EPIGENETIC TOXICITY OF PFOA IN HEPG2 CELLS AND ITS ROLE IN LIPID METABOLISM

BY

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THESIS

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ABSTRACT

Perfluorooctanoic acid (PFOA), a man-made stable perfluoroalkyl acid is used widely in the process of synthesizing fluoropolymers for the manufacturing of various daily products such as Teflon. PFOA is extremely persistent in the environment and can be detected in many organisms including humans. Different studies have shown the potential of PFOA causing diseases in animals. Previous study has suggested PFOA-induced hepatocellular damage over time with unusual fat deposit and liver enlargement. To closely study the underlying mechanisms of PFOA induced hepatocellular damage. Liver hepatocellular carcinoma cell line HepG2 was used as a model to study the PFOA induced inflammation *in vitro* on the cellular, epigenetic and metabolic levels. HepG2 cells were treated with 20 μM, 100 μM, 200 μM, 400 μM PFOA dissolved in DMSO for 48 hours before harvested for DNA, RNA and assays. The mRNA expression levels of cell cycle and proliferation genes are affected significantly, as well as the mRNA expression levels of *TET*s. Due to the unusual fat deposits in PFOA treated animals observed in prior studies, essential lipid metabolism gene were also studied and of which the mRNA expression levels are also changed, especially those regarding beta-oxidation pathway and lipid synthesis. Global methylation level of HepG2 cells was found to be in overall inverse proportion with different dosages of PFOA and had corresponding trends with mRNA expression of most genes of interest. Lipid quantification has also showed PFOA dose-dependent increase in the lipid accumulation in HepG2 cells. These evidences showed PFOA induced epigenetic changes play a major role in lipid metabolism genes activation and modulation in lipid metabolism, potentially causes abnormal fat deposit, liver inflammation and carcinogenesis.

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CHAPTER 1
INTRODUCTION

Perfluorooactanoic acid (PFOA) has been widely used as surfactant in industry in the process of synthesizing fluoropolymers to manufacture consumer product such as Gore-Tex and Teflon (Steenland et al., 2010). PFOA has a structure of eight-carbon chain therefore often also referred to as C8. As a member of perfllluoroalkyl substances (PFASs), PFOA is a very stable compound due to its strong carbon-fluorine bonds (Prevedorous et al., 2006). PFOA has also been found to be one of the most found perfluorinated compounds (PFCs) in the environment (Goosey & Harrad, 2011) and a very persistent one (Buck et al., 2011). PFOA are especially harmful to aquatic lives, imply toxicity to ecosystems (Rodea-Palomares et al., 2015; Giesy et al., 2010). The half-life of PFOA in serum is up to 8.5 years of human individuals exposed to PFOA contaminated water (Seals et al., 2010). With the average intrinsic elimination half-life of 2.4 years in humans (Russell, 2015), PFOA exhibits high bioaccumulation potential. PFOA has received a lot of concern since it has been detected in breast milk and cord serum (Apelberg et al., 2007; Llorca et al., 2010).

The accumulation of PFOA in animal bodies has led to multiple toxicities, such as genotoxicity, immunotoxicity, neurotoxicity and hepatotoxicity (DeWitt et al., 2008). Among those, hepatotoxicity was widely observed in different vertebrate animals, in the ways of unusual fat deposits, liver enlargement and hepatocarcinogenesis (Wu et al., 2018; Qazi et al., 2010; Benninghoff et al., 2011). Study also found positive association between PFOA exposure concentrations and serum alanine transaminase (ALT) level, which is a marker for hepatocellular damage (Gallo et al., 2012). Genotoxicity was also observed in human hepatoma cell line HepG2 exposed to PFOA (Yao & Zhong, 2005). The prior literature on the effects of PFOA exposure has suggested that vertebrates primarily suffer inflammation in liver, pancreas (Kennedy et al., 2004). Studies have also showed the potential causal relationship between PFOA and liver, pancreatic and other cancers (Biegel et al., 2001; Barry et al., 2013).

Due to its structural similarity to fatty acids, various studies have looked at correlation between PFOA exposure and different metabolism pathways, especially the peroxisome proliferator-activated receptors (PPARs). In a mouse study, even though study showed the requirement of PPARα in PFOA induced developmental toxicity, data showed large variability in

PPARs expression profile highly depending on the animal age (Abbott et al., 2012). Previous study also showed PFOA induced metabolic disruption in mouse neonates (Abbott et al., 2012). However, the underlying molecular mechanisms of PFOA-induced toxicity still remains unclear.

In non-human primates and rodents, PFOA has been found in relatively high concentration in liver tissue and have been related to liver enlargement. Study have shown association between hepatocellular adenoma and PFOA exposure in rats (Lau et al., 2007). Even though PFOA toxicity has been studies in many human studies, there is not thorough data on the toxicity of PFOA on human livers due to human study limitations (Lau et al., 2007). In order to better understand the mechanism associated with PFOA induced liver toxicity, our study explored the relationship between PFOA treatment and the expression of several rate-limiting lipid metabolism-related and β-oxidation pathway enzyme encoding genes and on the epigenetics level. Due to the benefits of controllable environment and relatively easy maintenance, we chose to explore the PFOA epigenetic toxicity on a human cell line *in vitro*. Originated from liver, hepatocellular carcinoma cell line HepG2 was chosen as our human liver *in vitro* model to carry out our experiments.

CHAPTER 2
MATERIALS AND METHODS

CHEMICALS AND TEST REAGENTS

The ammonium salt form of PFOA, Pentadecafluorooctanoic acid ammonium salt (CAS No. 3825-26-1) of ⩾98.0% purity purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) was used in the experiments.

For the *in vitro* exposure of human hepatocellular carcinoma HepG2 cells, Pentadecafluorooctanoic acid ammonium salt was dissolved in dimethyl sulfoxide (DMSO; CAS No. D8418) from Sigma-Aldrich to obtain a stock solution of 0.1 M. Largest equivalent volume of DMSO was spiked into the control experiment group. Different volumes of stock solution were added into the cell culture media such that the final concentrations of PFOA in media were 20 μM, 100 μM, 200 μM, 400μM, and the concentration of DMSO in all treatments and controls were lower than 0.4% v/v. It was empirically determined that 0.5% DMSO had negligible effect on cell response to MTT assay.

