic acid (PFOA) is an abundant perfluoroalkyl substance widely applied in industrial and consumer products. Among its potential health hazards, testicular toxicity is of major concern. To explore the potential effect of miRNA on post-translational regulation after PFOA exposure, changes in miRNAs were detected *via* miRNA array. Seventeen miRNAs were differentially expressed (eight upregulated, nine downregulated) in male mouse testes after exposure to 5 mg/kg/d of PFOA for 28 d (>1.5-fold and $P < 0.05$ compared with the control). Eight of these miRNAs were further selected for TaqMan qPCR analysis. Proteomic profile analysis indicated that many changed proteins after PFOA treatment, including intersectin 1 (ITSN1), serine protease inhibitor A3K (Serpina3k), and apolipoprotein a1 (APOA1), were involved in endocytosis and blood-testis barrier (BTB) processes. These changes were further verified by immunohistochemical and Western blot analyses. Endocytosis-related genes were selected for qPCR analysis, with many found to be significantly changed after PFOA treatment, including epidermal growth factor receptor pathway substrate 8 (Eps8), Eps15, cortactin, cofilin, espin, vinculin, and zyxin. We further predicted the potential interaction between changed miRNAs and proteins, which indicated that miRNAs might play a role in the post-translational regulation of gene expression after PFOA treatment in mouse testes. Among them, miR-133b-3p/clathrin light chain A (CLTA) was selected and verified *in vitro* by transfection and luciferase activity assay. Results showed that PFOA exposure affects endocytosis in mouse testes and that CLTA is a potential target of miR-133b-3p.

1. Introduction

Perfluoroalkyl substances (PFASs) are characterized by stable and strong carbon-fluoride bonds with unique water- and oil-repellent properties, resulting in their wide application in industrial and consumer products and environmental persistence (Calafat et al., 2007). Reports have shown that PFASs are not only found in various environmental matrices, but also in serum and tissue samples from human and animal populations (Giesy and Kannan, 2002). Among PFASs, eight-carbon-chain perfluorooctanesulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two of the most abundant. Studies have shown that PFOA is dose-dependently accumulated in laboratory animal samples, including in serum, liver, and testes (Yan et al., 2014; Zhang et al., 2014; Yan et al., 2015). In addition, PFOA has a relatively long half-life in human serum of 3.8 years (Olsen et al., 2007). Although many countries have restricted the industrial production of PFOA since 2000 (US EPA, U.E.P.A, 2016), its persistence as one of the most dominant PFASs in the environment has remained due to its continued production in countries without legal restriction and *via* degradation of its precursors (Prevedouros et al., 2006; Wang et al., 2009).

Both the environmental persistence and pervasive distribution of PFASs have increased concern in regards to their potential toxicity. Extensive research on the potential health hazards of PFOA exposure has been conducted (Butenhoff et al., 2004; Kennedy et al., 2004; Lau et al., 2004; Lau et al., 2007; Olsen and Zobel, 2007; Olsen et al., 2009). Among these potential health hazards, testicular toxicity is of major concern. Laboratory studies on adult male rats have shown that PFOA reduces testosterone and increases estradiol levels following exposure (Lau et al., 2007). A cross-sectional study reported negative associations between high combined levels of PFOA and PFOS and the proportion of morphologically normal spermatozoa in adult men (Joensen et al., 2009). In addition, luteinizing hormone and free testosterone were found to be positively correlated with plasma PFOA in men attending an *in vitro* fertilization clinic (Raymer et al., 2012).

Abbreviations: UTR, 3′ untranslated region; APOA1, Apolipoprotein a1; BTB, Blood-testis barrier; CLTA, Clathrin light chain A; ITSN1, Intersectin 1; iTRAQ, Isobaric tags for relative and absolute quantitation; miRNA, MicroRNA; PFOA, Perfluorooctanoic acid; PFASs, Perfluoroalkyl substances; Serpina3k, Serine protease inhibitor A3K.

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MicroRNAs (miRNA) are endogenous, short RNA molecules that recognize base-paired complementary sites on their target genes and block target gene translation or trigger degradation of target mRNA (Bartel, 2004). During spermatogenesis, accurately regulated gene expression is fundamental. Animal studies have shown significantly different testicular miRNA profiles between immature and mature animals (Yan et al., 2007; Luo et al., 2010), implying that post-transcriptional control of gene expression by miRNAs participates in spermatogenesis. In addition, among the more than 100 miRNA species found in mammalian testes, about 40% are differentially expressed between testicular and somatic tissues (Ro et al., 2007; Luo et al., 2010). Aberrant miRNA expression has been observed in many diseases (Wahid et al., 2010), suggesting the possibility that environmental pollutants could trigger these changes (Wang et al., 2015). We hypothesized that PFOA can trigger miRNA expression pattern alteration, which might represent a novel mechanism for the testicular toxicity of PFOA. In our previous study, we used isobaric tags for relative and absolute quantitation (iTRAQ) to determine proteomic profile changes in mouse testes after PFOA exposure (Zhang et al., 2014). In the present study, we identified differentially expressed miRNAs in mouse testes after PFOA exposure. Combined with the previous differentially expressed proteins, we explored testicular miRNA alteration and the potential interaction between miRNAs and changed proteins in testes after PFOA treatment to gain insight into the molecular mechanisms of the testicular toxicity of PFOA.

2. Materials and methods

2.1. Animals and treatment

Male BALB/c mice (aged 6–8 weeks) were randomly grouped ($n = 10$ per group) and dosed by oral gavage with Milli-Q water or 1.25, 5, or 20 mg/kg body weight/d of PFOA (Sigma-Aldrich, CAS No. 307-55-1, 99% purity, St. Louis, MO, USA) for 28 d. After treatment, the mice were sacrificed and their testes were either fixed in 4% paraformaldehyde or stored at −80 °C after being immediately frozen in liquid nitrogen. All procedures were approved by the Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences.

