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Acute PFOA exposure promotes epigenomic alterations in mouse kidney tissues

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ABSTRACT

Perfluorooctanoic acid (PFOA), a manufactured perfluorochemical is a common surfactant and environmental pollutant found in various consumer products and water sources. Epidemiological studies have demonstrated its association with kidney dysfunction. However, the mechanisms that trigger kidney dysfunction following PFOA exposure is a gap in the field. The work presented explores the potential epigenetic indicators of kidney disease due to exposure to PFOA. In this study, 30 days old CD-1 mice were exposed to 1, 5, 10, or 20 mg/kg/day of PFOA for 10 days. Following acute oral exposure, epigenetic alterations and expression levels of various markers of fibroblast activation were evaluated in kidney tissues. We noted that PFOA-exposed mice exhibited differential methylation yielding 879 differentially methylated regions compared to vehicle. The mRNA expression revealed significant increase in Dnmt1 with decreased Rasal1 expression at higher levels of PFOA exposure suggestive of Rasal1 hypermethylation (an early indicator of fibroblast activation in kidney). Like Dnmt1, we also observed significant increase in Hdac1, 3 and 4. These are class I & II HDACs which are known to be critically altered in some renal diseases. Further, the mRNA expression levels of TGF-β and α-SMA significantly increased compared to vehicle. The KEGG and Go enrichment pathway analysis of reduced representation bisulfite data also revealed pathways implicated in renal fibrosis. Our study shows clear evidence of epigenetic alterations (DNA methylation and HDAC expression changes) in tissues from mouse kidney following PFOA exposure. Our results also suggest that epigenetic alterations in kidney promote the expression of early markers of fibroblast activation.

1. Introduction

Poly- and Per fluoroalkyl substances (PFAS) are a family of chemicals containing a hydrophilic polar functional group and a long hydrophobic chain with fully saturated fluorine atoms (i.e. per fluoroalkyl chains) or at least hydrogen atoms in one of the carbon atoms replaced by fluorine atoms (i.e. polyfluoroalkyl substances) [1–3]. PFAS are extensively used in household products such as carpets, cleaners, nonstick cookware, wetting agents and in aqueous film forming foams [2,4]. Perflurooctanoate (PFOA) which belong to the PFAS family of chemicals is one of the most prominent contaminants in certain environments [5–7]. The major source of PFOA exposure in humans is through food, primarily fish products, drinking water, inhalation of dust and in consumer products [8–137]. Its elimination half-life inside human body is estimated to be about 4–5 years [14,15].

Earlier studies have shown that about 98 % of the US population have PFOA detected in their serum and it is distributed in kidneys and excreted without biotransformation [16–,17,18,19]. Exposure to PFOA has been known to cause considerable damage to various organs [19–21], including several pathways implicated in kidney disease and metabolic syndrome possibly associated with non-alcoholic fatty liver disease [22,23]. Examples include the peroxisome proliferator activated receptor activation [24], enhanced fibrotic and oxidative stress markers [25–30], induction of P450 [31,32] and disruption of efflux transporters like Bcrp which play important role in excretion of environmental chemical and drugs from liver and kidney [33]. According to the National Health and Nutrition Examination report [34], PFOA serum concentration has been found to be inversely proportional to the

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estimated glomerular filtration rate (eGFR). The increased PFOA serum levels considerably decreased the eGFR to < 60 mL/min/1.73 m2, a clinically relevant cut-off margin for chronic kidney disorder in adults [34,35]. Other large epidemiological studies of residents near the fluorochemical plant show that serum PFOA levels were positively correlated with decreased eGFR [36] and chronic kidney disease [37]. Increased concentrations of PFOA in serum were found to be associated with uric acid levels in serum [34,38,39], a marker known to be associated with a higher risk of chronic kidney disease [40]. Rats exposed to increased PFOA levels experienced hypertrophy, tissue proliferation and microvascular disease in kidney [20]. *In vitro* studies have also indicated that PFAS caused alterations in endothelial cell permeability [41,42]. These changes are among the fundamental mechanisms leading to ischemic renal failure in rats [43,44].

