Environmental Toxicology and Pharmacology 56 (2017) 136–144

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Research Paper

# The role of PPAR alpha in perfluorooctanoic acid induced developmental cardiotoxicity and L-carnitine mediated protection–Results of *in ovo* gene silencing

Meng Zhao[a,1], Qixiao Jiang[a,*,1], Min Geng[a], Li Zhu[b], Yunqiu Xia[a], Aashish Khanal[a], Chunbo Wang[a,*]

[a] Qingdao University Medical College, China
[b] The Affiliated Hospital of Qingdao University, China

ARTICLE INFO

*Keywords:*
Perfluorooctanoic acid
Developmental cardiotoxicity
*In ovo* gene silencing
Peroxisome proliferator-activated receptor alpha
L-Carnitine

ABSTRACT

Perfluorooctanoic acid (PFOA) is a persistent organic pollutant. This study established an *in ovo* peroxisome proliferator-activated receptor alpha (PPAR alpha) silencing model in chicken embryo heart, and investigated the role of PPAR alpha in PFOA induced developmental cardiotoxicity. The *in ovo* silencing was achieved by introducing lentivirus expressing PPAR alpha siRNA into ED2 chicken embryo *via* microinjection (0.05 ul/g egg weight). Transfection efficacy was confirmed by fluorescent microscopy and western blotting. To assess the developmental cardiotoxicity, cardiac function (heart rate) and morphology (right ventricular wall thickness) were measured in D1 hatchling chickens. 2 mg/kg (egg weight) PFOA exposure at ED0 induced significant elevation of heart rate and thinning of right ventricular wall thickness in D1 hatchling chickens. PPAR alpha silencing did not prevent PFOA-induced elevation of heart rate; however, it did significantly increase the right ventricular wall thickness as compared to PFOA exposed animals. Meanwhile, PPAR alpha silencing did not abolish the protective effects exerted by exposure to 100 mg/kg (egg weight) L-carnitine. In conclusion, PFOA-induced heart rate elevation is likely PPAR alpha independent, while the right ventricular wall thinning seems to be PPAR alpha dependent. The protective effects of L-carnitine do not require PPAR alpha.

## 1. Introduction

Perfluorooctanoic acid (PFOA), a perfluorinated compound (PFC), was first made into production and entered environment after World War II (Steenland et al., 2010). It is widely used in the production of myriad polymer products, such as non-stick plastic coatings, flame retardants and water repellent coatings (Begley et al., 2005). Beginning at late 1990s, PFOA was associated with various adverse health effects, such as endocrine disruption, immune toxicity, carcinogenicity and developmental toxicity (Biegel et al., 2001; Wolf et al., 2007; Dewitt et al., 2008; Vested et al., 2013). Moreover, PFOA is a highly stable compound both in environment and biota, resulting in persistent contamination (Fromel and Knepper, 2010). Although its production in the United States had been phased out in 2015 (Bjerregaard-Olesen et al., 2016), the production continues in other parts of the world (Liu et al., 2016), thus its effects throughout the world is expected to continue for a considerable amount of time.

Efforts had been made to elucidate the mechanism of toxicity for PFOA. From its chemical structure, it is generally accepted that PFOA could activate the peroxisome proliferator-activated receptor alpha (PPAR alpha) (Wolf et al., 2008). In a rodent model, PFOA exposure resulted in retarded development and higher mortality, while PPAR alpha knock out seems to abolish such effects (Abbott et al., 2007). However, PPAR alpha independent effects were also demonstrated in multiple studies as well (Buhrke et al., 2015; Filgo et al., 2015).

In our lab, the research focused on PFOA-induced developmental cardiotoxicity in chicken embryo. Previously, PFOA was found to alter the morphology and function of developing chicken embryo/hatchling chickens (Jiang et al., 2012), which was partially associated with PPAR alpha (Jiang et al., 2013). However, that study relied on PPAR alpha agonist WY 14,643, so further elucidation is still needed. In our recent study, PPAR alpha antagonist GW6471 was found to partially block the effects of PPAR alpha as well (Jiang et al., 2016). But again, the lack of specificity decreased the strength of such conclusions. Additionally, in Jiang et al. (2016), the potential role of L-carnitine in PFOA-induced developmental cardiotoxicity was highlighted. L-carnitine is an

* Corresponding authors.
*E-mail addresses:* jiangq08@126.com (Q. Jiang), cbwang666@126.com (C. Wang).
[1] These authors contributed equally.

http://dx.doi.org/10.1016/j.etap.2017.09.006
Received 20 May 2017; Received in revised form 4 September 2017; Accepted 14 September 2017
Available online 14 September 2017
1382-6689/ 

endogenous compound that participates in fatty acid beta oxidation as a fatty acid carrier (Reuter and Evans, 2012). In Jiang et al. (2016), PFOA exposure decreased the levels of L-carnitine and its short-chain derivatives in ED6 chicken embryo heart. Such results are consistent with previous reports that PFOA exposure could decrease L-carnitine level in other types of cells (Peng et al., 2013). However, the exact role of L-carnitine in PFOA induced developmental cardiotoxicity is yet to be elucidated.

To further elucidate the mechanism of toxicity of PFOA in developing chicken embryo heart, it is necessary to knock out/knock down PPAR alpha. However, to the best of our knowledge, no PPAR alpha knock out chicken model had been established previously. Readily available techniques for gene knock-out/silencing are often based on *in vitro* system (Kudryavtseva et al., 2016; Canver et al., 2017), which are inadequate for *in vivo* developmental study, as the *in vitro* system could not mimic the complex developing organisms completely. Nonetheless, it is indeed possible to introduce viral vectors into developing avian embryos (Zhang et al., 2012). With reference to the existing methods and necessary modifications, a new *in ovo* lentivirus-mediated gene silencing method was established in our lab. In this study, we took advantage of this technique to knock down PPAR alpha expression in developing chicken embryo heart, and further explored the role of PPAR alpha and L-carnitine in PFOA-induced development cardiotoxicity in chicken embryo.

## 2. Materials and methods

### 2.1. Materials

PFOA (77262, CAS 3825-26-1), and L-carnitine (C0158, CAS 541-15-1) were purchased from Sigma (St. Louis, MO, US). Fertile chicken eggs (*Gallus gallus*) were purchased from Linwen Trading Co. Ltd (Jining, Shandong, China). Lentivirus (Control lentivirus or PPAR alpha silencing lentivirus) were designed and produced by Genechem (Shanghai, China). Antibody against PPAR alpha was purchased from Biorbyt (Cambridge, UK). Antibody against GAPDH was purchased from ZSbio (Beijing, China). Other chemicals and consumables were of the highest grade obtainable.

### 2.2. Methods

#### 2.2.1. Chicken embryo incubation

Upon arrival, fertile chicken eggs were cleaned with povidone iodide solution and dipped dry with tissue papers. Then eggs were candled to mark the air cell area with pencil, weighed and assigned to groups evenly based on egg weight. Eggs were incubated in a Keyu incubator (Dezhou, Shandong, China). Temperature and humidity were automatically controlled by the incubator. Basically, the temperature starts at 37.9° Celsius and gradually decreased to 37.1° Celsius as incubation progressed, while the humidity starts at 50% and gradually increased to 70%. Eggs were turned every 3 h until two days before hatch. One day before hatching, eggs were moved to individual small hatch boxes. Hatched chickens were kept in a warmed box supplied with water until experiments. All procedures followed national institutes of health guide for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Qingdao University.