2.2. Protein network

Proteomic profile (iTRAQ) analysis was performed on testes from the control and 5 mg/kg/d PFOA-treated animals (Zhang et al., 2014). Proteins with altered expression were selected for further analysis of endocytosis and blood-testis barrier (BTB)-related networks, and this analysis was a secondary analysis based on a previous proteomic profile. All detailed iTRAQ assay data, differentially expressed protein identification, and Pathway Studio analysis *via* the ResNet database (version 6.5, Ingenuity Systems, Inc.) are shown in our previous study (Zhang et al., 2014).

2.3. Western blot analysis

Intersectin 1 (ITSN1), serine protease inhibitor A3K (Serpina3k), and apolipoprotein a1 (APOA1) were analyzed by Western blot assay using testicular protein extracts from the control and 1.25, 5, and 20 mg/kg/d PFOA treatment groups. Protein bands were analyzed with Quantity One software (v 4.6.3, Bio-Rad, USA) and the data were normalized to GAPDH levels.

2.4. qPCR of mRNA

To verify that PFOA treatment altered endocytosis in the mouse testes, genes involved in endocytosis were selected for real-time quantitative PCR analysis. Total RNAs from the testes of the control and the 1.25, 5, and 20 mg/kg/d PFOA treatment groups were isolated using the RNeasy plus Mini Kit (Qiagen, USA). cDNA synthesis and qPCR were performed as per our previous description (Wang et al., 2015), and 18S rRNA was used as the internal control. Primer sequences are listed in Supplementary Table S1. The qPCR data were analyzed with MxPro qPCR software, and the comparative CT ($2^{-\Delta\Delta CT}$) method was used to calculate the fold change of mRNA levels (Livak and Schmittgen, 2001).

2.5. MiRNA array

To be consistent with the doses used in our previous iTRAQ assay (Zhang et al., 2014), animals from the control and 5 mg/kg/d PFOA exposure group were chosen for miRNA array. Testes from two random individuals in the same group were pooled into one sample, and three pooled samples from each group were used for hybridization in the mouse miRNA arrays (Agilent Technologies, Santa Clara, CA, USA). MiRNAs with more than 1.5-fold alteration ($P < 0.05$) were deemed to be significantly changed by PFOA treatment.

2.6. TaqMan qPCR analysis of miRNA

Eight significantly changed miRNAs (miR-133b-3p, miR-365-3p, miR-17-3p, miR-193-5p, miR-191-5p, miR-184-3p, miR-410-3p, and miR-187-3p) from the miRNA analysis were selected for TaqMan qPCR assay (Life Technologies-Applied Biosystems, CA, USA) to confirm the results and detect the miRNA levels in the 1.25, 5, and 20 mg/kg/d PFOA exposure groups. U6 snRNA served as the internal control. All processes were performed according to the manufacturer's protocols, and the comparative CT ($2^{-\Delta\Delta CT}$) method was used to calculate the fold change of miRNA levels (Livak and Schmittgen, 2001).

2.7. miRNA-target network prediction

The networks between altered miRNAs and their potential targets from the differentially expressed proteins were constructed using the online TargetScan database and drawn using Cytoscape (v 3.2.1) software.

2.8. Plasmid transfection and dual-luciferase reporter assay

The 3′ untranslated region (UTR) of CLTA mRNA contained sequences complementary to the seed sites of miR-133b-3p. We amplified this relevant 3′ UTR region from the mouse genomic DNA, as well as its mutated sequence in the putative binding site with miR-133b-3p, and inserted it into psiCHECK-2 dual-luciferase reporter plasmid (Promega, Madison, WI, USA), designated as psi-CLTA 3′ UTR-wt and psi-CLTA 3′ UTR-mut, respectively. Details on the primers used for the above amplification are shown in Supplementary Table S2. The HEK 293T cells were transfected with 1 μg of the above psiCHECK-2 recombinant vector and 50 nM miR-133b-3p agomir (miR-133b-3p) or 50 nM agomir Negative Control (NC) (RiboBio Co., Ltd. Guangzhou, China), and then measured with Synergy™ 2 multi-mode readers 133 (BioTek, Vermont, USA) at 48 h after transfection.

2.9. Immunohistochemical analysis

Testes from the control and three treatment groups were fixed in 4% paraformaldehyde at room temperature (RT) for 1 d, dehydrated in 30% sucrose solution, and then embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA). Using a Leica CM1900 cryostat (Heidelberger, Nussloch, Germany), frozen sections (5 μm) were sliced, then air dried and fixed in 4% paraformaldehyde at RT for 10 min. The slices were rinsed in PBS-T buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.5% TritonX-100), and then incubated with 3% H_2O_2 in the dark at RT for 15 min to block endogenous peroxidase reaction. After blocking, the slices were incubated with rabbit antibodies of CLTA and peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Delaware Avenue, CA, USA). Brown colored hybrid proteins were

developed using 3, 3″-diaminobenzidine (DAB), and the sections were observed by microscopy (Nikon Instruments Inc., Japan).

2.10. Statistical analysis

Comparisons between groups were performed using analysis of variance (ANOVA), followed by Tukey's HSD test or independent-samples *t*-test using SPSS software (Version 18, SPSS, Inc., Chicago, IL, USA). All data were presented as means ± SE, and $P < 0.05$ was considered significant.