Despite these attempts, there is a paucity of information on the role of epigenetic mechanisms and programming on PFOA-induced renal injury. Therefore, in this study we investigated the possible mechanisms through which PFOA triggers indicators of renal injury. Specifically, in the present study we hypothesized that PFOA induces epigenetic changes in the kidney thus regulating the genes responsible for fibroblast activation. We focused on epigenetic alteration due to the fact that epigenetic modifications comprising of DNA methylation changes and histone modifications play a key role in the development of kidney fibrosis ultimately leading to chronic kidney disorder. According to previous work hypermethylation of specific genes by DNA methyl transferase (DNMTs) activate kidney fibrosis [45,46]. Like DNA methylation, several studies have also revealed that histone acetylation participates in experimental renal fibrosis [47,48]. However, previous studies have shown that PFAS exposure induced renal dysfunction at higher concentrations, therefore in our studies we exposed mice to lower, median and higher concentration of PFOA to evaluate the concentration dependent effects on epigenetic alterations in mechanism that govern kidney function.

2. Materials and methods

2.1. Chemicals

PFOA (99 % purity) was obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of PFOA (13.57, 6.785, and 3.39 mg/mL) were prepared by diluting PFOA in 0.5 % tween (Sigma-Aldrich, St.Louis, MO). The stock solutions were diluted to make doses of 1, 5, 10 and 20 mg/kg/day of PFOA. The concentrations of PFOA were selected based on environmental presence and previously published studies. These concentrations were selected based on the mean serum concentrations of PFOA in occupationally exposed workers which were in the range of 1000 − 2000 ng/ml. The highest concentration of serum PFOS and PFOA following occupational exposure was about 15,000 ng/ml and 13,500 ng/ml respectively [49,50]. In mice studies, a serum level of 171 μg PFOA/ml was acquired after 17 days of 20 mg/kg/day oral gavage [51]. Therefore, in our studies considering both community and occupational exposure we chose to expose mice at low, median, and high concentration (1, 5, 10 and 20 mg/kg/day).

2.2. Animals and dosing paradigm

Female adult CD-1 mice were obtained from Charles River, USA and kept in polysulfone, ventilated cages at 25 °C on 12 L:12D cycles. The mice were fed Teklad Rodent Diet 8604 (Harlan) and provided with purified water ad libitum. All animal protocols were approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC protocol#19037) per guidelines set forth by the National Institute of Health for the Care and Use of Laboratory Animals. CD-1 female mice (30 days of age) were consecutively dosed orally for 10 days with either vehicle control (water) or PFOA (1, 5, 10, or 20 mg/kg/day). Mice were euthanized during diestrus cycle after 10 days of dosing and kidney samples were collected for further studies.

2.3. Reduced representation bisulfite sequencing

2.3.1. Library construction and sequencing

Two samples (n = 2) from control and two samples from the high dose group were used for the Reduced Representation Bisulfite Sequencing (RRBS) analysis. Genomic DNA from the mouse kidney tissues were extracted and purified with a Purelink genomic DNA mini kit (Thermofisher, Waltham, MA, USA) per manufacturer's instructions. An additional step comprised of RNase A treatment as suggested by the manufacturer. The concentrations of extracted DNA were measured by a NanoDrop spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the quality check of the extracted DNA was performed by DNA electrophoresis gel. Construction of libraries and sequencing on the Illumina HiSeq 4000 were performed at the Roy J. Carver Biotechnology Center at the University of Illinois at Urbana-Champaign. RRBS libraries were constructed with the Ovation RRBS Methyl-Seq kit from Nugen. Briefly, 100 ng of high molecular weight DNA was digested with MspI, ligated to sequencing adaptors, treated with bisulfite and amplified by PCR. The final libraries were quantitated with Qubit (ThermoFisher, MA) and the average size was determined on a Fragment Analyzer (Agilent, CA). The libraries were then diluted to 10 nM and further quantitated by qPCR on a CFX Connect Real-Time qPCR system (Biorad, Hercules, CA) for accurate pooling of barcoded libraries and maximization of number of clusters in the flowcell. The pooled barcoded shotgun libraries were then loaded on a NovaSeq lane for cluster formation and sequencing. They were sequenced for 100 nt from one side of the DNA fragments. The typical output per lane in the NovaSeq is 400 million reads (SP flowcell) and 2 billion reads (S4 flowcell). The FASTQ read files were generated and demultiplexed with the bcl2fastq v2.20 Conversion Software (Illumina, San Diego, CA).

2.3.2. RRBS data analysis

RRBS allows for the enrichment of sequences with relatively high CpG content (i.e., promoter and CpG islands [CGI] regions) due to digestion of the entire genome by restriction enzyme Mspl. Following sequencing after bisulfite treatment, the analysis of the methylation status of each base site is performed [52,53]. Although only restriction fragments are sequenced, this analysis covers predominately CpG-rich regions, thus identifying methylation state of the whole genome from RRBS results.