CELL CULTURE

HepG2 cell line, Hep G2 [HEPG2] (ATCC® HB-8065™) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HepG2 cells were cultured in the culture medium of 89% v/v Dulbecco's modified Eagle's medium with L-Glutamine and high glucose (ATCC 30-2002; Manassas, VA, USA), 10% v/v Fetal bovine serum (Gibco™ 10082147; Thermo Fisher Scientific; Waltham, MA, USA), and 1% v/v Penicillin Streptomycin Solution, 100X (REF 30-002-Cl; CORNING; Corning, NY, USA) They are maintained in a humidified cell culture incubator at 37 °C as recommended.

Three groups of experiment were performed for each assay. All groups of experiment were performed at different time to achieve independence between groups. All experiments utilized cell cultures within a window of 6 passages.

MTT ASSAY

The [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] (MTT) assay was used to determine the cell viability. The MTT reagent purchased from Invitrogen (Cat. No. M6494) was prepared by dissolving 4 mg MTT per 1 mL sterile phosphate-buffered saline (Gibco™ 10010023; Thermo Fisher Scientific;), then sterilized by filtering through a MILLEX GP 0.22 μm filter (Catalog No. SLGP033RS; MilliporeSigma; Burlington, MA, USA) and stored in darkness at 4 °C. MTT solvent was prepared by adding 0.33% v/v IGEPAL CA-630 in isopropanol, following by the pH adjusted to between 3 and 4 with 65 mM HCl aqueous solution.

For treatment experiment to investigate the effect of different concentrations of PFOA on HepG2 cell growth and proliferation, HepG2 cells were dissociated with trypsin (Gibco™ 25300-062; Thermo Fisher Scientific; Waltham, MA, USA) and seeded into a 24-well plate at a density pf 20 000 cells per well. The number of cells provided high stability in results as optimal resolution in the linear range of MTT assay. Cells were cultured in appropriate DMEM based media for about 24 hours to achieve 30%-40% confluence before adding the corresponding concentration of PFOA dissolved in DMSO. Cultures were then allowed to grow for 48 hours. At this time the control cell culture was grown to 80%-90% confluence. Afterward the medium was substituted with phenol red free DMEM medium (Gibco™ 31053028; Thermo Fisher Scientific; Waltham, MA, USA) with 10% v/v Fetal bovine serum, and 1% v/v Penicillin Streptomycin Solution, 100X added.

MTT reagent was then added at 1: 10 to the existing medium, and incubated for another 4 hours at 37 °C. The MTT solvent solution was added to stop the reaction at 1 to 1 ratio to the medium and incubated for 2 hours at room temperature. Absorbance was recorded at 570 nm wavelength with microplate reader (Synergy HT, BioTek; Winooski, VT, USA).

Cell viability is expressed as the percentage of absorbance values at different doses to their vehicle control. It was calculated with the formula $[OD_{PFOA} - OD_{blank}] / [OD_{0.4\%DMSO} - OD_{blank}] \times 100\%$

LIPID STAINING AND QUANTIFICATION

For the Oil Red O staining to determine the fat deposit of PFOA treated cells, the HepG2 cell culture process is the same as MTT assay, except cells were cultured on poly-L-lysine coated cover glasses (GG-12-PLL, Neuvitro Corporation; Vancouver, WA, USA). After the 48 hours treatment, all cells were washed with PBS and fixed with 4% paraformaldehyde for 1 hour. Then the cells were stained with freshly prepared 0.5% Oil Red O solution in isopropanol for 2 hours. Afterward the cells were washed with molecular biology grade water (REF 46-000-CM, Corning; Manassas, VA, USA).

The slides were prepared and observed with Cytoviva darkfield microscope with 100X oil immersion lens and subsequently photographed with hyperspectral camera. The level of lipids was quantified with ImageJ Software and ENVI 4.8 software.

GENE EXPRESSION ANALYSIS

For Gene expression analysis, we looked at the RNA expression profile on eight cell proliferation genes, six DNA methylation related genes, and seven lipid metabolism related genes. All genes and their encoding proteins are listed in Table 1. HepG2 cells were seeded 10 000 per cm2 in T25 flasks and cultured till 20% - 30% confluence before adding the 20 μM, 100 μM, 200 μM, 400μM of PFOA dissolved in DMSO into experimental groups and DMSO to vehicle control group. Cultures were then allowed into grow for 48 hours before cells were dissociated with tyrpsin for RNA extration.

The total RNA was isolated with TRIzol™ Reagent (Invitrogen™ 15596026; Thermo Fisher Scientific; Waltham, MA, USA). The quantity and quality of RNA extracted was tested with NanoDrop One (ND-ONE-W, Thermo Fisher Scientific; Waltham, MA, USA). Complete cDNA synthesis was achieved by performing reverse transcription with High Capacity cDNA Reverse Transcription Kit (Applied biosystems REF 4368814; Waltham, MA, USA) and 1 μg total RNA. Quantitative real-time polymerase chain reaction (qRT-PCR) mix was comprised of SYBR Green PCR Master Mix reagents (Applied biosystems REF 4367659; Waltham, MA, USA), synthesized cDNA and corresponding primers. All primers are designed using NCBI RefSeq Transcripts and BLAST and are listed in Table 2. qRT-PCR was performed on a Step

One Plus Real-Times PCR System (Applied biosystems, Waltham, MA, USA) with 20 µl per well of total reaction mixture in a 96-well plate. Reactions were run in duplicate with the following conditions. Started with initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturing at 95 °C for 15 s, annealing at 58 °C for 35 s, extension at 72 °C for 60 s. Finally, a melting curve stage consisting of 15 s at 95 °C, 60 s at 60 °C and a temperature ramp from 60 °C to 95 °C in increment of 0.3 °C for 15 s. The melting curve of each sample was examined to ensure the specify of each run with the StepOne software v2.3. After normalizing to the RNA expression of GAPDH. The relative RNA expression of each target gene in terms of ΔCT was calculated. Quantification of the expressed transcripts was performed with $2^{-\Delta\Delta CT}$ method according to Schmittgen & Livak.

GLOBAL METHYLATION ASSAY

Genomic DNA from HepG2 was extracted immediately after PFOA treatment period with PureLink Genomic DNA mini kit (Invitrogen K182002; Thermo Fisher Scientific; Waltham, MA, USA). The quantity and quality of DNA extracted was tested with NanoDrop One spectrophotometer (Thermo Fisher Scientific). The whole-genome methylation profile was tested with EpiGentek MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) according to the manufacturer's manual. It was performed as a 96-well plate based colorimetric immunoassay. In each well, 100 ng of total DNA was attached to the well bottom surface, following by the DNA methylation marks labeling with monoclonal antibodies. Subsequently developed color was normalized with a set of standards with known methylation percentage.