3. Results

3.1. PFOA treatment changed endocytosis and BTB proteins in the mouse testes

Several proteins in the testes that changed after 5 mg/kg/d PFOA treatment were involved in endocytic internalization and the BTB. Therefore, all significantly changed proteins after PFOA treatment were further analyzed to determine the networks related to endocytosis and the BTB. In the networks, 18 proteins were associated with endocytosis (Fig. 1A) and 17 proteins were associated with BTB processes (Fig. 1B). Proteins involved in both cell processes included Serpina3k, isoform 1 of mitogen-activated protein kinase 14 (MAPK14), apolipoprotein A-I (APOA1), alpha-1-antitrypsin 1–3 (SERPINA1), carbonic anhydrase 3 (CA3), calreticulin (CALR), isoform 2 of drebrin-like protein (DBNL), isoform 1 of intersectin-1 (ITSN1), annexin A5 (ANXA5), and barrier-to-autointegration factor (BANF1). Among them, Serpina3k, ITSN1, and APOA1 were validated by Western blot analysis. Consistent with the iTRAQ assay results, their expressions were significantly reduced in the 5 mg/kg/d PFOA group compared with control group (Fig. 2A and B). For Serpina3k, a dose-dependent decrease after PFOA treatment was observed. We also detected Serpina3k and APOA1 mRNA levels using qPCR assay, but no significant changes were observed after PFOA treatment (Supplementary Fig. S1). In addition, these verification results were not restricted to the altered protein levels observed by iTRAQ assay. Additional endocytosis-related genes were included in the broad verification test using qPCR assay. Many of these selected genes showed an upregulated, dose-dependent tendency in testes after PFOA treatment, and included epidermal growth factor receptor pathway substrate 8 (Eps8), Eps15, cortactin; cofilin, espin, vinculin, and zyxin. These results indicate that PFOA extensively altered the endocytosis process in the mouse testes (Fig. 3).

3.2. PFOA exposure altered miRNA profiles in mouse testes

To study the miRNA profiles, whole testes from the control and 5 mg/kg/d PFOA treatment group were examined by miRNA array. Nine and eight miRNAs were down and upregulated, respectively (at least 1.5-fold alteration, $P < 0.05$) (Table 1). Among these miRNAs, miR-187-3p, miR-410-3p, miR-223-3p, miR-127-3p, and miR-532-3p were biologically conserved among mice, rats, and humans. To further confirm the microarray data, eight significantly altered miRNAs, including four upregulated (miR-133b-3p, miR-365-3p, miR-17-3p, and miR-193-5p) and four downregulated miRNAs (miR-191-5p, miR-184-3p, miR-410-3p, and miR-187-3p) were selected for TaqMan qPCR analysis. Four groups (control and the 1.25, 5, and 20 mg/kg/d PFOA-treated groups) were used, and all eight selected miRNAs showed the same tendency as the microarray results after PFOA treatment (Fig. 4). The four downregulated miRNAs were significantly decreased under all three PFOA treatment doses using the qPCR confirmation assay. Although three (miR-133b-3p, miR-365-3p and miR-193-5p) among the four upregulated miRNAs were not changed in 1.25 mg/kg/d group compared with control group, they were significantly upregulated in 5 and 20 mg/kg/d PFOA treatment groups. For miR-17-3p, it was significantly upregulated after 1.25 and 5 mg/kg/d PFOA treatment, and increased nearly 1.3-fold after 20 mg/kg/d PFOA treatment compared with those in 1.25 and 5 mg/kg/d PFOA treatment groups.