The methylation profiling was calculated based on methylation level and CpG density in a specific area. The CpG density is defined for each CpG site within a window of 200 bp. The sliding window method was used for the study of differentially methylated regions (DMRs).

The workflow for processing the data is a modified version of the nf-core pipeline [54] and is available on Github (https://github.com/HPCBio/methylseq/tree/NuGen). In short, sequences were tagged with their associated Nugen-based UMI barcode, then trimmed using Trim_galore [55]. Trimmed reads were preprocessed according to the NuGen protocol to remove the diversity adapters (https://github.com/nugentechnologies/NuMetRRBS), followed by alignment to the mouse reference genome (Gencode release GRCm38, release M19) using Bismark v 0.18.1 [55]. Aligned sequences were dereplicated via the tagged UMI sequences using the available NuGen script (https://github.com/nugentechnologies/nudup) to remove PCR duplicates. Methylation calling was performed for all methylation contexts using Bismark and the bismark_methylation_extractor script.

2.3.3. CpG and DMR analysis

All analyses utilized the R/Bioconductor methylkit package [56]. Methylation differences were calculated using the 'calculateDiffMeth' method in methylkit, selecting CpGs with a minimum read depth of 10 across samples, a q-value < = 0.01, and methylation change of at least

25 %. Differentially methylated regions (DMRs) used the above CpGs, a window size and step size of 1 kb, with the same q-value and methylation change (ie. 25 %). Hyper/hypomethylated regions were annotated using the R genomation package [57] to assess location of differentially methylated CpG or regions relative to genic regions. EntrezGene IDs for these genes were accessed using biomaRt [58] and used for KEGG pathway and Gene Ontology enrichment analyses performed with the Bioconductor limma and clusterProfiler packages [59,60] using default parameters.

2.4. Isolation of RNA and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from the mouse kidney samples using Triazol method (Ambion, Thermofisher, Waltham, MA, USA) followed by DNAase treatment and purification with RNeasy mini kit (Qiagen Inc., Germantown, MD, USA). The RNA was reverse transcribed to cDNA using the high capacity cDNA synthesis kit (Applied Biosystems, Thermofisher, Waltham, MA, USA). The variations in target genes expression were assessed with 20 μl of Powerup SYBR Green PCR master mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) in a quantitative real-time PCR system (StepOnePlus Real-Time PCR Systems; v 2.0 Applied Biosystems, USA). Data analysis was done using ΔΔCt method along with normalization of transcription to the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene.

2.5. Statistical analyses

SPSS statistical software (SPSS Inc., Chicago, IL) was used for data analysis. Multiple comparisons between normally distributed experimental groups were performed using the one-way analysis of variance. Dunnett post hoc comparisons were done when equivalent variances were assumed. Whereas, when the non-equivalent variants were assumed Games–Howell post hoc comparisons were applied. If the data was not normally distributed, Kruskal–Wallis H test was used for comparison among groups. This was followed by Mann-Whitney U two-independent sample test. Statistical values were considered significant at $P < 0.05$.

3. Results

3.1. Acute oral exposure of PFOA promotes methylation changes in the mouse kidney

To determine the global DNA methylation levels in mouse kidney following PFOA exposure, the 5-methyl cytosine (5mC) levels and 5-hydroxy methylation (5hmC) levels (Epigentek, NY, USA) were quantified in kidney samples. No significant changes in 5mC (Supp. Fig. 1A) or 5hmC (Supp. Fig. 1B) were noted. Since global methylation levels did not show any significant changes, we performed RRBS to identify specific methylation changes. RRBS was performed with the DNA extracted from kidney tissues of vehicle and mouse exposed to PFOA. The differential methylation at the CpG level (single nucleotide) were sorted by q-value (adjusted p-value) with a q-value cutoff of 0.01. In our preliminary work we identified CpG sites exhibiting significant differences based on the above cutoff (Fig. 1A) value. Since individual base methylation changes have little functional relevance, only differentially methylated regions (DMRs) were considered. We defined DMRs as windows of 1000 bp that contain at least 4 cytosines, more than 10 reads per cytosine were required, which differed in methylation by more than 25 % and a q-cutoff value of ≤0.01. About 879 DMRs were identified in total within CpG context. Results show that 879 genes were differentially methylated between vehicle and PFOA exposed mice (20 mg/kg/day). We specifically analyzed both hypomethylation and hypermethylation in the defined DMRs. Though most of the hypo and hypermethylation regions were found in the gene body, the promoter has about 12 % and 16 % of hypo and hypermethylated regions respectively (Fig. 1B and C). This indicates the effect of PFOA on differential methylation changes in exon, intron, and specifically in the promoter compared to control.