CHAPTER 3
RESULTS

CELL VIABILITY

To determine the cytotoxicity at different dosage of PFOA on HepG2 cells, combined with prior knowledge on PFOA cytotoxicity of cell line (1), we started with concentrations of 10 μM, 20 μM, 40 μM, 100 μM ,200 μM, and 400 μM, 600 μM. However, we found no significant difference between 10 μM, 20 μM, and 40 μM treatments in terms of both cell viability assay and gene expression of TETs and DNMTs. Therefore, we decided to only experiment with 20 μM at lower concentrations. At 600 μM, most cells were dead due to the high dosage. So, it was also not included in the subsequent study. Initially we also studied results from HepG2 cells treat with different concentrations of PFOA for 24 hours, 48 hours and 72 hours. For 24 hours treatment give less significant separation in results corresponding different dosage probably due to insufficient time, and 72 hours treatment also gives less separation in results probably due to cell overgrowth and cell death, we did all subsequent studies with 48 hours incubation of PFOA. MTT assay was performed on PFOA treated HepG2 cells, representing the effect of different dosage of PFOA on HepG2 cell viability. There is no significant difference between negative control without any treatment and vehicle control with the same quantity of DMSO added as it was in the 400 μM PFOA treated media.

From 0 μM, 20 μM to 100 μM, cell viability increased in a dose-dependent fashion. While from 100 μM, 200 μM, to 400 μM, cell viability decreased in a dose-dependent fashion. According to MTT assay, cell viability was at highest after 100 μM PFOA treatment for 48 hours, which was increased by about 40%. While cell viability was at lowest after 400 μM PFOA treatment for 48 hours, which was decreased by about 20% comparing with control (Figure 1).

EFFECT ON CELL APOPTOSIS, PROLIFERATION AND CYCLE GENES

Due to the strong influence PFOA had on cell viability and reported influences PFASs have on cell metabolism and proliferation (Hagenaars et al., 2013; Peng et al., 2013; Yan et al., 2015), we had a closer look at several genes related to apoptosis, cell proliferation and cell cycle via relative RNA expression profiles. We have seen a slight increase in lower concentration of

PFOA treatment and strong dose-dependent decrease in higher concentration PFOA treatment in *BAX* expression in HepG2 cells. Cell cycle genes *CCNA2*, *CCNE1*, *CCNB1* were also tested with their gene expression profile. All those cyclin encoding genes showed similar trends in gene expression. At lower concentration of PFOA, there were small increases. As the dosage increases, the gene expression of those genes decreased significantly and mostly dose-dependently. Further we examined the expression of four genes, *CDKN1A*, *CDK4*, *RPS6*, *KITLG*, related to cell proliferation. Cyclin-dependent kinase inhibitor 1 encoding *CDKN1A* increased significantly with higher concentration of PFOA treatment, including over 6-fold increase at 200 μM and over 8-fold increase at 400 μM. Cyclin-dependent kinase 4 encoding *CDK4* expression profile has shown minor changes at different concentrations but mostly decreased at higher dosages. *RPS6* expression was decreased at 20 μM PFOA and increased at all higher concentrations of PFOA. The expression of Kit Ligand encoding gene *KITLG* has also decreased with all concentrations of PFOA. All detailed relative mRNA expressions of cell apoptosis, cell cycle, and cell proliferation related genes are presented in Figure 2.

EFFECT ON LIPID METABOLISM AND TRANSPORT GENES

The structural similarity between PFOA backbone and fatty acids suggests that they may interfere with the cellular lipid metabolism and transportation (Luebker et al., 2002; Ophuang & Singer, 1980; Wang et al., 2013; Ye et al., 2009). To better understand the effect of PFOA on lipid metabolism, we analyzed the expression of selected genes involved in liver cell lipid metabolism and transport. We also examined intracellular lipid accumulation via lipid staining and quantification.

From a pool of lipid metabolism related genes, we selected those that are largely expressed in liver tissue since HepG2 was our model of the study. Out of those genes, a few of them have substantial alternation in RNA expression corresponding to the change in PFOA treatment. Among the beta-oxidation involved acyl-CoA dehydrogenase enzyme encoding genes, *ACAD11* and *ACADM* have a small increase in expression with the PFOA dosage increment. Along with the increased expression of catabolism related genes, the expression of a lipid synthesis related gene *ACSL1* has increased much more significantly in a dose-dependent manner. At 400 μM PFOA treatment level, the expression of *ACSL1* was increased by about 6-fold. Furthermore,

FABP1 which encodes fatty acid binding protein and responsible for fatty acid uptake, transport and metabolism had decreased expression has decrease in expression along of PFOA increments. *ACOX2* encodes acyl-CoA oxidase 2 involved in the fatty acids degradation in peroxisomes had decreased expression. The expression of HMGCR also decreased in a dose-dependent fashion. Overall, we observed increased expression of lipid metabolism genes in different aspects (Figure 5).

EFFECT ON INTRACULLULAR LIPID STORAGE

Next we had a closer look on the intracellular lipid storage in PFOA treated HepG2 cells. After treatment, cells grown on coverslips were immediately fixed with 10% neutral buffered formalin. Cells were subsequently stained using 0.3% Oil Red O in 60% isopropanol solution. Following we observed the cells with dark field microscope equipped with 100X oil immersion lens and imaged with hyperspectral camera. Hyperspectral data were analyzed to calculate the average intensity at 663nm wavelength. Higher intensity indicates higher density of lipid deposit. From Figure 6. we can see that lipid deposit decreased at 20 μM PFOA treatment and increased at 100 μM, 200 μM and 400 μM PFOA treatments.

EPIGENETIC TOXICITY OF PFOA: EFFECT ON GENOME-WIDE METHYLATION AND ITS MECHANISM

Since we have seen strong dose-dependent changes in some selected genes. For better understanding the effect of PFOA on genome-wide gene expression status, we analyzed the global methylation for HepG2 after different concentration treatment of PFOA. The quantification of genome-wide 5-mC content in treated cells showed significant dose-dependent reduction of methylation at higher concentrations of PFOA from 100 μM to 400 μM. While there was a minor increase in global methylation level in 20 μM PFOA treated HepG2, compared to control (Figure 7).