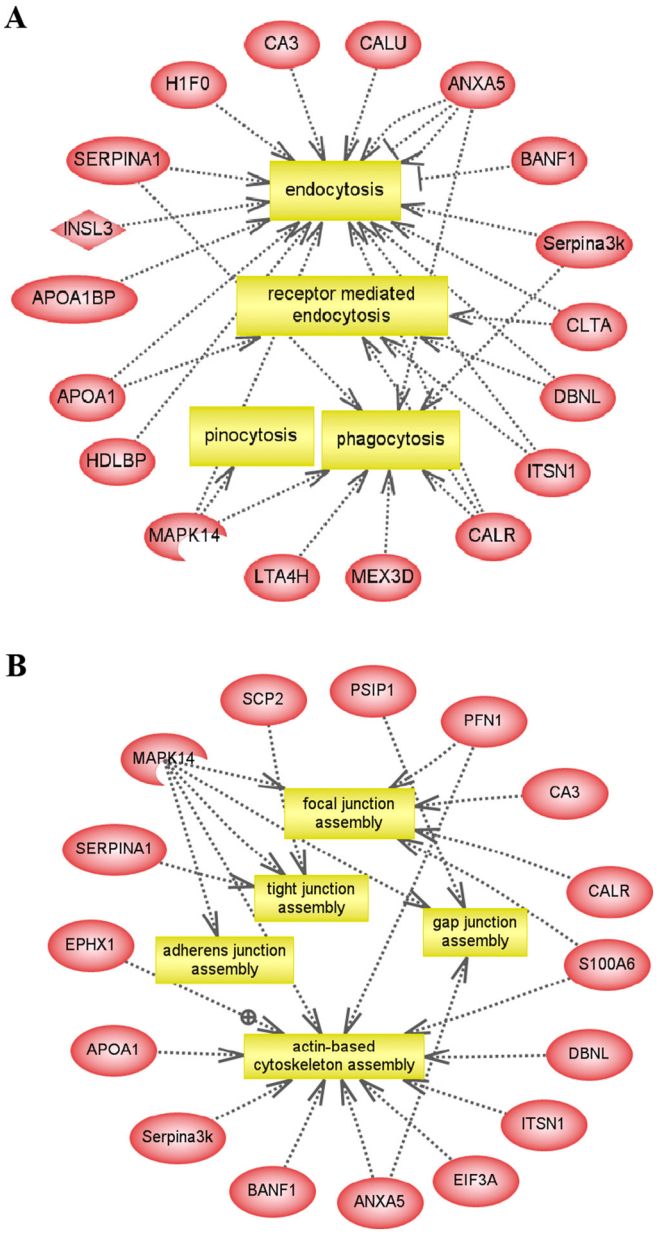

Fig. 1. Network of differentially expressed proteins in mouse testes after PFOA treatment. (A) Network of proteins related to endocytosis. (B) Network of proteins related to the BTB. Network analysis of differentially expressed proteins was performed using Pathway Studio (v 7.0) software. Full names of the proteins in the figure are as follows: CA3, carbonic anhydrase 3; CALU, calumenin; ANXA5, annexin A5; BANF1, barrier-to-autointegration factor 1; Serpina3k, serine protease inhibitor A3K; CLTA, clathrin light chain A; DBNL, isoform 2 of drebrin-like protein; ITSN1, isoform 1 of intersectin-1; CALR, calreticulin; MEX3D, RNA-binding protein MEX3D; LTA4H, leukotriene A-4 hydrolase; MAPK14, isoform 1 of mitogen-activated protein kinase 14; HDLBP, vigilin; APOA1, apolipoprotein A-I; APOA1BP, apolipoprotein A-I-binding protein; INSL3, insulin-like 3; SERPINA1, alpha-1-antitrypsin 1–3; H1f0, putative uncharacterized protein; S100a6, protein S100-A6; Eif3a, eukaryotic translation initiation factor 3 subunit A; Ephx1, epoxide hydrolase1; Scp2, isoform SCPx of non-specific lipid-transfer protein; Psip1, isoform 1 of PC4 and SFRS1-interacting protein; Pfn1, profilin-1.

3.3. Potential miRNA targets among changed proteins

Importing miRNAs and proteins identified by the miRNA and iTRAQ assays, respectively, we built a network between miRNAs and target genes using TargetScan. More than 10 miRNAs and their target pairs

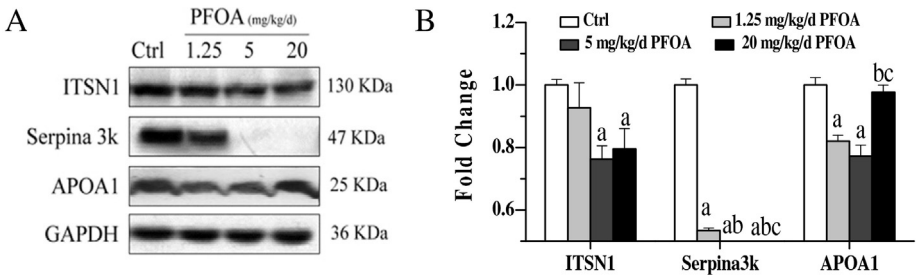

Fig. 2. Western blotting results of proteins related to endocytosis and the BTB in mouse testes. Western blots (A) and band densities (B) of protein ITSN1, Serpina3k, and APOA1. All three proteins were changed in mouse testes after PFOA exposure, as determined by iTRAQ assay. Testis homogenates from the control and PFOA-treated mice (1.25, 5 and 20 mg/kg/d, 28 d) were used for Western blot analysis. GAPDH served as the internal reference. Results are presented as means ± SE ($n = 6$), $^{a}P < 0.05$ compared with the control, $^{b}P < 0.05$ compared with 1.25 mg/kg/d group, $^{c}P < 0.05$ compared with 5 mg/kg/d group by ANOVA and Tukey's HSD test.

were predicted, including miR-133b-3p/zinc finger CCCH domain-containing protein 11A (Zc3h11a), miR-133b-3p/DBNl, miR-133b-3p/Clta, miR-193-5p/Itsn1, miR-17-3p/Itsn1, miR-532-3p/Itsn1, miR-410-3p/RNA-binding protein MEX3D (Mex3d), miR-410-3p/glycylpeptide N-tetradecanoyltransferase 1 (Nmt1), miR-467d-5p/eukaryotic translation initiation factor 5 (Eif5), miR-467f/calumenin (Calu), miR-233-3p/WD repeat-containing protein 62 (Wdr62), miR-530-5p/solute carrier family 2, and facilitated glucose transporter member 3 (Slc2a3) (Fig. 5). These results suggest that the genes of the changed proteins were potential targets of the changed miRNAs. Among the miRNA-target pairs, miR-133b-3p/CLTA was selected for further verification by luciferase activity assay after psi-CLTA 3′ UTR vector and miR-133b-3p agomir co-transfection in HEK 293T cells. The 3′ UTR of CLTA mRNA contained potential binding sites with miR-133b-3p (Fig. 6A). In addition, these potential binding sequences with miR-133b-3p in the 3′ UTR of CLTA were conserved among many mammals (Fig. 6B). A significant decrease in firefly luciferase activity was observed in psi-CLTA 3′ UTR-wt and miR-133b-3p agomir co-transfected HEK 293T cells compared with that in the control (psi-CLTA 3′ UTR-wt and agomir NC co-transfected), whereas no significant change was observed in psi-CLTA 3′ UTR-mut and miR-133b-3p agomir co-transfected cells (Fig. 6C). These results imply that miR-133b-3p bound with the 3′ UTR of CLTA and inhibited the level of luciferase protein. The *in vivo* results (Fig. 4B) showed that compared with the control, miR-133b-3p increased about 1.5-fold after 5 mg/kg/d PFOA exposure, which was similar to the miRNA array results; and a dominant increase in miR-133b-3p was also observed after 20 mg/kg/d PFOA treatment (Table 1). In contrast, CLTA proteins showed a decreasing tendency in the testes by immunohistochemical analysis (Fig. 6D), which was consistent with the change observed by iTRAQ assay. The *in vivo* miR-133b-3p and CLTA results support that CLTA might be a target of miR-133b-3p.