3.2. PFOA induced mRNA expression changes of the major epigenome players

DNMTs and Ten eleven translocation (TETs) enzymes are the major enzymes that regulate DNA methylation levels. First, we quantified mRNA expression levels of *Dnmts*. There are different isoforms of DNMTs. Here we mostly focused on DNMT1, DNMT3a, and DNMT3b primarily. This is because early in the development, the *de novo* DNA methyl transferases namely *Dnmt3a* and *Dnmt3b* plays a key role in establishing the genomic 5mC patterns and these patterns were maintained by DNMT namely *Dnmt1*. The PFOA exposure significantly reduced *Dnmt3a* expression levels in higher concentration; thus, affecting the de novo DNA methylation (Fig. 2A). However, no significant changes in *Dnmt3b* levels were observed expect at higher exposure levels of PFOA (Fig. 2A; 20 mg/kg/day). Unlike *Dnmt3a* and *3b*, *Dnmt1* show dramatic increase in their expression levels with increased exposure levels (Fig. 2A; 20 mg/kg/day).

Next, we assessed the mRNA expression levels of *Tets*. TET1 is a family of three proteins, that includes TET1, TET2, and TET3. These three proteins catalyze the oxidation of 5mC to 5hmC, 5-formylcytosine and finally to 5-carboxylcytosine [61,62] thus resulting in demethylation. *Tet1* expression was significantly altered with minimal to no change in *Tet2* expression (Fig. 2B). However, increased *Tet3* expression levels at higher concentration of PFOA exposure (Fig. 2B; 20 mg/kg/day) was observed. Our data thus indicates that at higher concentration there is a concentration dependent effect on the expression of major enzymes that regulate DNA methylation, providing strong evidence that direct exposure to PFOA triggers epigenetic alterations in the tissues of the kidney.

Similar to DNA methylation, histone modification also plays a key role in epigenetic regulation of the DNA and hence gene expression. Histone acetylation acts by reducing the overall positive charge on histones thus facilitating DNA transcription by weakening their interaction with DNA. The acetylation process occurs when the acetyl group (COCH3) is transferred from acetyl-coenzyme A (acetyl-CoA) to the lysine residues of the histone tail, through a process controlled by histone acetyltransferases. Acetylated residues are then recovered by histone deacetylases (HDACs) [63]. In order to assess the involvement of histone modifications in the PFOA induced epigenetic changes, we quantified mRNA expression levels of various *Hdacs*. Expression levels of *Hdac 1, 2, 3, 4, 5, 6, 8* and *10* were evaluated. A significant increase in *Hdac1* expression levels was noted at higher exposure levels. A similar trend with *Hdac3* and *Hdac4* (Fig. 3) was also observed. However, no significant changes in *Hdac 2, 5, 6, 8* and *10* was noted.

3.3. Acute oral exposure of PFOA modifies mRNA expression of the genes involved in fibrotic fibroblast activation

To determine whether PFOA triggers fibroblast activation in kidney, mRNA expression levels of specific marker genes known to be triggered during fibroblast activation was measured. One such target gene is *Rasal1*. RASAL1 is a member of the RAS-GAP family, which causes inactivation of Ras by catalyzing GTP-Ras to GDP-Ras [64]. The growth factor–independent Ras hyperactivity is known to cause autonomous cell proliferation within cancer cells [65,66]. It is known that epigenetic silencing of RASAL1 plays a major role in the activation of fibrotic fibroblast. Therefore, we measured the mRNA levels of *Rasal1* gene. The decrease of RASAL1 mRNA could suggest fibroblast activation due to exposure to PFOA (Fig. 4). Next, we measured the expression of α-smooth muscle actin (*α-Sma*). This is because fibroblast activation normally shows an increased proliferative activity followed by increased extracellular matrix constituents and expression of *α-Sma*. A