To study the mechanism of how the methylation level changes and the possible involvement of Tet Methylcytosine Dioxygenases and DNA Methyltransferases, we evaluated

the mRNA relative expression profiles of corresponding genes *TET1*, *TET2*, *TET3*, and *DNMT1*, *DNMT3A*, *DNMT3B* in PFOA treated HepG2 cells. We have observed significant dose-dependent changes in all three *TET*s, which was also proportional to global methylation level changes. *TET1* expression had an overall trend of decreasing with the increasing dosage of PFOA. While *TET2* and *TET3* had overall trend of increasing with the increasing dosage of PFOA (Figure 3). But the behavior is opposite at 20 μM PFOA. However, there was not a definite trend in the expression of all three *DNMT*s (Figure 4).

CHAPTER 4
DISCUSSION

From the MTT assay results in Figure 1. we can see that PFOA increases the cell viability or metabolism rates in a dose-dependent fashion from no PFOA to 100 μM PFOA treatment after two days. While from 100 μM to higher dosage of PFOA, the viability of HepG2 cells decreases. Since we did not observe much cell death at treatment lower than 200 μM and cells start to die more at 400 μM. This indicates lower dose exposure of PFOA increases the proliferation of HepG2 cells and higher dose exposure may cause apoptosis.

Due to the strong influence PFOA had on cell viability and reported influences PFASs have on cell metabolism (Hagenaars et al., 2013; Peng et al., 2013; Yan et al., 2015) and our observation in the MTT assay, we had a closer look at the expression of several genes related to apoptosis, cell metabolism and cell cycle. Apoptosis regulator BAX plays its role in mitochondrial apoptotic process (Edlich et al., 2011). We have seen a strong dose-dependent decrease in *BAX* expression in PFOA treated HepG2 cells. Counteract with what we have observed in MTT assay, which shows possible apoptosis activation at higher dosage of PFOA, *BAX* express less as the PFOA dosage increases. This could indicate other mechanism that is causing cell death at higher PFOA treatment. Cell cycle genes *CCNA2*, *CCNE1*, *CCNB1* were tested with their gene expression profile. *CCNA2* encodes a cyclin that controls both G1/S and G2/M transition phases (Pagano et al., 1992). *CCNE1* encodes a cyclin that is essential for the G1/S cycle transition control (Ohtsubo et al., 1995). Lastly *CCNB1* encodes an essential cyclin that controls G2/M cell cycle transition (Brown et al., 2007). All those cyclin encoding genes showed similar trends in gene expression. At 20 μM PFOA, there were small increases in gene expression in two of these cell cycle genes. As the dosage increases, the gene expression of those genes decreased significantly and mostly dose-dependently. These could signify the irregular modulation of cell cycles due to PFOA exposure. Cyclin-dependent kinase inhibitor 1 encoding *CDKN1A* increased significantly with higher concentration of PFOA treatment, including over 8-fold increase at 400 μM and over 11-fold increase at 200 μM. Previous study has shown that *CDKN1A* may be involved in responding to DNA damage via p53/TP53 mediated inhibition of cellular proliferation (Ducoux et al., 2001). Cyclin-dependent kinase 4 encoding *CDK4* expression profile has shown minor changes at different concentrations. RPS6 encodes 40S

ribosomal protein S6, which may play important role in controlling cell growth and proliferation via controlling the translation of certain classes of mRNA (Ruvinsky & Meyuhas, 2006). The expression of RPS6 was increased at higher concentrations of PFOA. The expression of Kit Ligand encoding gene *KITLG* has also decreased significantly with any concentration of PFOA (Figure 2). Overall, we can see the possible association between cell proliferation activation and PFOA exposure.

From a pool of lipid metabolism related genes, we selected those that are largely expressed in liver tissue since HepG2 was our model of the study. Out of those genes, a few of them have substantial alternation in RNA expression corresponding to the change in PFOA treatment. *ACAD11* encodes acyl-CoA dehydrogenase enzyme preferring lipids with carbon chain length range from 20 to 26 (He et al., 2011). The enzyme is involved in beta oxidation pathway and have some unknown properties (He et al., 2011). Generally, the expression of *ACAD11* has increased with the treatment of PFOA. *ACADM* encodes the medium chain specific preferring acyl-CoA, which catalyzes the initial step of mitochondrial fatty acid beta-oxidation pathway (Malecki et al., 2014). Its expression has also increased. Besides the increased expression of two catabolism related genes, the expression of *ACSL1*, a fatty acid coenzyme A ligase isozyme encoding gene, has increased significantly in a dose-dependent manner. ASCL1 works in the activation of long chain fatty acids synthesis (Lobo et al., 2009). The vast increase in expression of *ACSL1* at higher dosages of PFOA signifies the increase of lipid anabolism at higher PFOA exposure. Furthermore, *FABP1* which encodes fatty acid binding protein and responsible for fatty acid uptake, transport and metabolism had decreased expression. Therefore, *FABP1* expression decrease provides possible evidence that ACOX2 involved in the fatty acids degradation in peroxisomes had decreased expression. To sum up, we observed increased expression of lipid metabolism genes in different aspects. But out of all the expression of lipid synthesis related gene was most elevated, indicating a possible increased storage of lipids. (Figure 5) This hypothesis was further supported by the lipid quantification in HepG2 cells after Oil Red O staining and darkfield microscope hyperspectral imaging. With hyperspectral data, we can not only decide the lipid quantity based on the overall area stained but more precisely estimate the lipid quantity at each lipid storage droplet by looking at the average intensity. Between the two lipid droplets with same ORO stained area, higher average intensity at 663 nm wavelength indicates higher density of lipid stored. From our ORO stained cell images, we can

clearly see the increase in intracellular lipid storage at after higher concentration PFOA treatment.

From the previously mentioned gene expression we can see that different genes have responded to PFOA treatment differently. But we saw especially strong expression increase in lipid metabolism genes and cell proliferation genes. Together with large alternation in *TET1*, *TET2*, *TET3* expression levels, we suspected epigenetic changes as a major game player in changing the cell proliferation and increase the lipid storage induced by PFOA. But not all *TET*s expression act the same way. In the future, we would want to examine how PFOA affect different *TET*s differently and what each TET Methylcytosine Dioxygenase does to lipid metabolism pathway activation. Nonetheless, our global methylation assay also showed overall trend of decrease in methylation with the increase of PFOA, indicating abnormal gene expression activation due to PFOA exposure.