4. Discussion

4.1. PFOA treatment changed endocytosis and BTB remodeling in mouse testes

Testosterone stimulates spermatogenesis and supports the development of immature spermatozoa. Exposure to PFOA, which acts as a

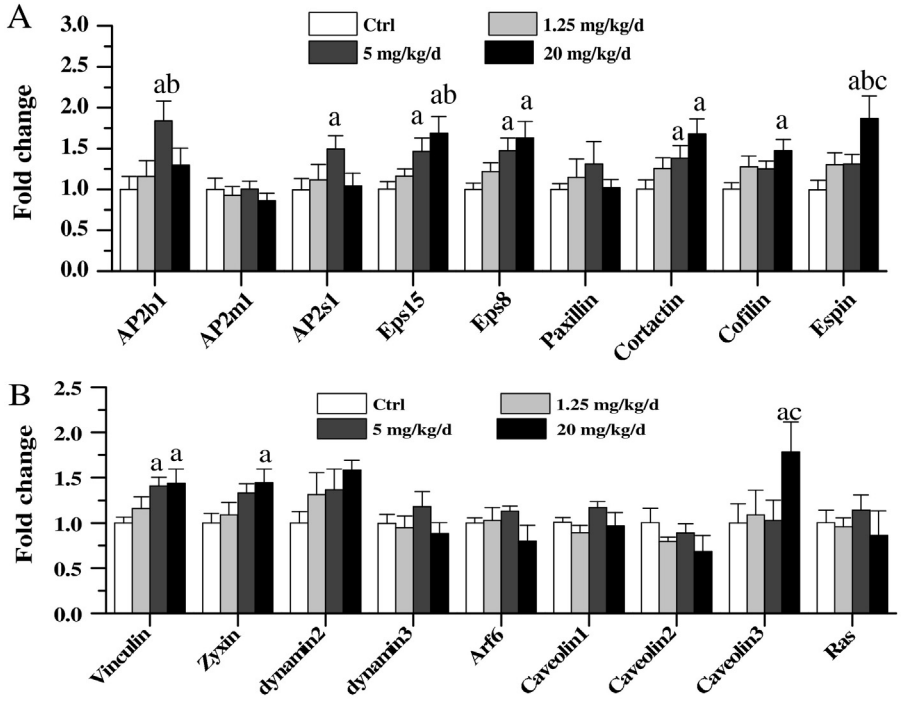

Fig. 3. Fold change of mRNAs in genes related to endocytosis in the testes of control and PFOA-treated mice (1.25, 5 and 20 mg/kg/d, 28 d). The 18S gene was used as the internal reference. Results are presented as means ± SE ($n = 6$), $^{a}P < 0.05$ compared with the control, $^{b}P < 0.05$ compared with 1.25 mg/kg/d group, $^{c}P < 0.05$ compared with 5 mg/kg/d group by ANOVA and Tukey's HSD test. Full name of the genes in the figure are as follows: AP2b1, adaptor-related protein complex 2, beta 1 subunit; AP2m1, adaptor-related protein complex 2, mu 1 subunit; APs1, adaptor-related protein complex 2, sigma 1 subunit; Eps, epidermal growth factor receptor pathway substrate; Arf6, ADP-ribosylation factor 6.

Table 1
Changed miRNAs in mouse testes treated with PFOA.

Downregulated miRNAs	Fold change	*P* value	Upregulated miRNAs	Fold change	*P* value
miR-674-5p	0.02	1.10E-05	miR-467f	1.77	0.005
miR-187-3p[a]	0.16	0.035	miR-467d-5p	1.81	0.029
miR-337-5p	0.23	0.002	miR-680	1.82	0.049
miR-410-3p[a]	0.32	0.019	miR-532-3p	1.82	0.047
miR-191-5p	0.53	0.002	miR-133b-3p	1.83	0.029
miR-184-3p	0.53	0.002	miR-365-3p	2.21	0.022
miR-503-5p	0.54	0.031	miR-17-3p	7.15	0.039
miR-223-3p[a]	0.64	0.041	miR-193-5p	8.6	0.005
miR-127-3p[a]	0.64	0.042			

These miRNAs were consistently changed 1.5-fold or more between the 5 mg/kg/d PFOA treatment and control testis sample replicates, and were statistically significant (Student's *t*-test, $P < 0.05$).

[a] miRNAs with conserved sequences among mice, rats, and humans.

suspected endocrine disruptor, results in lower testosterone in laboratory animals (Shi et al., 2007; White et al., 2011). Testosterone inhibition by PFOA has also been reported in isolated Leydig cells (Biegel et al., 1995; Zhao et al., 2010). Under our exposure dose, the body weight of mice in the 5 and 20 mg/kg/d dose groups significantly decreased by 4.6% and 20.5%, respectively, compared with the control group (Yan et al., 2014). Absolute testis weight was significantly diminished by 26.8% in the 20 mg PFOA/kg/d group, but no change was observed in 1.25 and 5 mg/kg/d dose groups. Testis weight relative to body weight was also calculated, and none of the PFOA doses significantly altered this parameter (Zhang et al., 2014). Our previous study showed that accompanied with a dose-dependent decrease in PFOA-treated group testosterone levels, sperm quality was reduced and adhesion between germ cells and Sertoli cells was lost, suggesting that PFOA can induce functional lesions in the testes (Zhang et al., 2014). However, the molecular mechanism behind these pathological lesions in testes was not clear. To uncover the global proteomic alteration in mouse testes after PFOA treatment, we previously conducted iTRAQ analysis and identified 93 proteins that differed in expression level (Zhang et al., 2014). Among the significantly changed proteins, bioinformatics analysis showed that 18 were associated with endocytosis. Endocytosis, which describes the phenomenon in which internal membranes are produced from plasma membrane *de novo*, plays a prominent role not only in cell nutrient uptake, but also in cell signaling and shaping (Doherty and McMahon, 2009). Several endocytic pathways were defined, including clathrin-mediated endocytosis, caveolae-/caveolin1-dependent endocytosis, and Arf6 dependent endocytosis. The endocytosis disturbance effect of PFOA in testes was further verified by qPCR assay, with the mRNA alteration of genes, including AP2b1, APs1, Eps8, Eps15, cofilin, espin, cortactin, vinculin, and zyxin. All the above genes were involved in the endocytic pathway of the seminiferous epithelium (Vogl et al., 2014).