#### 2.2.2. Air cell injection

As described in Jiang et al. (2012), the fertile chicken eggs were injected with vehicle (sunflower oil), PFOA or PFOA along with L-carnitine. Dosing suspensions containing PFOA 20 mg/ml or PFOA 20 mg/ml + L-carnitine 1000 mg/ml were prepared in sunflower oil so that the desired final doses (PFOA 2 mg/kg egg weight or PFOA 2 mg/kg egg weight + L-carnitine 100 mg/kg egg weight) could be achieved at 0.1 ul/g (egg weight) injection. To improve the solubility and stability of L-carnitine in suspension, 20% distilled water and 10% ethanol were added to the suspension containing L-carnitine (oil volume was correspondingly decreased to ensure identical total volume).

Briefly, the air cell area was sterilized with 70% ethanol, then a hole approximately 1 mm in diameter was opened with a metal probe, and then the dosing suspensions were injected with fine pipette. All dosing solutions were vortexed prior to injection. After the injection, the hole was sealed with melted paraffin. The dose selection of PFOA and of L-carnitine was based on prior studies in the lab (Jiang et al., 2012; Jiang et al., 2016), along with the reference dose set by FDA on marketed L-carnitine preparations for human consumption (50–100 mg/kg/day for infants and children) (USFDA, 2015).

#### 2.2.3. Establishment of in ovo lentivirus transfection method

The *in ovo* transfection method was developed based upon previous egg injection experiences, with some reference to a previous publication (Zhang et al., 2012). For all the methods tested, sterile condition was maintained. A microinjector (1–5 ul) (Anting Scientific Instrument, Shanghai, China) was used to inject lentivirus into the eggs. The lentivirus stock solution (5–6 $\times$ $10^8$ TU/ml) was diluted to 3 $\times$ $10^8$ TU/ml with sterile saline, and the injection volume was 0.05 ul/g (egg weight).

In preliminary tests, direct injection into subgerminal cavity at ED0 or into the embryo at ED2 after making a hole 3 mm in diameter in the center of the air cell area and tearing open the inner membrane resulted in extremely high embryo mortality (data not shown), thus a new method was utilized instead: a hole approximately 1.5 mm in diameter was made in the center of the air cell area, then the lentivirus solution was injected underneath the center of inner membrane but not too deep into the yolk (approximately 1.5–2 mm underneath the inner membrane). Subsequently, the hole was sealed with a small piece of tape. This method maintained acceptable survival rate of embryos and was used for the following experiments.

Two time points were tested with the transfection method: ED0 and ED2. Since the lentivirus used will express enhanced green fluorescent protein (EGFP) in the host organism, fluorescent microscopy for EGFP was performed in ED15 chicken embryo hearts (ED15 was the earliest time point to get acceptable freezing sectioning of the heart) to confirm the transfection efficacy. Briefly, ED15 embryos were taken out of the shell, quickly decapitated, and the hearts were dissected out, rinsed in cold phosphate buffered saline, and freeze-embedded with O.C.T. Compound (Sakura, Tokyo, Japan). A Leica CM1860 cryomicrotome (Leica, Wetzlar, Germany) was used to section the hearts at 20 um thickness, and the resulting sections were visualized directly under an Olympus BX50 fluorescent microscope (Olympus, Tokyo, Japan) with excitation wavelength 460–490 nm and emission wavelength 515 nm. At least three independent embryos were assessed per treatment. Based on the transfection efficacy, ED2 time point was selected for the following experiments.

#### 2.2.4. Compatibility of air cell injection and lentivirus transfection

To confirm that the air cell injection method is compatible with the lentivirus transfection method, one additional group of embryos were air cell injected with vehicle (sunflower oil) at ED0 and transfected with lentivirus at ED2. On ED2, the paraffin seal on top of the hole made during air cell injection was carefully removed with fine forceps and enlarged to 1.5 mm in diameter, allowing the lentivirus injection. Then the hole was sealed with tape and eggs were returned to incubator. The transfection efficacy was checked with fluorescent microscopy as described in 2.2.3.

#### 2.2.5. Confirmation of PPAR alpha silencing

With the method described in 2.2.3, three PPAR alpha silencing lentivirus and the control lentivirus designed and manufactured by Genechem (details of siRNA sequences were provided in Table 1, vector information available at http://www.genechem.com.cn:8080/Zaiti.aspx?zt=GV248) were transfected into ED2 chicken embryo. Sterile

**Table 1**
siRNA sequences of the PPAR alpha silencing lentivirus used in transfection.

| Virus ID | 5′ | STEM | Loop | STEM | 3′ |
|---|---|---|---|---|---|
| PPARalpha Virus-1-a | Ccgg | CTGGGAAATGGTCCAGGATCT | CTCGAG | AGATCCTGGACCATTTCCCAG | TTTTTg |
| PPAR alpha Virus-1-b | aattcaaaaa | CTGGGAAATGGTCCAGGATCT | CTCGAG | AGATCCTGGACCATTTCCCAG | |
| PPAR alpha Virus-2-a | Ccgg | GATGATATAGGTAGTCCTTTA | CTCGAG | TAAAGGACTACCTATATCATC | TTTTTg |
| PPAR alpha Virus-2-b | aattcaaaaa | GATGATATAGGTAGTCCTTTA | CTCGAG | TAAAGGACTACCTATATCATC | |
| PPAR alpha Virus-3-a | Ccgg | CTGTGATATAATGGAGCCAAA | CTCGAG | TTTGGCTCCATTATATCACAG | TTTTTg |
| PPAR alpha Virus-3-b | aattcaaaaa | CTGTGATATAATGGAGCCAAA | CTCGAG | TTTGGCTCCATTATATCACAG | |

PPAR alpha silencing lentivirus were designed and manufactured by Genechem (Shanghai, China). The detailed sequences information were included in this table.
The lentivirus vector was GV248, with the components: hU6-MCS-Ubiquitin-EGFP-IRES-puromycin. For details, please refer to http://www.genechem.com.cn:8080/Zaiti.aspx?zt=GV248.