CHAPTER 5
CONCLUSION

This study has showed alternation in cell viability, gene expression, global methylation status of HepG2 cells treated with different concentrations of PFOA. We have observed different impacts of PFOA on HepG2 cells at low and high dosages. Overall, cell proliferation increases at lower doses of PFOA and decreases at higher doses, which was backed up with the increase in expression of cell proliferation genes. PFOA also causes increase in both lipid synthesis and degradation. But the overall impact of such alteration resulted into increased lipid deposit. Global methylation decreases at higher dosages of PFOA in a dose-dependent manner, which could indicate strong epigenetic toxicity of PFOA. While *DNMT*s may not be the crucial reason why such changes happen, *TET*s may play an important role in the global methylation alteration and abnormal lipid deposit in HepG2 cells due to PFOA exposure. However, additional experiments are needed to further determine the epigenetic toxicity of PFOA and its impact on lipid metabolism and liver diseases.

TABLE AND FIGURES

Table 1. Genes and their encoding proteins, and the primer sequences designed for quantitative real-time polymerase chain reaction

Gene	Protein encoded	Primer sequence (5'-3')	
TET1	Methylcytosine dioxygenase TET1	Forward:	CAGAACCTAAACCACCCGTG
		Reverse:	TGCTTCGTAGCGCCATTGTAA
TET2	Methylcytosine dioxygenase TET2	Forward:	GATAGAACCAACCATGTTGAGGG
		Reverse:	TGGAGCTTTGTAGCCAGAGGT
TET3	Methylcytosine dioxygenase TET3	Forward:	TCCAGCAACTCCTAGAACTGAG
		Reverse:	AGGCCGCTTGAATACTGACTG
DNMT1	DNA (cytosine-5)-methyltransferase 1	Forward:	TACCTGACGACCCTGACCTC
		Reverse:	CGTTGGCATCAAAGATGGACA
DNMT3A	DNA (cytosine-5)-methyltransferase 3A	Forward:	TATTGATGAGCGCACAAGAGAGC
		Reverse:	GGGTGTTCCAGGGTAACATTGAG
DNMT3B	DNA (cytosine-5)-methyltransferase 3B	Forward:	GGCAAGTTCTTCGAGGTCTCTG
		Reverse:	TGGTACATGGCTTTTGGATAGGA
BAX	Apoptosis regulator BAX	Forward:	CAAACTGGTGCTCAAGGCCC
		Reverse:	GGGCGTCCCAAAGTAGGAGA
CCNA2	Cyclin-A2	Forward:	AGTAAACAGCCTGCGTTCACC
		Reverse:	GAGGGACCAATGGTTTTCTGG
CCNE1	G1/S-specific cyclin-E1	Forward:	AAATGGCCAAAATCGACAGG
		Reverse:	CGAGGCTTGCACGTTGAGTT
CCNB1	G2/mitotic-specific cyclin-B1	Forward:	ATGACATGGTGCACTTTCCTCC
		Reverse:	GCCAGGTGCTGCATAACTGG
CDKN1A	Cyclin-dependent kinase inhibitor 1	Forward:	TACCCTTGTGCCTCGCTCAG
		Reverse:	GGCGGATTAGGGCTTCCTCT
CDK4	Cyclin-dependent kinase 4	Forward:	CTGTGCCACATCCCGAACTG
		Reverse:	GCCTCTTAGAAACTGGCGCA
RPS6	40S ribosomal protein S6	Forward:	CGATGAACGCAAACTTCGTA
		Reverse:	TTCGGACCACATAACCCTTC
KITLG	Kit ligand	Forward:	GGCAAATCTTCCAAAAGACTACATG
		Reverse:	CTACCATCTCGCTTATCCAACAATG
ACAD11	acyl-CoA dehydrogenase family member 11	Forward:	CAGGGTCGAATCTTCCGTGAT
		Reverse:	GTCCTGATGAGCTGCAGCTT
ACADM	acyl-CoA dehydrogenase medium chain	Forward:	GAGCAGGCTCTGATGTAGCTG
		Reverse:	TTCCTGGGGTATCTGCTTCC

Table 1. continued

Gene	Protein encoded	Primer sequence (5'-3')	
ACSL1	acyl-CoA synthetase long chain family member 1	Forward:	GACCTCTCCATGCAGTCAGT
		Reverse:	GACACCTGTATTCCCCTCTGG
FADS2	fatty acid desaturase 2	Forward:	CTGACCTGGAATTCGTGGGC
		Reverse:	CATGTCCTCAGCCGTCTTCC
FABP1	fatty acid binding protein 1	Forward:	TACAGTGGACAGTCTGGTCGG
		Reverse:	GGCAGACCGATTGCCTTCATG
ACOX2	acyl-CoA oxidase 2	Forward:	CTTTCTGGAGACGTGGCCTT
		Reverse:	GGCCCTGAAGATATGTCCCATG
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	Forward:	TGGGAATGCAGAGAAAGGTGC
		Reverse:	AAGCTCCCATCACCAAGGAG
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Forward:	GAAGGTGAAGGTCGGAGTC
		Reverse:	GAAGATGGTGATGGGATTTC