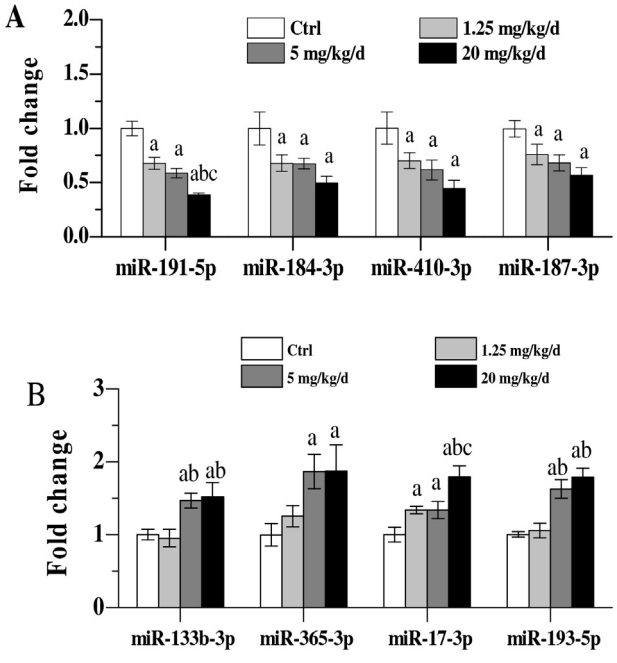

Fig. 4. qPCR of miRNA. RNAs from the control and PFOA-treated mouse (1.25, 5 and 20 mg/kg/d, 28 d) testes were prepared and fold changes of differentially expressed miRNAs were determined by TaqMan miRNA assay. U6 was used as the internal reference. Results are presented as means ± SE ($n = 6$), [a]$P < 0.05$ compared with the control, [b]$P < 0.05$ compared with 1.25 mg/kg/d group, [c]$P < 0.05$ compared with 5 mg/kg/d group by ANOVA and Tukey's HSD test.

Seventeen differentially expressed proteins were associated with the BTB. The BTB creates an immunological barrier to prevent exogenous chemicals from entering the apical compartments, thereby providing a suitable environment for spermatogenesis (Steinberger and Klinefelter, 1993; Siu et al., 2009). The BTB is sensitive to environmental toxicants, including PFASs (Zhang et al., 2008; Qiu et al., 2013; Wan et al., 2014). Our previous study demonstrated BTB damage after PFOA treatment, as observed by impermeable biotin fluorescence passing through the BTB to the lumen tubular of the testes and the decrease in transepithelial electrical resistance in Sertoli cells *in vitro*. In addition, the levels of important structure proteins in gap junctions and tight junctions, such as connexin-43 and claudin-11, were significantly reduced in the testes after PFOA treatment. These results indicate that BTB disturbance between germ cells and Sertoli cells might be crucial to PFOA-induced male reproductive dysfunction in mice (Lu et al., 2016).

Among the 17 proteins associated with BTB, 10 were also involved in endocytosis. Endocytosis and BTB remodeling are closely related cell processes. Endocytosis plays an important role in the regulation of protein levels and turnover at cell junctions (Maxfield and McGraw, 2004; Ivanov et al., 2005; Vogl et al., 2014). More importantly, endocytic vesicle-mediated protein trafficking is crucial to spermatogenesis during the epithelial cycle. During the intermittent phases, the junctions between Sertoli cells disassemble and reassemble to facilitate preleptotene and/or leptotene spermatocytes transiting cross the Sertoli-Sertoli cell interface without compromising barrier function (Mruk and Cheng, 2004; Yan et al., 2008; Wong et al., 2009; Wong et al., 2010; Smith and Braun, 2012; Su et al., 2013; Vogl et al., 2013). Studies have shown that the endocytosis protein dynamin (McNiven et al., 2000; Sever et al., 2000), which was identified as differentially expressed in our iTRAQ assay, is also structurally associated with integral membrane proteins at the BTB (Kamitani et al., 2002; Lie et al., 2006). Among the proteins involved in endocytosis and BTB remodeling, ITSN1, Serpina3k, and APOA1 were validated by Western blotting and found to be significantly reduced in the 5 mg/kg/d PFOA treatment group. ITSN1 is a widely expressed, evolutionarily conserved multidomain scaffold protein, which is mainly engaged in clathrin-mediated endocytosis and cell signaling (O'Bryan, 2010; Tsyba et al., 2011). ITSN1 interacts with many endocytic proteins, including dynamin, AP2 (adaptor protein 2), and epsin family members. ApoA-I protects ABCA1 from intracellular proteolysis after ABCA1 clathrin-mediated endocytotic internalization (Lu et al., 2008). Serpina3k is a serine protease inhibitor, which prevents the hypoxia-induced decrease of the tight junction protein occludin and protects the integrity in the corneal endothelial junctional barrier (Zhang et al., 2009). Altered expression of two endocytosis and BTB remodeling proteins, CALR and MAPK14, was also verified previously (Zhang et al., 2014).