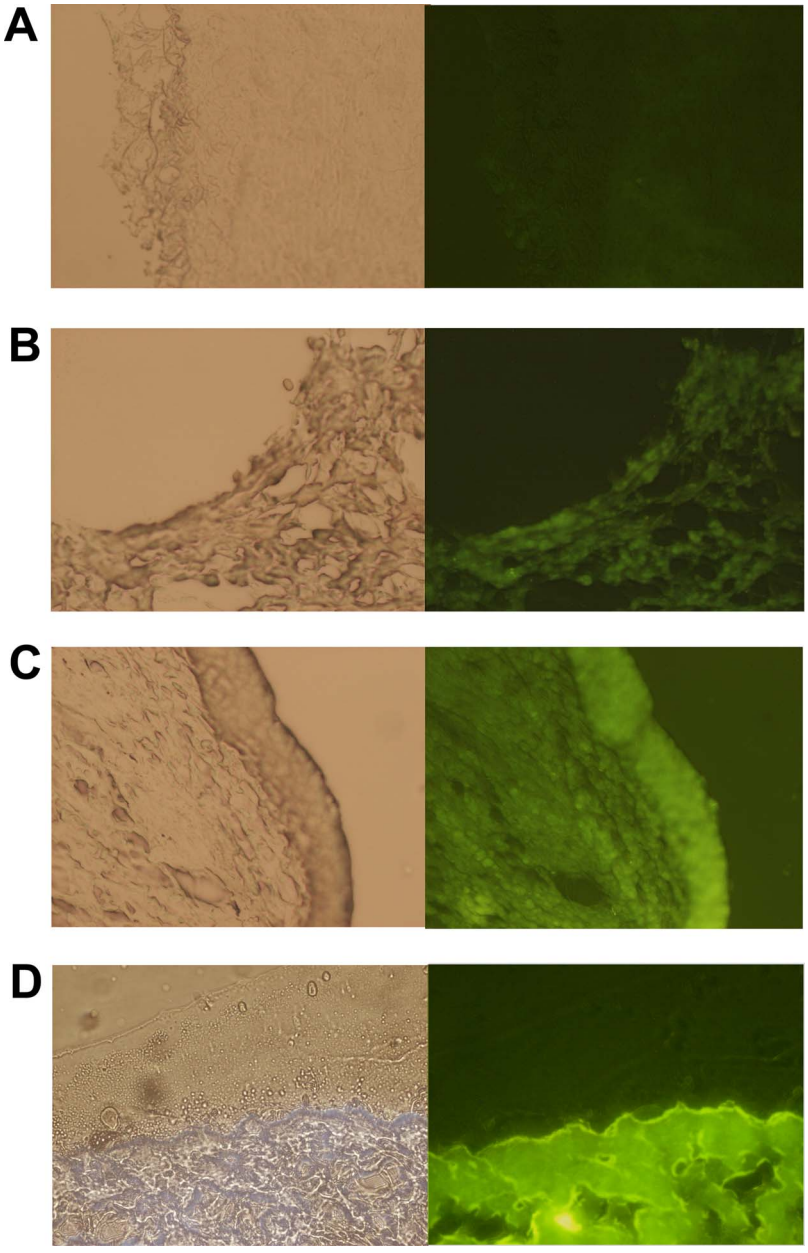


**Fig. 1.** Representative fluorescent microscopy pictures of ED15 chicken embryo hearts.
Chicken embryos were microinjected with lentivirus expressing enhanced green fluorescent protein at ED0 or ED2, and incubated to ED15. The hearts were sectioned at a cryomicrotome (Leica CM1860) at 20 um and directly visualized with an Olympus BX50 fluorescent microscope (Excitation: 460–490 nm, emission: 515 nm, magnitude: 200X).
A. Representative pictures from vehicle control embryo. Left panel: brightfield picture; right panel: fluorescent picture.
B. Representative pictures from embryos microinjected with lentivirus at ED0. Left panel: brightfield picture; right panel: fluorescent picture.
C. Representative pictures from embryos microinjected with lentivirus at ED2.Left panel: brightfield picture; right panel: fluorescent picture.
D. Representative pictures from embryos air cell injected with vehicle (sunflower oil) at E0, and then microinjected with lentivirus at E2. Left panel: brightfield picture; right panel: fluorescent picture.

saline was used as vehicle control. To confirm the PPAR alpha silencing efficacy, embryos were returned to incubator and allowed to develop until ED15, then embryos were taken out of shell, quickly decapitated, and the hearts were collected for western blotting. The western blotting protocol was as described in Wang et al. (2014) with slight modifications. The concentration of anitbodies used was 1:1000 for PPAR alpha and 1:3000 for GAPDH. Images were visualized with ECL chemoluminscence kit (Solarbio, Beijing, China), acquired with UVP 810 (UVP, Upland, CA, US), and analyzed with ImageJ (NIH, US). Semi-quantification was performed by first normalizing the density of PPAR alpha bands to the corresponding GAPDH bands, and then normalizing each bands to control. Three blots from three independent groups of samples

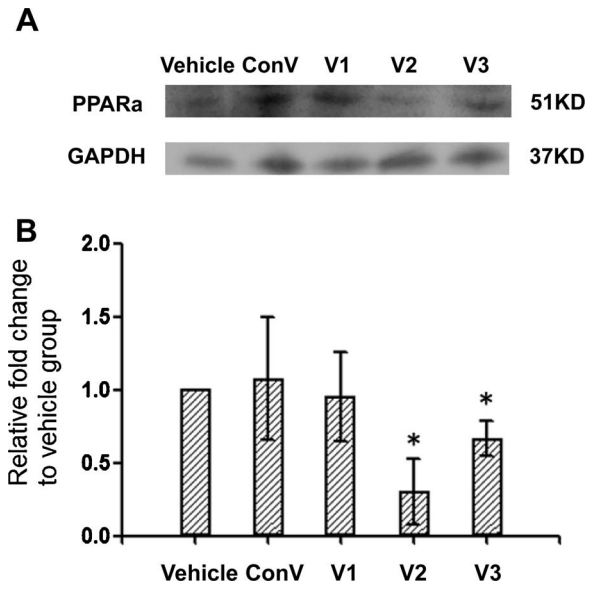


**Fig. 2.** Western blotting for PPAR alpha in ED15 chicken embryo hearts.
Chicken embryos were microinjected with three types of PPAR alpha silencing lentivirus (refer to Table 1 for detail) at ED2, incubated to ED15, and then the hearts were dissected out, protein samples were extracted with radio immunoprecipitation assay (RIPA) buffer and subjected to western blotting for PPAR alpha. N = 3 per group. Conv: Control lentivirus. V1, V2, V3: The three lentivirus with different designs.
*: Statistically different from vehicle control group ($P < 0.05$).
A. Representative western blotting picture for PPAR alpha.
B. Quantification for PPAR alpha expression normalized to GAPDH.

were performed. The lentivirus with best silencing efficacy (Virus-2 in Table 1) was selected for subsequent experiments.

#### 2.2.6. Assessment of hatchling chicken heart rate

Fertile chicken eggs were evenly assigned into eight groups based on egg weight: vehicle (sunflower oil); vehicle + control lentivirus; vehicle + PPAR alpha silencing lentivirus; PFOA 2 mg/kg (egg weight); PFOA 2 mg/kg + control lentivirus; PFOA 2 mg/kg + PPAR alpha silencing lentivirus; PFOA 2 mg/kg + L-carnitine 100 mg/kg and PFOA 2 mg/kg + L-carnitine 100 mg/kg + PPAR alpha silencing lentivirus. Vehicle/PFOA/L-carnitine were air cell injected at ED0, while control/PPAR alpha silencing lentivirus were injected at ED2 as described in 2.2.3. Treated embryos were incubated until hatch. Hatchling chicken heart rates were assessed as described in Jiang et al. (2016).

#### 2.2.7. Assessment of hatchling chicken heart morphology

After the functional assessment, hatchling chickens (anaesthetized with 33 mg/kg pentobarbital, intraperitoneal injection) were sacrificed. Hearts were processed, and the morphology was assessed as described in Jiang et al. (2012). Briefly, seven measurements were equally spaced radially along the right ventricular wall; the mean of the seven measurements was then normalized to the whole heart weight. For details, please refer to Supplementary Fig. 1.

### 2.3. Statistical analysis

Statistical analysis was performed with SPSS 17.0. One way analysis of variance (ANOVA) was used to detect differences. When ANOVA returned positive results, post-hoc least significant difference (LSD) tests were used to compare among groups. Results were considered statistically significant when $P < 0.05$.

## 3. Results

### 3.1. Establishment of lentivirus transfection method

Fluorescent microscopy revealed higher transfection efficacy in embryos transfected at ED2 than those transfected at ED0, as indicated by brighter green fluorescence on the heart tissue (Fig. 1A–C), thus ED2 was selected as the final transfection time point. Additionally, the transfection efficacy did not appear to be affected by air cell injection at ED0 and then lentivirus transfection at ED2 (Fig. 1D), indicating that the lentivirus transfection method is compatible with the classical air cell injection method.