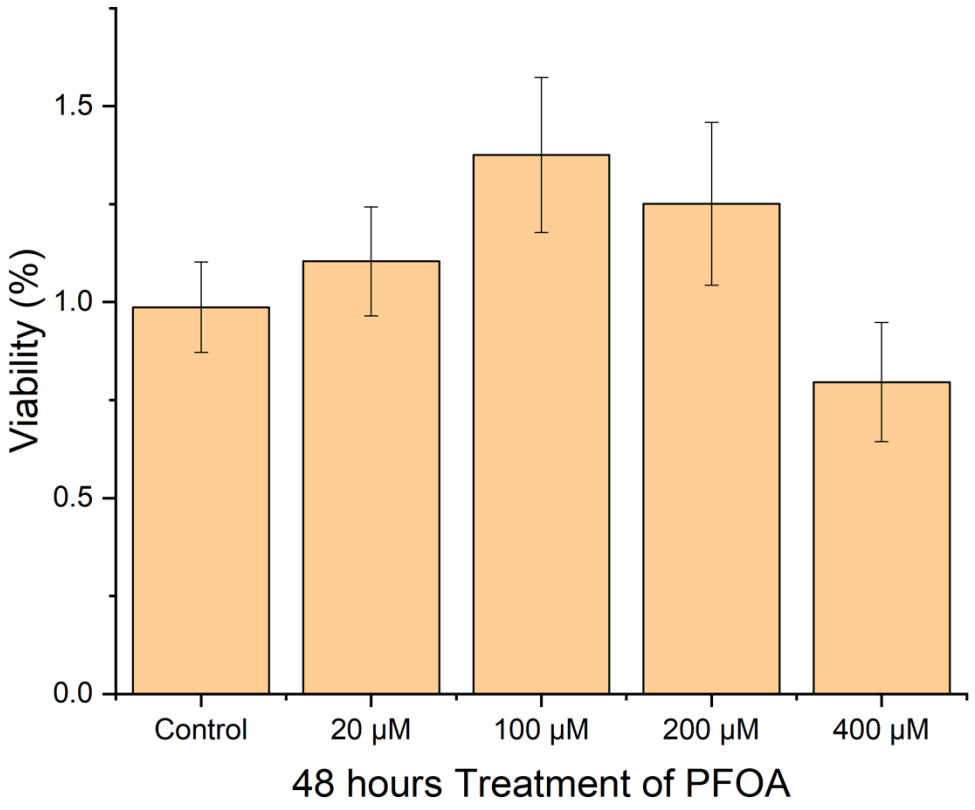

Figure 1. Evaluation of PFOA induced cytotoxicity in HepG2 cells. The cytotoxicity in HepG2 cells was determined as the cell viability measured with MTT assay. Low concentration of PFOA caused increases in cell viability. While higher concentrations of PFOA caused dose-dependent decreases in cell viability. Data are shown as mean ± sample standard deviation of nine independent replicates.

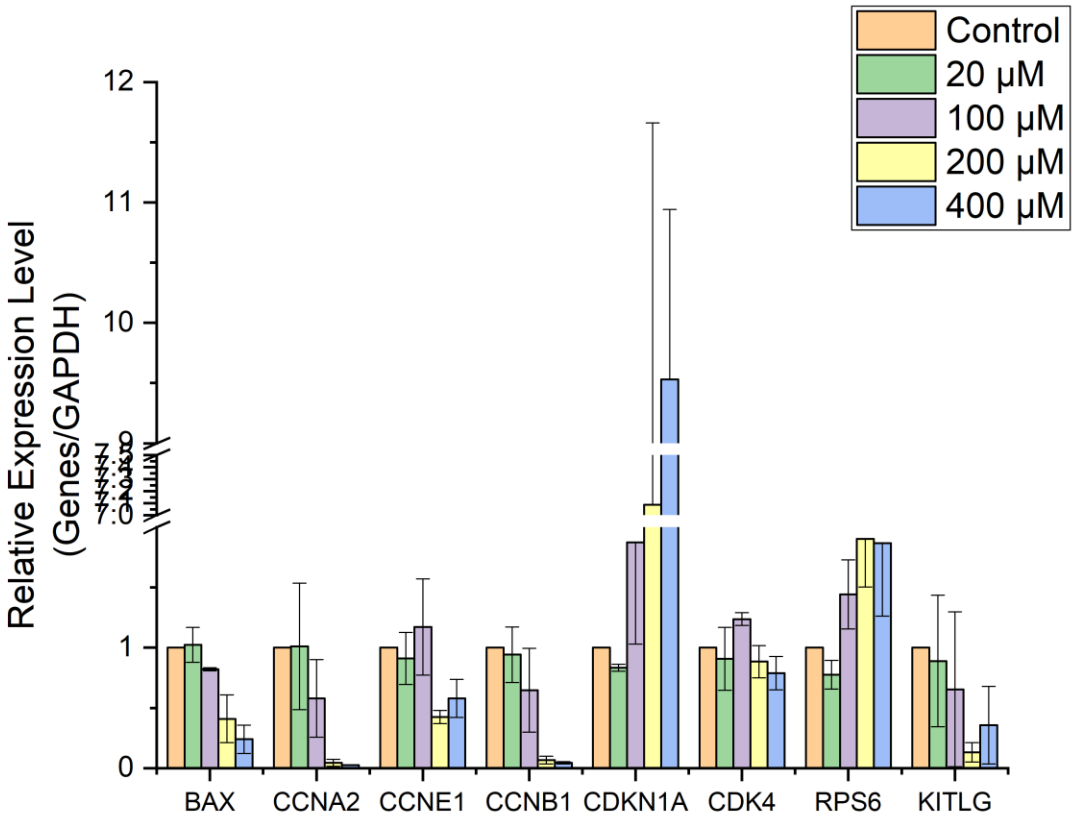

Figure 2. Effect on the expression of cell apoptosis, cycle and proliferation. Relative expression of cell apoptosis, cycle and proliferation genes. Data are shown as mean ± sample standard deviation of three independent replicates.