Although levels of many endocytosis proteins was altered in the testes after PFOA treatment, histological endpoints showing endocytosis change will be more helpful. The testes tissue was not prepared for electron microscope observation after PFOA treatment, and a more detailed histological and immunochemical analysis focused on selected endocytosis pathways will be necessary in the future.

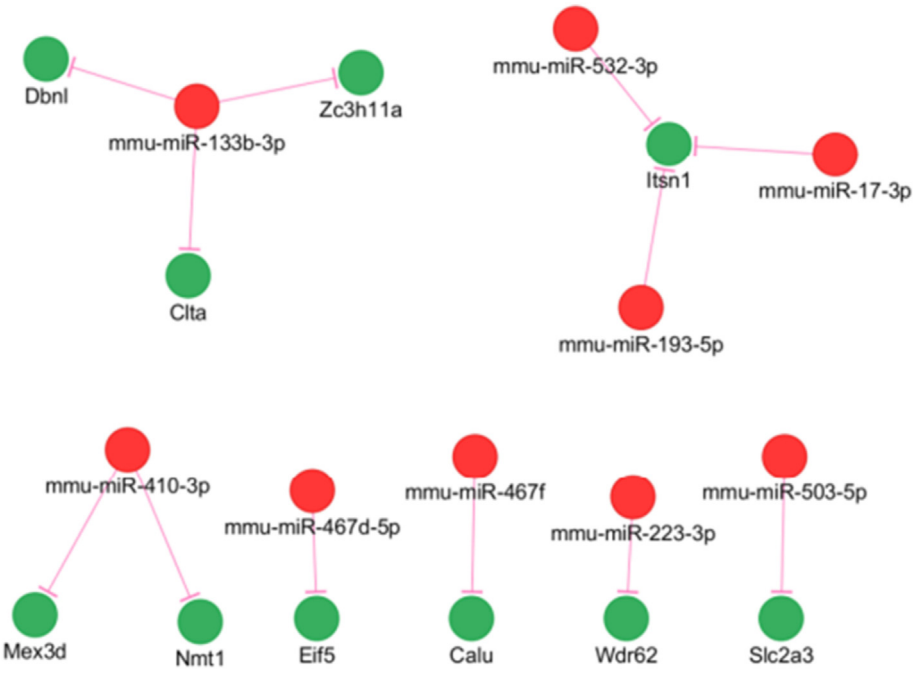

Fig. 5. Predicted miRNA targets from differentially expressed proteins in mouse testes after PFOA exposure. Target prediction was performed using TargetScan, and the networks were drawn using Cytoscape (v 3.2.1). Full names of the proteins in the figure are as follows: Calu, calumenin; Dbnl, isoform 2 of drebrin-like protein; Zc3h11a, zinc finger CCCH domain-containing protein 11A; Itsn1, isoform 1 of intersectin-1; Mex3d, RNA-binding protein MEX3D; Nmt1, glycylpeptide N-tetradecanoyltransferase 1; Eif5, eukaryotic translation initiation factor 5; Clta, clathrin light chain A; Wdr62, WD repeat-containing protein 62; Slc2a3, solute carrier family 2, facilitated glucose transporter member 3.

4.2. MiRNA profile alteration in testes might be involved in the toxicological effect of PFOA

MiRNAs are RNA molecules 21–23 nucleotides in length, which repress target genes at the post-transcriptional level in animals (Lee et al., 1993). MiRNAs regulate target mRNAs mainly through the recognition and binding of mRNA 3′ UTR motifs, which are complementary to its seed region (nucleotides 2–7) (Lewis et al., 2003). One reasonable explanation for the inconsistency between mRNAs and their corresponding proteins is that miRNAs might be involved in the post-transcriptional regulation of their targets (Novotny et al., 2007a; Novotny et al., 2007b). In addition, compared with somatic cells, higher abundances of some miRNAs are found in primordial germ cells, germ cells, and germ line stem cells (Buchold et al., 2010). In the present