### 3.2. Confirmation of PPAR alpha silencing

The efficacy of PPAR alpha silencing was demonstrated by western blotting on ED15 chicken embryo heart protein extracts. Three lentiviruses were tested (as indicated in Table 1). While virus-1 did not exhibit significant impacts, virus-2 and virus-3 significantly decreased the expression levels of PPAR alpha in ED15 chicken embryo heart by 68.4% and 34.6%, respectively (Fig. 2). Thus, virus-2 was selected for subsequent experiments.

### 3.3. General parameters of hatchling chickens treated with PFOA and/or lentivirus

The slim body weight (calculated by subtracting the yolk weight from whole body weight), heart index (heart weight/slim body weight), liver index (liver weight/slim body weight) and hatchability (total number hatched/total number reached ED19) were reported in Fig. 3. No statistical significance was detected among the groups.

### 3.4. Effects of PPAR alpha knockdown on the hatchling chicken heart rate

Consistent with previous studies in the lab (Jiang et al., 2016), PFOA 2 mg/kg (egg weight) air cell injection at ED0 induced significant heart rate elevation in hatchling chickens comparing to those treated with vehicle, while co-treatment with control lentivirus or PPAR alpha silencing lentivirus did not significantly alleviate such changes. Meanwhile, 100 mg/kg (egg weight) L-carnitine co-treatment effectively reverted the changes in heart rate regardless of lentivirus treatment. Control or PPAR alpha silencing lentivirus treatment alone did not alter heart rates remarkably (Fig. 4).

### 3.5. Effects of PPAR alpha knockdown on the hatchling chicken heart morphology

Changes to the right ventricular wall thickness of chicken embryo hearts were reported by our previous studies (Jiang et al., 2012). In the current study, the D1 hatchling chicken heart right ventricular wall thickness normalized to heart weight (um/g) was significantly decreased following PFOA 2 mg/kg exposure at ED0, while co-treatment with 100 mg/kg L-carnitine protected the hearts from such changes. Such results were consistent with previous results (Jiang et al., 2016). Additionally, co-treatment with control lentivirus had no significant impact. However, co-treatment with PPAR alpha silencing lentivirus at ED2 did significantly alleviate such changes. Additionally, 100 mg/kg L-carnitine treatment together with PFOA exposure and PPAR alpha silencing lentivirus effectively reversed the thinning of right ventricular wall, with a higher mean, but this result did not statistically differ from those of the PFOA + PPAR alpha silencing group (Fig. 5).

## 4. Discussion

The involvement of PPAR alpha in many PFOA-induced effects had been demonstrated. However, due to the lack of available gene

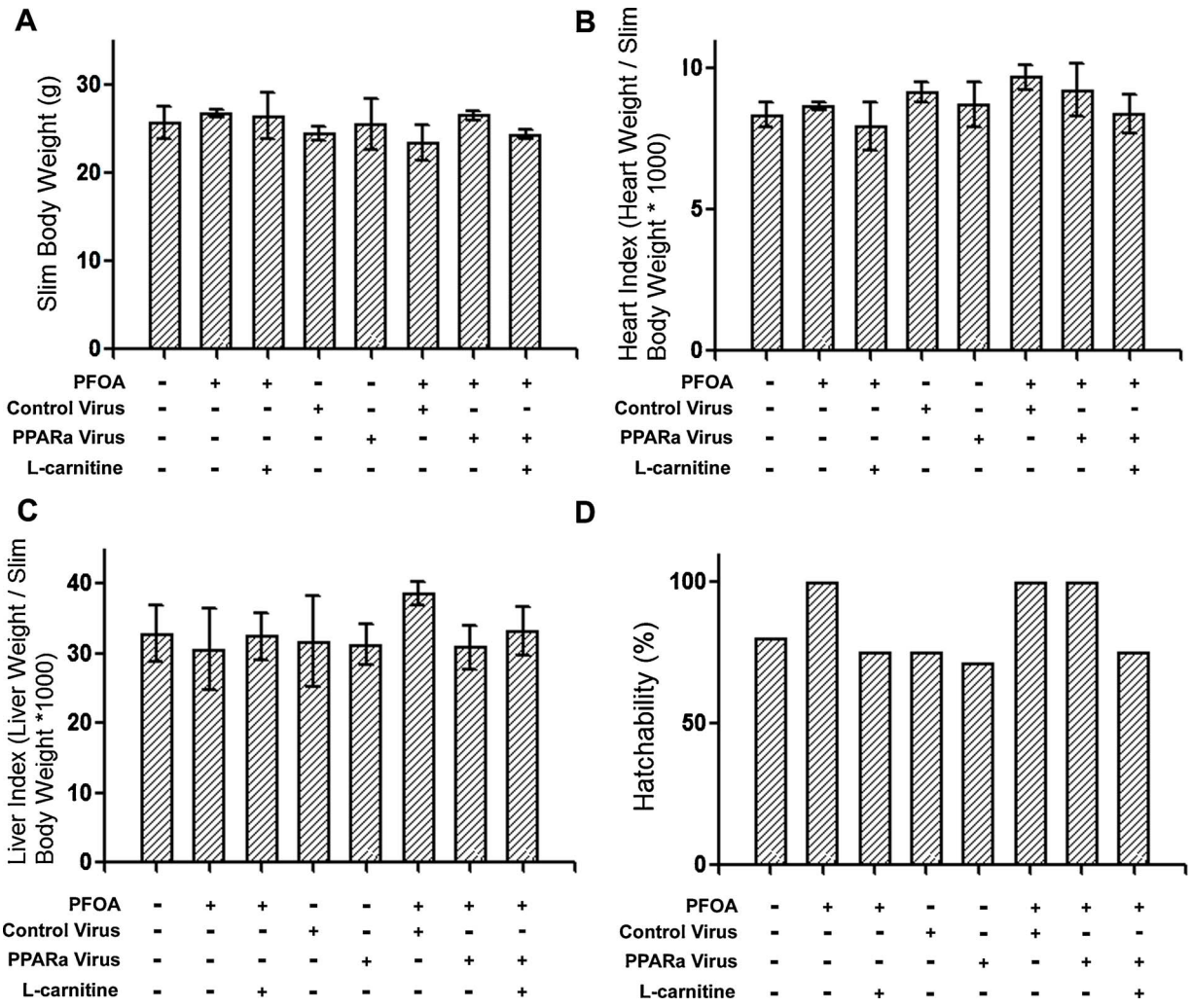


**Fig. 3.** General toxicity parameters of the D1 hatchling chickens.

Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), 2 mg/kg PFOA (egg weight), or 2 mg/kg PFOA and 100 mg/kg L-carnitine (egg weight) at ED0, then microinjected with control lentivirus or PPAR alpha silencing lentivirus at ED2, and then incubated to hatch. Incubation condition was 37.9–37.1° celesus, 50%–70% humidity. Slim body weight (whole body weight − yolk weight), heart index (heart weight/slim body weight * 1000), liver index (liver weight/slim body weight * 1000) and hatchability for the hatchling chickens (number of chickens hatched/number of embryos reached ED19) were recorded. Data were presented as mean + standard derivation. N = 3–5 per group.