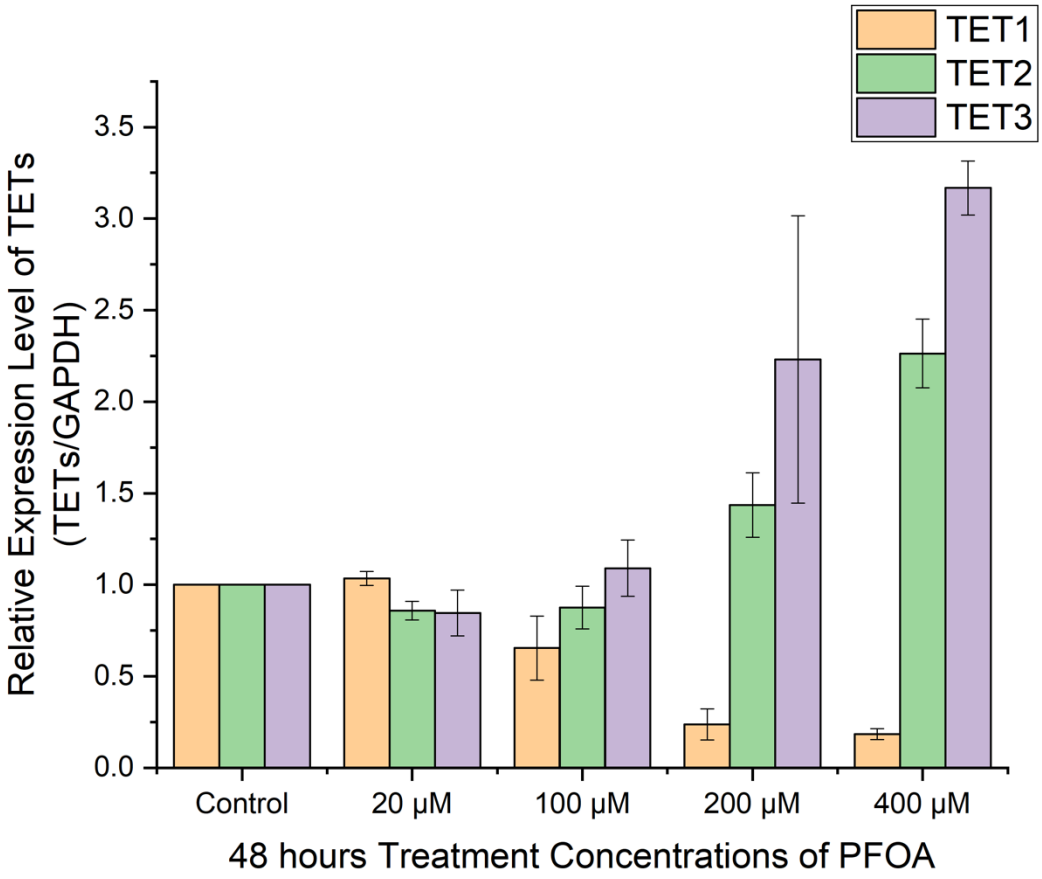

Figure 3. Effect on the expression of *TET*s. Relative expression of *TET1*, *TET2*, and *TET3*. Data are shown as mean ± sample standard deviation of three independent replicates.

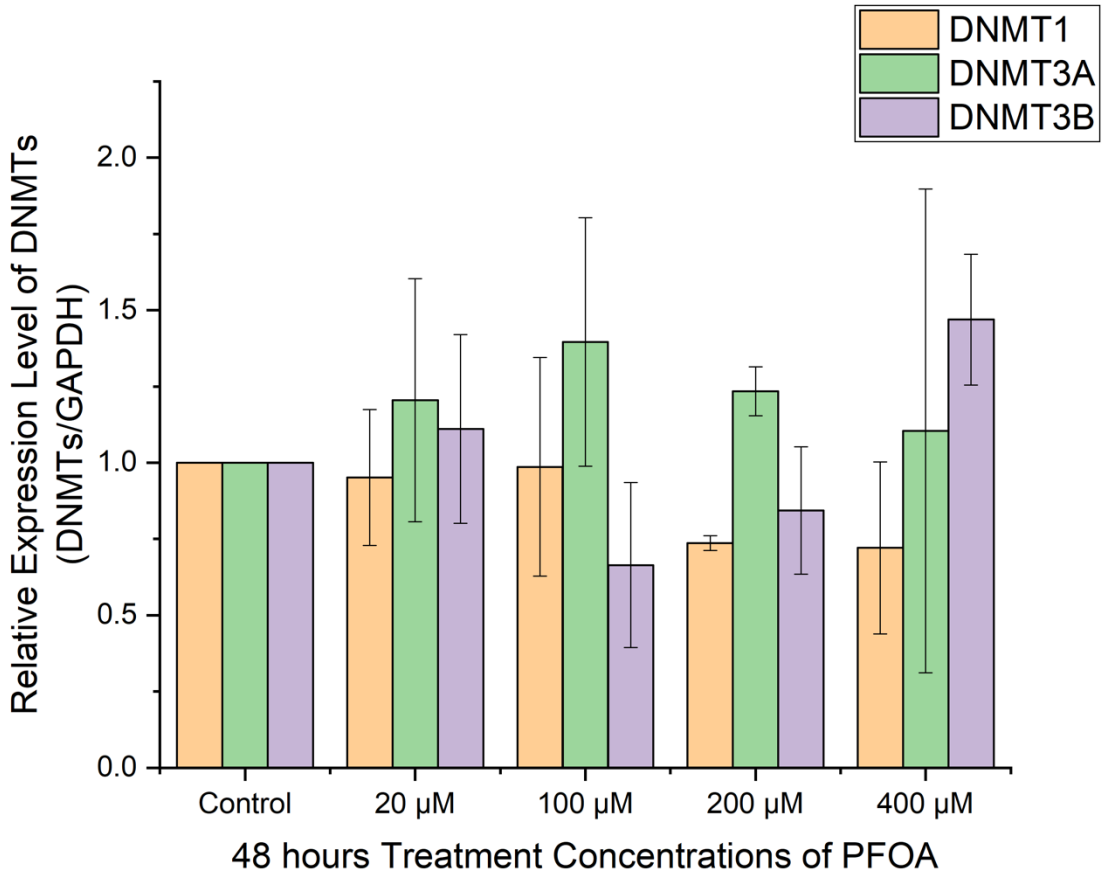

Figure 4. Effect on the expression *DNMT*s. Relative expression of *DNMT1*, *DNMT3A*, *DNMT3B*. Data are shown as mean ± sample standard deviation of three independent replicates.