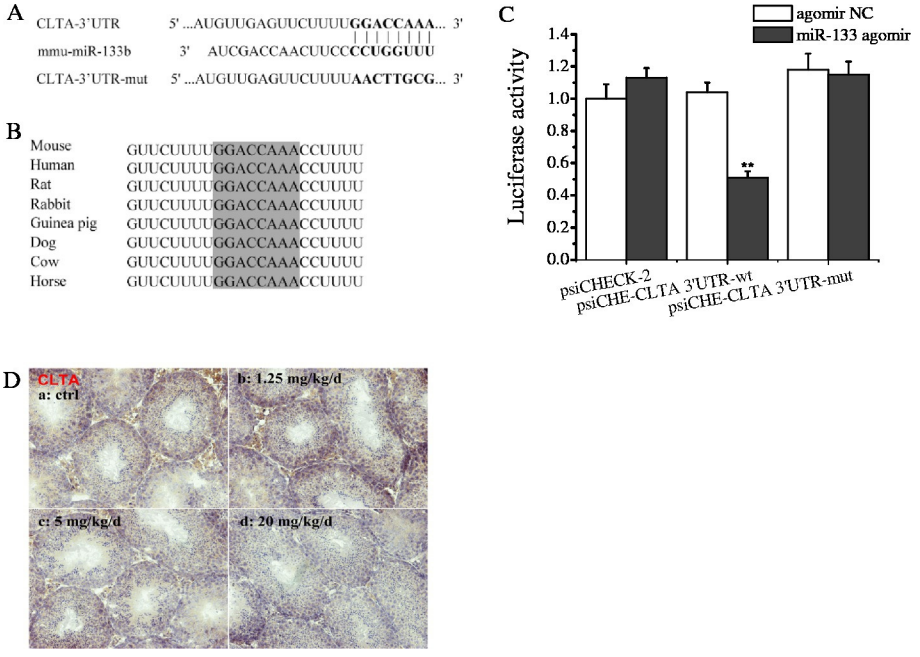

Fig. 6. CLTA as a potential target of miR-133b-3p. (A) Predicted binding site of miR-133b in the 3″ UTR of CLTA. (B) Predicted binding site of miR-133b in the 3″ UTR of CLTA is highly conserved among many mammals. (C). Luciferase activity. The psiCHECK-2, psi-CLTA-3″ UTR-wt, or psi-CLTA-3″ UTR-mut vectors were co-transfected with miR-133b agomir or agomir NC into HEK 293T cells, and normalized Renilla luciferase activity was determined. Results are presented as means ± SE, $^{**}P < 0.01$. (D) Immunohistochemical analysis of CLTA protein in testes of the control and PFOA-treated mice (1.25, 5 and 20 mg/kg/d, 28 d). Micrographs using frozen sections of testes were observed following CLTA antibody incubation.

study, PFOA exposure changed miRNA expression profiles in the mouse testes, with eight upregulated and nine downregulated, implying that the complicated effects of PFOA on testes might be partially exerted through miRNAs. Among the seventeen altered miRNAs, eight (four upregulated and four downregulated) was further confirmed by TaqMan qPCR assay. Significant changes of miRNAs profile were also observed in our previous studies in liver and serum after PFAS treatment (Yan et al., 2014; Wang et al., 2015). However, the liver miRNAs showed totally different profiles to that of the testes, implying divergent regulation between the two organs.

Although the regular population has serum PFOA levels of no more than 10 ng/mL, occupational workers can possess very high serum PFOA levels. For example, in one of our previous studies of occupational participants from a fluorochemical plant in Jiangsu China, we reported a median serum level of 1.64 μg/mL (Wang et al., 2012). In our current subacute toxic study, a relatively high PFOA dosage was selected, with 1.25 mg/kg/day PFOA exposure leading to PFOA testicular levels of 0.01 μg/g wet weight and in 5 and 20 mg PFOA/kg/day groups reaching 5.37 and 8.06 μg/g wet weight (Zhang et al., 2014). Our previous study showed that serum PFOA levels was 23.83 μg/mL, 56.24 μg/mL and 105.29 μg/mL in the 1.25, 5 and 20 mg PFOA/kg/day groups, respectively (Yan et al., 2014). Although the internal serum exposure concentration in the current animal study was 1–2 orders of magnitude higher than that in occupational workers, given the fact that PFOA is persistent and bioaccumulative, with a nearly 3.8 year half-life in humans, the testes proteome and miRNA profile alteration in mice after 28 day exposure can be served as a sort of alarm revealing the potential hazards of high PFOA burden.

4.3. CLTA is a potential target of miR-133b-3p

Due to the unavailability of effective high-throughput methods and the laborious nature of experiments, computational prediction of miRNA targets based on features, including 5′ seed matches, conservation and secondary structure of mRNA sequences are a popular choice (Lewis et al., 2003; Bartel, 2009; Min and Yoon, 2010). More than 10 miRNAs and protein targets pairs were built using TargetScan, implying that miRNAs might play a role in the regulation of post-translational protein repression in mouse testes after PFOA treatment. Among these miRNAs and protein targets pairs, miR-133b-3p/CLTA was selected and further verified by luciferase activity assay. Results showed that miR-133b-3p bound with the 3′ UTR of CLTA and inhibited the level of recombinant luciferase protein. As observed in the *in vivo* results, miR-133b-3p was increased after 5 mg/kg/d PFOA treatment compared with that in the control, whereas CLTA proteins showed a decreasing tendency in testes by immunohistochemical assay, supporting that CLTA might be a target of miR-133b-3p. Studies on miR-133 have shown that it is mainly involved in muscle development, and its aberrant expression is linked to diseases such as heart failure, cardiac hypertrophy, and muscular dystrophy (Townley-Tilson et al., 2010; Yu et al., 2014). In addition, miR-133b has been found consistently downregulated in eight tumor types (including testicular), implying its important role in cancer pathogenesis and development (Navon et al., 2009). Clathrin-mediated endocytosis is the principal way by which mammalian cells internalize cell surface receptors (Doherty and McMahon, 2009), and clathrin is a major protein in coat membranes of vesicles during clathrin-mediated endocytosis and sorting in the trans-Golgi network/endosomal system (Traub, 2005; Brodsky, 2012). The miR-133b-3p regulation of CLTA, an important molecule in the self-assembly of the clathrin coat, suggests that miRNA is involved in the disturbance of endocytosis in testes following PFOA exposure.

5. Conclusions

Results demonstrated that PFOA treatment resulted in dominant alteration of proteins involved in endocytosis and BTB remodeling in the testes. miRNA profiles in mouse testes were changed after PFOA treatment, with eight upregulated and nine downregulated. MiRNA-protein interaction prediction supported the concept that miRNAs might play a role in testicular toxicity induced by PFOA treatment. The regulation of CLTA by miR-133b-3p was verified *in vitro* with transfection and luciferase activity assay.

Conflict of interest

We declare that we have no conflict of interest.

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Appendix A. Supplementary data

Primer sequences for qPCR are shown in Table S1. Primer sequences for 3′ UTR cloning are listed in Table S2. Serpina3k and APOA1 qPCR results are shown in Supplementary Fig. 1. Supplementary data associated with this article can be found in the online version, at doi 10.1016/j.taap.2017.01.014.

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