A. Slim body weight of D1 hatchling chickens.

B. Heart index of D1 hatchling chickens.

C. Liver index of D1 hatchling chickens.

D. Hatchability.

manipulation methods, all previous data regarding to the role of PPAR alpha in PFOA-induced developmental cardiotoxicity in chicken embryo was based on PPAR alpha agonist/antagonist, which only provided limited information (Jiang et al., 2013, 2016). In the current study, a method for *in ovo* silencing of PPAR alpha in developing chicken embryo heart was established. With such a model, more details have been revealed regarding the role of PPAR alpha in this specific endpoint.

### *4.1. In ovo lentivirus mediated PPAR alpha knockdown in developing chicken embryo heart*

Lentivirus belongs to the family of retrovirus which operates by integrating DNA segments into host genomes, making it a suitable tool for introducing exogenous genes into target organisms (Naldini et al., 1996). While the most common utilization is *in vitro* transfection, *in vivo* application is also available (Sugiyama et al., 2005). Regarding to the *in ovo* application, a couple of reports exist (Zhang et al., 2012; Semple-Rowland and Berry, 2014), but no readily available methods were found for the specific application in the current study. The method was designed based on existing reports and preliminary studies in the lab.We found that the lentivirus applied in this study could effectively transfect the developing chicken embryo heart, as indicated by fluorescent microscopy results. The transfection occurred quite ubiquitously in the heart, making the model suitable to study roles of specific genes in heart development. Due to limitations to the instrument and availability of samples, tissues from other organs were not evaluated for transfection efficacy, but may be performed in future studies. Interestingly, transfection at ED2 resulted in considerably higher transfection efficacy than transfection at ED0. A possible explanation is that the lentivirus could be carried throughout embryo through blood, and heart receives the highest volume of perfusion throughout the embryo. Since no blood circulation is present at ED0, no massive amount of lentivirus was carried to the developing heart tissue *via* blood, and the lentivirus will not stay active for more than a few hours under the warm and humid condition in the incubator, according to the manufacturer.

The method is stable, practical and cost-effective. Lentivirus transfection resulted in persistent expression of siRNA specifically targeting

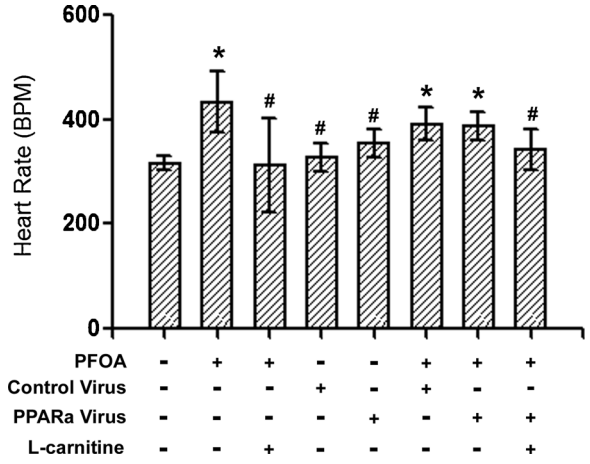


**Fig. 4.** Functional changes in hatchling chickens.
Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), 2 mg/kg PFOA (egg weight), or 2 mg/kg PFOA and 100 mg/kg L-carnitine (egg weight) at ED0, then microinjected with control lentivirus or PPAR alpha silencing lentivirus at ED2, and then incubated to hatch. D1 Hatchling chickens were anaesthetized with 33 mg/kg pentobarbital, electrocardiography was recorded with BL-420E+ (Taimeng, Chengdu, China) and heart rates were calculated by 60/RR interval. N = 3–5 per group.
*: Statistically different from vehicle control group ($P < 0.05$).
#: Statistically different from PFOA 2 mg/kg group ($P < 0.05$).

PPAR alpha, resulting in long-lasting downregulation of PPAR alpha with minimal other effects. In contrast, while antagonists could be equally or even more potent suppressing the target activity, specificity and stability often has space of improvement (El-Jamal et al., 2013). Better stability and specificity of lentivirus revealed more definitive information regarding to the role of PPAR alpha in the current study. However it does have drawbacks that need to be improved in future: most importantly, transfection at ED2 did not cover the first two days of embryo development, which could be very important. A critical exposure window experiment for PFOA has been planned in near future to confirm the importance of the first two day exposure. Additionally, this method only decreased PPAR alpha expression instead of completely knock it out, so part of it (approximately 30% according to results of western blotting) still exists in the embryo tissue. Complete knock out of PPAR alpha *via* CRISPR-Cas9 method will be performed in the future.

### 4.2. Effects of PPAR alpha silencing

With the *in ovo* PPAR alpha silencing model, the role of PPAR alpha in PFOA-induced developmental cardiotoxicity was investigated. In previous studies, the two major observed effects following developmental exposure to PFOA was the functional changes (higher heart rate and other altered functional parameters) and morphological changes (thinned right ventricular wall) (Jiang et al., 2012, 2016). Interestingly, we observed distinct differences between these two endpoints in PPAR alpha knockdown animals.

For the functional changes (heart rate), PPAR alpha silencing did not exhibit significant protection against PFOA induced elevation of heart rate in D1 hatchling chickens, suggesting that PPAR alpha is not involved in this effects. In one of our previous study, PPAR alpha agonist WY 14,643 induced heart rate elevation (Jiang et al., 2013). However, the extent of PPAR alpha activation by WY 14,643 was much higher for WY 14,643 than PFOA in that study, while achieving similar heart rate changes, suggesting that the significant heart rate change induced by PFOA is likely independent of PPAR alpha, which is consistent with the current study. Furthermore, in another study, PPAR alpha antagonist GW6471 failed to protect against PFOA induced heart rate elevation in hatchling chickens (Jiang et al., 2016). Results of the current study helped to exclude the possibility that GW6471 was depleted during the incubation, and further confirmed that the functional changes induced by PFOA is PPAR alpha independent. Additionally, PPAR alpha silencing alone did not induce any remarkable changes to heart rate in the current study, which is consistent with Guellich et al. (2013), in which PPAR alpha knockout did not significantly impact the heart rate. Notably, other functional parameters seem to be altered in Guellich et al. (2013). When possible, echocardiography will be performed on the PPAR alpha silenced hatchling chickens.

On the other hand, PPAR alpha silencing effectively alleviated the morphological changes in PFOA-exposed hatchling chicken hearts, reverting the average thickness of right ventricular wall to a level comparable to vehicle control, which is consistent with previous results as well, in which PPAR alpha antagonist GW6471 induced similar effects (Jiang et al., 2016). It is highly likely that PPAR alpha is involved in PFOA-induced morphological changes in the hatchling chicken heart. The comparison between the current study and Jiang et al. (2016) revealed more information: the right ventricular wall thickness is approximately 84% of vehicle control in PPAR alpha silenced animals, while the treatment of GW6471 resulted in approximately 99% of control. Since these are not completely parallel studies, it is difficult to compare statistically. Nevertheless, this difference suggests that the morphological effects of PFOA are likely mediated through the activation of PPAR alpha, rather than interactions of PPAR alpha protein with other physiological components. The partial alleviation following PPAR alpha silencing is likely the results of incomplete silencing. Additionally, this effect seems to be specific to PFOA-induced effects, since PPAR alpha silencing solo treatment did not result in any significant changes in the right ventricular wall morphology.

Different outcomes regarding to PPAR alpha suggests that the morphological and functional changes induced by PFOA in developing chicken embryo hearts could actually be mechanistically independent to each other: the functional change is independent to PPAR alpha, while the morphological change involves PPAR alpha signaling.