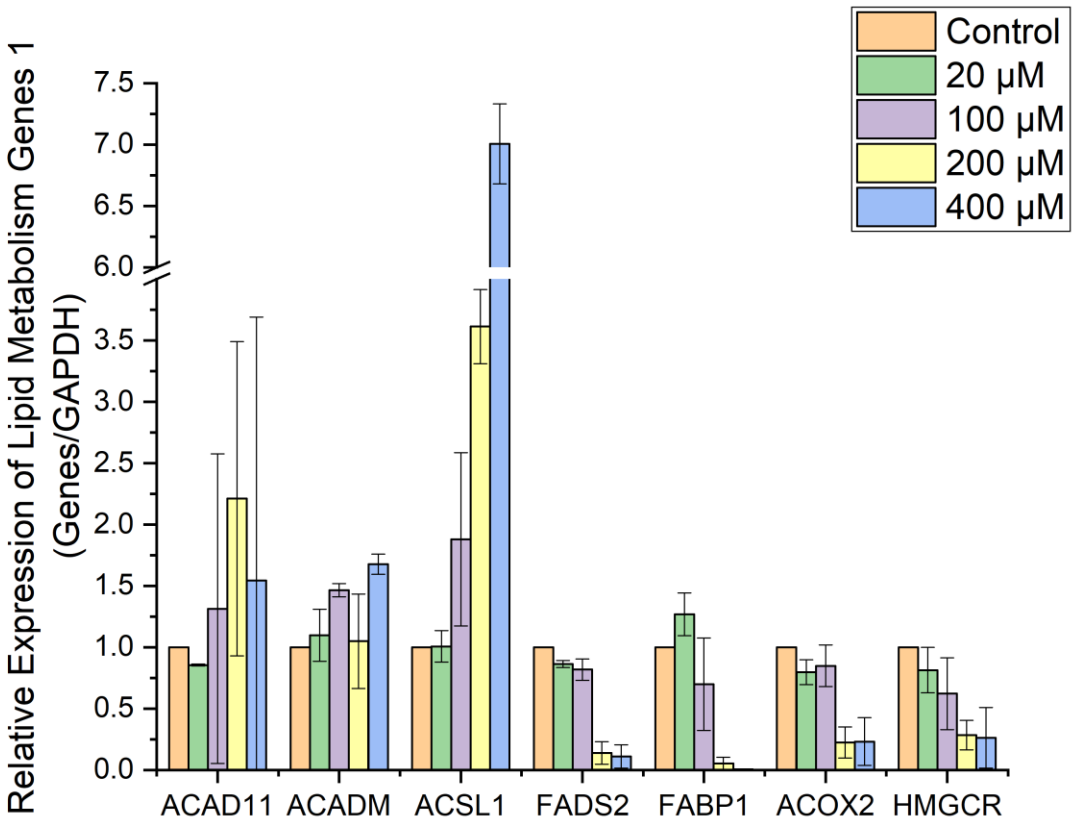

Figure 5. Effect on lipid metabolism and transport. Relative expression of cell lipid metabolism and transport genes. Data are shown as mean ± sample standard deviation of three independent replicates.

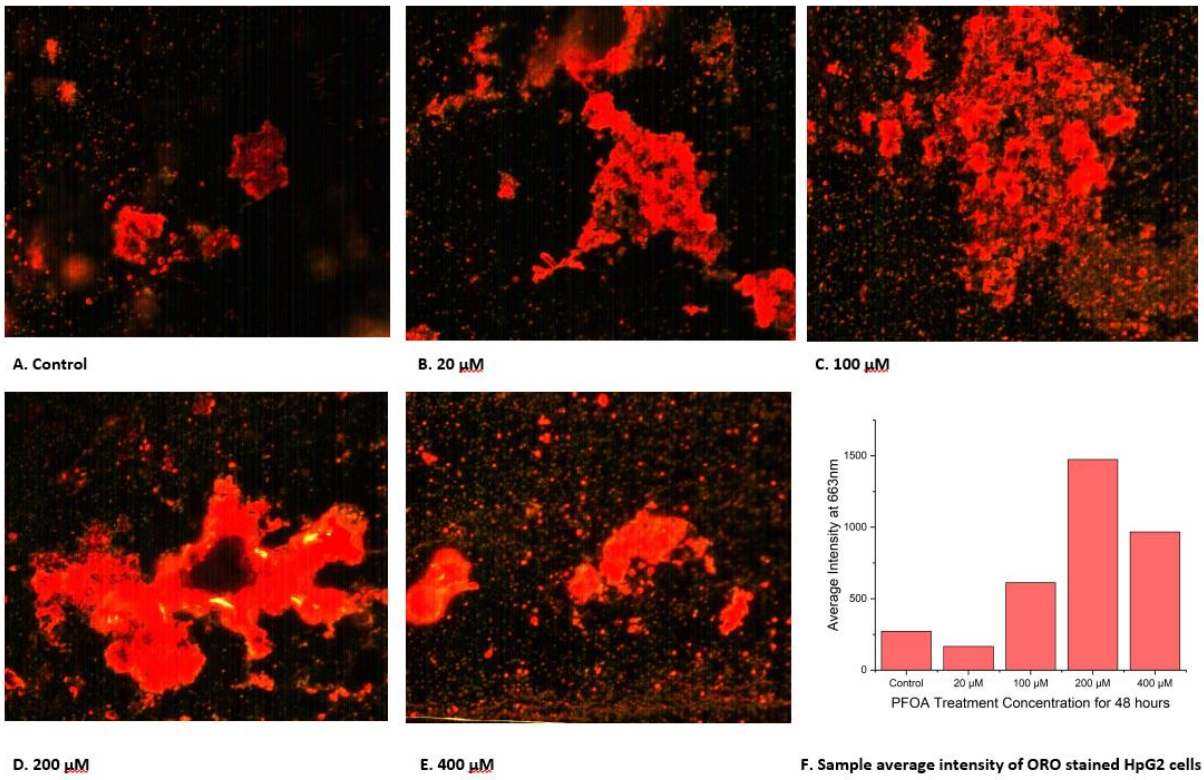

Figure 6. Quantification of intracellular lipid storage. (A-E) Oil Red O stained HepG2 imaged by dark field microscope hyperspectral camera. (F) Quantification of intracellular lipid in HepG2 cells based on average intensity at 663 nm wavelength.

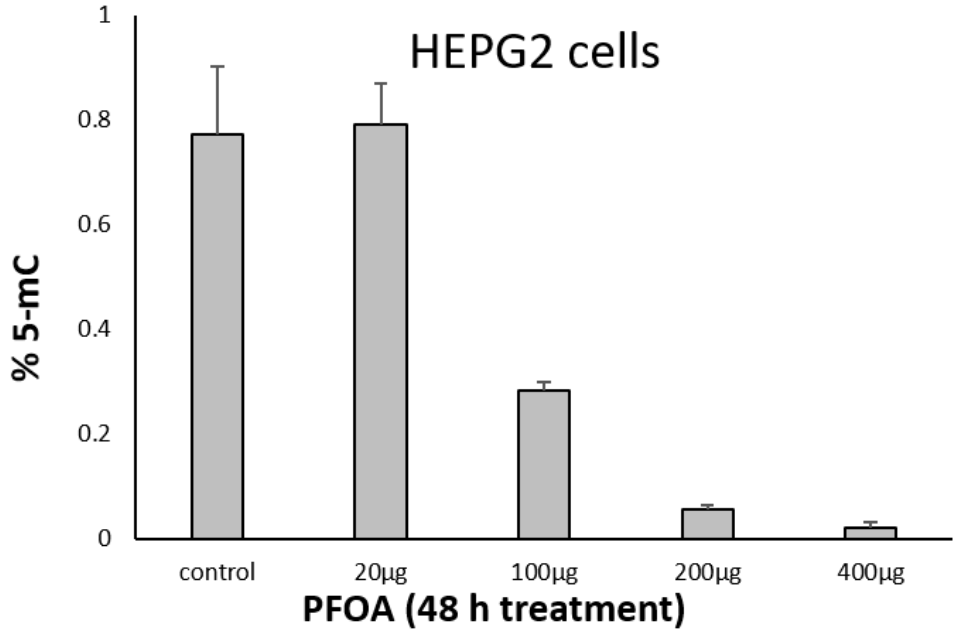

Figure 7. Global Methylation Assay. Global DNA methylation levels of HepG2 after different concentration PFOA treatments. Data are shown as mean ± sample standard deviation of three independent replicates.

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