### 4.3. PPAR alpha and L-carnitine

In the previous study, L-carnitine was found to exert protective effects in PFOA-induced developmental cardiotoxicity in chicken embryo (Jiang et al., 2016). L-carnitine is the shuttle molecule that facilitates the entry of long-chain fatty acids into mitochondria for beta-oxidation (Kerner and Hoppel, 2000). L-carnitine and its short-chain derivatives such as acetyl-L-carnitine and propionyl-L-carnitine are known to modulate muscle metabolism and exert cardioprotective effects (Stephens et al., 2007; Andreozzi et al., 2008). On the other hand, PPAR alpha activation was known to regulate genes involved in fatty acid oxidation (Ajith and Jayakumar, 2016),

including important genes of carnitine shuttle/metabolism (Navidshad and Royan, 2016). It is theoretically possible that L-carnitine and PPAR alpha crosstalk to each other in the cardioprotective effects. In the current study, L-carnitine effectively reverted the heart rate changes as well as the morphological changes induced by PFOA in hatchling chicken hearts, further confirming our previous studies (Jiang et al., 2016), however, no statistical significant heart rate changes were observed between the PFOA + L-carnitine group and the PFOA + L-carnitine + PPAR alpha silencing group, indicating that PPAR alpha is not required for L-carnitine to exert protective effects for the heart rate.

Regarding to the morphological changes, PPAR alpha silencing together with L-carnitine treatment resulted in effective protection against the right ventricular wall thinning effect induced by PFOA. While it does not statistically differ from PFOA + L-carnitine treatment group or PFOA + PPAR alpha silencing group, the mean value seems to be higher comparing to PFOA + PPAR alpha silencing group. Observed effects could be the results of simple additive effects of PPAR alpha silencing and L-carnitine protection, or unknown interactions between PPAR alpha and L-carnitine. Decreased endogenous L-carnitine levels

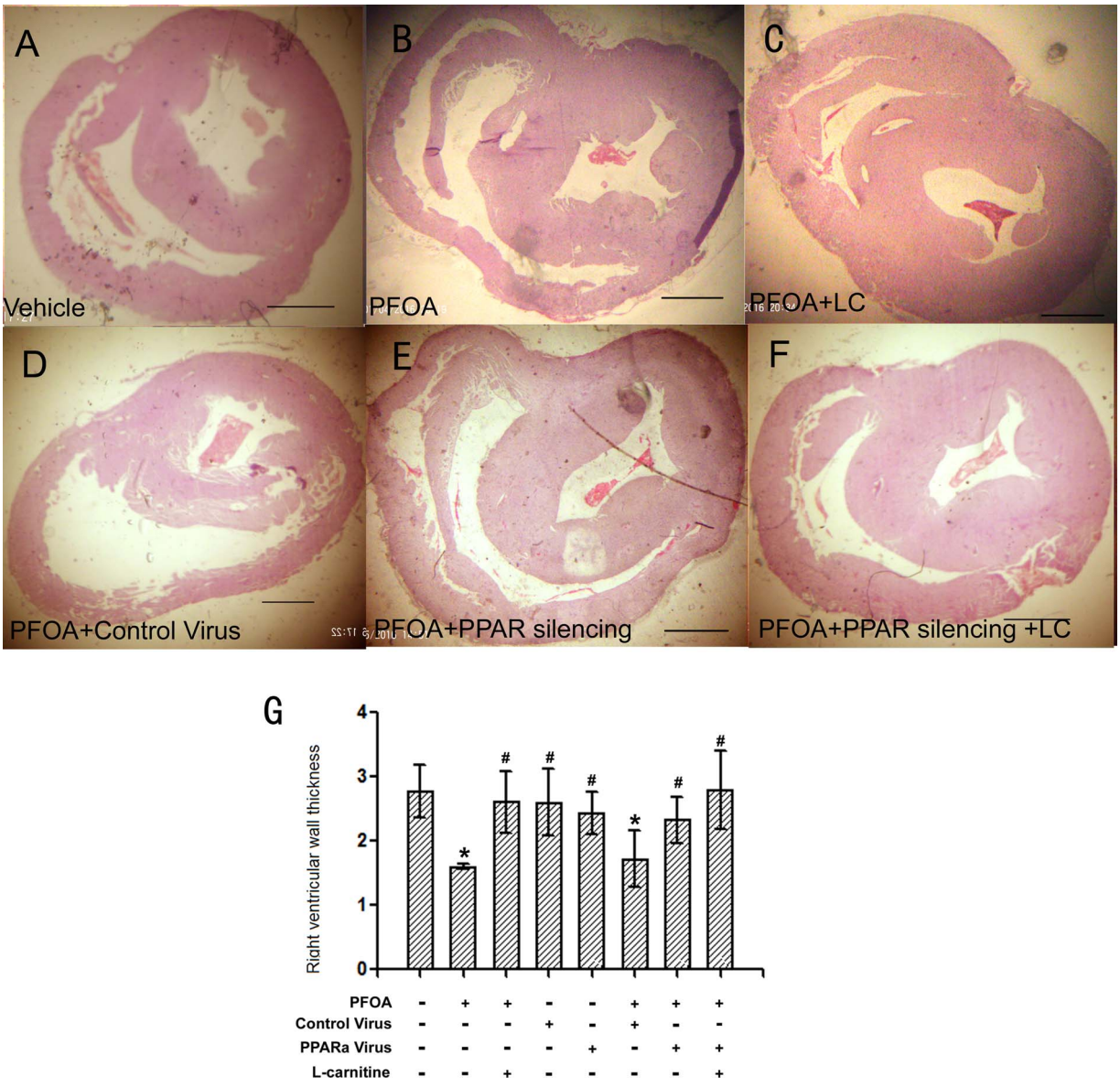


**Fig. 5.** Morphological changes in hatchling chickens treated with PFOA and/or L-carnitine.
Fertile chicken eggs were cleaned, candled and air cell injected with vehicle control (sunflower oil), 2 mg/kg PFOA (egg weight), or 2 mg/kg PFOA and 100 mg/kg L-carnitine (egg weight) at ED0, then microinjected with control lentivirus or PPAR alpha silencing lentivirus at ED2, and then incubated to hatch. Hatchling chickens were anaesthetized with 33 mg/kg pentobarbital (intraperitoneal injection) and sacrificed, hearts were collected and processed for histological assessment. N = 3–5 per group.
*: Statistically different from vehicle control group ($P < 0.05$).
#: Statistically different from PFOA 2 mg/kg group ($P < 0.05$).
LC: L-carnitine
PPAR silencing: PPAR alpha silencing lentivirus
A. Representative picture of hatchling chicken heart histology pictures from vehicle control group. Scale bars represent 1000 um.
B. Representative picture of hatchling chicken heart histology pictures from PFOA 2 mg/kg group. Scale bars represent 1000 um.
C. Representative picture of hatchling chicken heart histology pictures from PFOA 2 mg/kg + L-carnitine 100 mg/kg group. Scale bars represent 1000 um.
D. Representative picture of hatchling chicken heart histology pictures from PFOA 2 mg/kg + control lentivirus group. Scale bars represent 1000 um.
E. Representative picture of hatchling chicken heart histology pictures from PFOA 2 mg/kg + PPAR alpha silencing lentivirus group. Scale bars represent 1000 um.
F. Representative picture of hatchling chicken heart histology pictures from PFOA 2 mg/kg + L-carnitine 100 mg/kg + PPAR alpha silencing lentivirus group. Scale bars represent 1000 um.
G. Quantification of the hatchling chicken right ventricular wall thickness normalized to heart weight.

were observed following PFOA developmental exposure in the previous study (Jiang et al., 2016), which is consistent with other reports such as Peng et al. (2013), in which PFOA decreased L-carnitine levels in liver cells. Our speculation is that PPAR alpha might participate in that endpoint, affecting endogenous L-carnitine levels, which in turn affected heart development. In that case, L-carnitine supplement could completely overcome such effects, while PPAR alpha silencing could alleviate the effects, but the protective effects were somewhat less prominent. Further study on the endogenous L-carnitine levels in chicken embryo hearts with PPAR alpha silenced has been planned.

### 4.4. Other potential mechanisms

Our data suggest that L-carnitine seems to be protective against PFOA-induced developmental cardiotoxicity, while PPAR alpha silencing had limited effects. However, it is worth noting that other

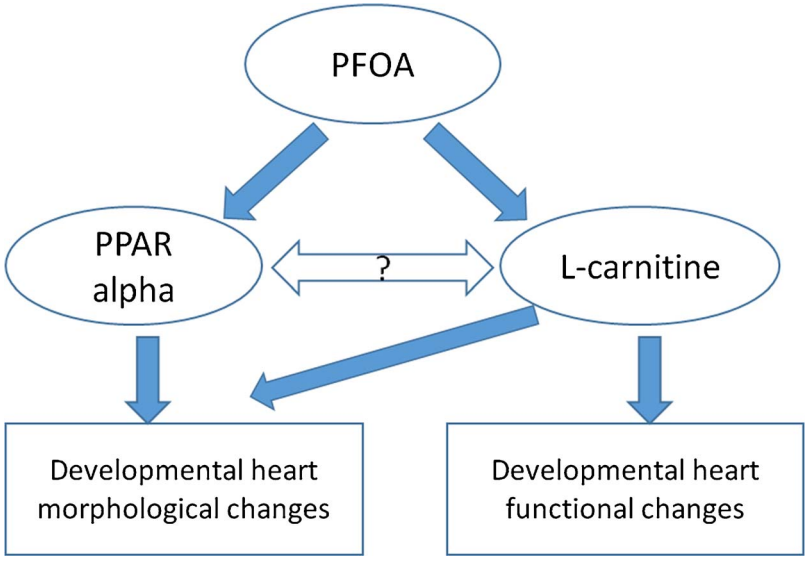


**Fig. 6.** Mechanism of toxicity in PFOA-induced developmental cardiotoxicity.
The known information regarding to the mechanism of PFOA-induced developmental cardiotoxicity is summarized in a chart. Solid arrows represent known information; hollow arrow represents unknown interactions.

mechanisms may exist as well, which will be further pursued. First of all, the knockdown technique resulted in decreased PPAR alpha expression, but not complete absence. PPAR alpha may still play a role even in those knock-down animals. To our best knowledge, the complete PPAR alpha knock out model in chicken is not available yet, which will be a short-term goal of our team. Secondly, in the current study, the *in ovo* and *in vivo* work were inevitably affected by variability, along with a relatively small margin of difference (percentage change approximately 20%–40%). While changes to vital parameters such as heart rate usually were in the observed range (Bakkehaug et al., 2016), it is possible that other unknown factors may contribute, such as oxidative stress (Chen et al., 2017) or other signaling pathways such as NF-KB (Zhang et al., 2014; Miao et al., 2015). We will continue to reveal more information regarding to PFOA-induced developmental cardiotoxicity.

## 5. Conclusion

In the current study, an *in ovo* PPAR alpha silencing chicken embryo model has been established. PPAR alpha silencing could not counteract the heart rate elevation in hatchling chickens following developmental exposure to PFOA, but could alleviate the thinning of right ventricular wall. On the other hand, L-carnitine effectively reverted both functional and morphological changes induced by PFOA. Available information so far had been summarized in Fig. 6. In conclusion, PPAR alpha is partially involved in PFOA-induced developmental cardiotoxicity in chicken embryo, while L-carnitine is likely a key molecule in the mechanism of toxicity.

## Conflict of interests

There are none.

## Acknowledgement

This work was supported by the National Natural Science Foundation of China (Grant No. 81502835).

## 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.etap.2017.09.006.

## References

Abbott, B.D., Wolf, C.J., Schmid, J.E., Das, K.P., Zehr, R.D., Helfant, L., Nakayama, S., Lindstrom, A.B., Strynar, M.J., Lau, C., 2007. Perfluorooctanoic acid induced developmental toxicity in the mouse is dependent on expression of peroxisome proliferator activated receptor-alpha. Toxicol. Sci. 98, 571–581.
Ajith, T.A., Jayakumar, T.G., 2016. Peroxisome proliferator-activated receptors in cardiac energy metabolism and cardiovascular disease. Clin. Exp. Pharmacol. Physiol. 43, 649–658.
Andreozzi, G.M., Leone, A., Laudani, R., Martin, R., Deinit, G., Cataldi, V., 2008. Levo-propionyl-carnitine improves the effectiveness of supervised physical training on the absolute claudication distance in patients with intermittent claudication. Angiology 59, 84–89.
Bakkehaug, J.P., Naesheim, T., Torgersen Engstad, E., Kildal, A.B., Myrmel, T., How, O.J., 2016. Reversing dobutamine-induced tachycardia using ivabradine increases stroke volume with neutral effect on cardiac energetics in left ventricular post-ischaemia dysfunction. Acta Physiol. 218, 78–88.
Begley, T.H., White, K., Honigfort, P., Twaroski, M.L., Neches, R., Walker, R.A., 2005. Perfluorochemicals: potential sources of and migration from food packaging. Food Addit. Contam. 22, 1023–1031.
Biegel, L.B., Hurtt, M.E., Frame, S.R., O'Connor, J.C., Cook, J.C., 2001. Mechanisms of extrahepatic tumor induction by peroxisome proliferators in male CD rats. Toxicol. Sci. 60, 44–55.
Bjerregaard-Olesen, C., Bach, C.C., Long, M., Ghisari, M., Bossi, R., Bech, B.H., Nohr, E.A., Henriksen, T.B., Olsen, J., Bonefeld-Jorgensen, E.C., 2016. Time trends of perfluorinated alkyl acids in serum from Danish pregnant women 2008–2013. Environ. Int. 91, 14–21.
Buhrke, T., Kruger, E., Pevny, S., Rossler, M., Bitter, K., Lampen, A., 2015. Perfluorooctanoic acid (PFOA) affects distinct molecular signalling pathways in human primary hepatocytes. Toxicology 333, 53–62.
Canver, M.C., Bauer, D.E., Dass, A., Yien, Y.Y., Chung, J., Masuda, T., Maeda, T., Paw, B.H., Orkin, S.H., 2017. Characterization of genomic deletion efficiency mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 nuclease system in mammalian cells. J. Biol. Chem. 292, 2556.
Chen, Y., Zhou, L., Xu, J., Zhang, L., Li, M., Xie, X., Xie, Y., Luo, D., Zhang, D., Yu, X., Yang, B., Kuang, H., 2017. Maternal exposure to perfluorooctanoic acid inhibits luteal function via oxidative stress and apoptosis in pregnant mice. Reprod. Toxicol. 69, 159–166.
Dewitt, J.C., Copeland, C.B., Strynar, M.J., Luebke, R.W., 2008. Perfluorooctanoic acid-induced immunomodulation in adult C57BL/6: J or C57BL/6N female mice. Environ. Health Perspect. 116, 644–650.
El-Jamal, N., Dubuquoy, L., Auwerx, J., Bertin, B., Desreumaux, P., 2013. In vivo imaging reveals selective PPAR activity in the skin of peroxisome proliferator-activated receptor responsive element-luciferase reporter mice. Exp. Dermatol. 22, 137–140.
Filgo, A.J., Quist, E.M., Hoenerhoff, M.J., Brix, A.E., Kissling, G.E., Fenton, S.E., 2015. Perfluorooctanoic acid (PFOA)-induced liver lesions in two strains of mice following developmental exposures: pPARalpha is not required. Toxicol. Pathol. 43, 558–568.
Fromel, T., Knepper, T.P., 2010. Biodegradation of fluorinated alkyl substances. Rev. Environ. Contam. Toxicol. 208, 161–177.
Guellich, A., Damy, T., Conti, M., Claes, V., Samuel, J.L., Pineau, T., Lecarpentier, Y., Coirault, C., 2013. Tempol prevents cardiac oxidative damage and left ventricular dysfunction in the PPAR-alpha KO mouse. Am. J. Physiol. Heart Circ. Physiol. 304, H1505–H1512.
Jiang, Q., Lust, R.M., Strynar, M.J., Dagnino, S., DeWitt, J.C., 2012. Perfluorooctanoic acid induces developmental cardiotoxicity in chicken embryos and hatchlings. Toxicology 293, 97–106.
Jiang, Q., Lust, R.M., DeWitt, J.C., 2013. Perfluorooctanoic acid induced-developmental

cardiotoxicity: are peroxisome proliferator activated receptor alpha (PPARalpha) and bone morphorgenic protein 2 (BMP2) pathways involved? J. Toxicol. Environ. Health A 76, 635–650.
Jiang, Q., Wang, C., Xue, C., Xue, L., Wang, M., Li, C., Deng, Z., Wang, Q., 2016. Changes in the levels of l-carnitine, acetyl-l-carnitine and propionyl-l-carnitine are involved in perfluorooctanoic acid induced developmental cardiotoxicity in chicken embryo. Environ. Toxicol. Pharmacol. 48, 116–124.
Kerner, J., Hoppel, C., 2000. Fatty acid import into mitochondria. Biochim. Biophys. Acta 1486, 1–17.
Kudryavtseva, A.V., Fedorova, M.S., Zhavoronkov, A., Moskalev, A.A., Zasedatelev, A.S., Dmitriev, A.A., Sadritdinova, A.F., Karpova, I.Y., Nyushko, K.M., Kalinin, D.V., Volchenko, N.N., Melnikova, N.V., Klimina, K.M., Sidorov, D.V., Popov, A.Y., Nasedkina, T.V., Kaprin, A.D., Alekseev, B.Y., Krasnov, G.S., Snezhkina, A.V., 2016. Effect of lentivirus-mediated shRNA inactivation of HK1, HK2, and HK3 genes in colorectal cancer and melanoma cells. BMC Genet. 17, 156.
Liu, Z., Lu, Y., Wang, P., Wang, T., Liu, S., Johnson, A.C., Sweetman, A.J., Baninla, Y., 2016. Pollution pathways and release estimation of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in central and eastern China. Sci. Total Environ. 580, 1247–1256.
Miao, C., Ma, J., Zhang, Y., Chu, Y., Li, J., Kuai, R., Wang, S., Peng, H., 2015. Perfluorooctanoic acid enhances colorectal cancer DLD-1 cells invasiveness through activating NF-kappaB mediated matrix metalloproteinase-2/-9 expression. Int. J. Clin. Exp. Pathol. 8, 10512–10522.
Naldini, L., Blomer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., Trono, D., 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272, 263–267.
Navidshad, B., Royan, M., 2016. Peroxisome proliferator-activated receptor alpha (PPARalpha), a key regulator of lipid metabolism in avians. Crit. Rev. Eukaryot. Gene Expr. 26, 303–308.
Peng, S., Yan, L., Zhang, J., Wang, Z., Tian, M., Shen, H., 2013. An integrated metabonomics and transcriptomics approach to understanding metabolic pathway disturbance induced by perfluorooctanoic acid. J. Pharm. Biomed. Anal. 86, 56–64.
Reuter, S.E., Evans, A.M., 2012. Carnitine and acylcarnitines: pharmacokinetic, pharmacological and clinical aspects. Clin. Pharmacokinet. 51, 553–572.
Semple-Rowland, S.L., Berry, J., 2014. Use of lentiviral vectors to deliver and express bicistronic transgenes in developing chicken embryos. Methods 66, 466–473.
Steenland, K., Fletcher, T., Savitz, D.A., 2010. Epidemiologic evidence on the health effects of perfluorooctanoic acid (PFOA). Environ. Health Perspect. 118, 1100–1108.
Stephens, F.B., Constantin-Teodosiu, D., Greenhaff, P.L., 2007. New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle. J. Physiol. 581, 431–444.
Sugiyama, O., Sung An, D., Kung, S.P., Feeley, B.T., Gamradt, S., Liu, N.Q., Chen, I.S., Lieberman, J.R., 2005. Lentivirus-mediated gene transfer induces long-term transgene expression of BMP-2 in vitro and new bone formation in vivo. Mol. Ther. 11, 390–398.
USFDA, 2015. USFDA Website. http://www.accessdata.fda.gov/drugsatfda_docs/label/2015/018948s026,019257s012,020182s013lbl.pdf (Accessed 20 May 2017).
Vested, A., Ramlau-Hansen, C.H., Olsen, S.F., Bonde, J.P., Kristensen, S.L., Halldorsson, T.I., Becher, G., Haug, L.S., Ernst, E.H., Toft, G., 2013. Associations of in utero exposure to perfluorinated alkyl acids with human semen quality and reproductive hormones in adult men. Environ. Health Perspect. 121, 453–458.
Wang, X., Jiang, Q., Wang, W., Su, L., Han, Y., Wang, C., 2014. Molecular mechanism of polypeptides from Chlamys farreri (PCF)'s anti-apoptotic effect in UVA-exposed HaCaT cells involves HSF1/HSP70, JNK, XO, iNOS and NO/ROS. J. Photochem. Photobiol. B 130, 47–56.
Wolf, C.J., Fenton, S.E., Schmid, J.E., Calafat, A.M., Kuklenyik, Z., Bryant, X.A., Thibodeaux, J., Das, K.P., White, S.S., Lau, C.S., Abbott, B.D., 2007. Developmental toxicity of perfluorooctanoic acid in the CD-1 mouse after cross-foster and restricted gestational exposures. Toxicol. Sci. 95, 462–473.
Wolf, C.J., Takacs, M.L., Schmid, J.E., Lau, C., Abbott, B.D., 2008. Activation of mouse and human peroxisome proliferator-activated receptor alpha by perfluoroalkyl acids of different functional groups and chain lengths. Toxicol. Sci. 106, 162–171.
Zhang, Z., Sun, P., Yu, F., Yan, L., Yuan, F., Zhang, W., Wang, T., Wan, Z., Shao, Q., Li, Z., 2012. Transgenic quail production by microinjection of lentiviral vector into the early embryo blood vessels. PLoS One 7, e50817.
Zhang, W., Wang, F., Xu, P., Miao, C., Zeng, X., Cui, X., Lu, C., Xie, H., Yin, H., Chen, F., Ma, J., Gao, S., Fu, Z., 2014. Perfluorooctanoic acid stimulates breast cancer cells invasion and up-regulates matrix metalloproteinase-2/-9 expression mediated by activating NF-kappaB. Toxicol. Lett. 229, 118–125.