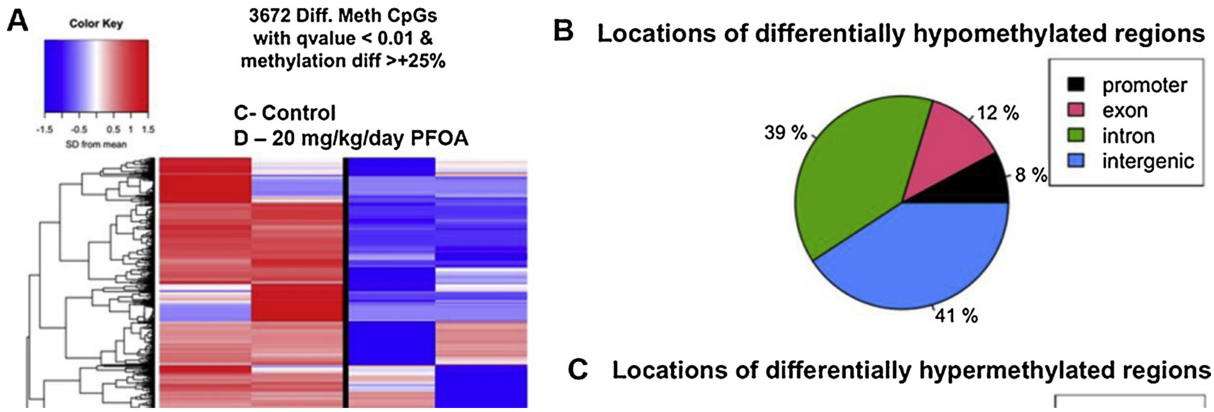

Fig. 1. Oral exposure of PFOA induce methylation changes in kidney samples. Histograms quantifying percentage of (A) Heat map showing differential methylation pattern following PFOA exposure in kidney samples. There were more than 3672 differentially methylated CPGs following PFOA exposure. Percentage of DMRs being (B) hypo methylated (C) hyper methylated in CPG context.

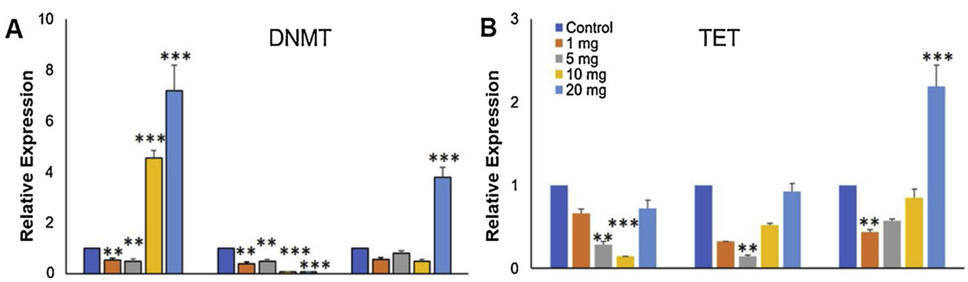

Fig. 2. PFOA induced mRNA expression changes of major epigenome players namely *Dnmts* and *Tets*. Histograms quantifying (A) *Dnmts* and (B) *Tets* mRNA expression levels. The expression levels were normalized with GAPDH/ actin b. *Dnmt3a* expression levels were significantly reduced with increased concentration of PFOA whereas *Dnmt1* expression levels significantly increases with increased PFOA concentration (n = 3 for each concentration, ** $P < 0.001$, * $P < 0.05$).

significant increase in *α-Sma* mRNA levels (Fig. 4) was noted at increased levels of PFOA exposure suggesting a fibroblast activation. We also tested other two genes known to be involved in kidney development namely *Lrnf2* and *Dlg2* which shows no significant changes with PFOA exposure. Based on our data it is evident that PFOA has the potential to trigger key biomarkers relevant to fibroblast activation in kidney. Next, we measured *TGF-β* as its increased mRNA expression is one of the characteristic features of kidney fibroblast activation. As the level of PFOA exposure increased, the expression of *TGF-β* increased significantly (Fig. 4) suggestive of fibroblast activation due to PFOA exposure.

3.4. PFOA exposure exhibits differential methylation of genes/pathways known to be involved in kidney fibrosis

Next, to investigate whether the pathways or cellular process implicated in kidney fibrosis or

chronic kidney diseases are differentially methylated, we applied Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway analysis. For both KEGG and GO, the hypergeometric test was used to identify the enriched pathways, determining the differentially expressed genes after comparing with the entire genome. For both analyses, the p-value was set at ≤ 0.05 with DMR TSS region Gene IDs of 25 % difference and q-value ≤ 0.01. In the KEGG analysis we identified smooth muscle, RAP1, MAPK, Wnt, P53 as well as ferroptosis pathway (Fig. 5A). These pathways are known to be one of the major players in kidney fibrosis and chronic kidney disease. Similarly, with GO analysis we identified the following cell process namely ammonium ion metabolism, G protein coupled receptor signaling, regulation of cell fate commitment, and smooth muscle proliferation (Fig. 5B). These are known to be altered in renal diseases.

4. Discussion

The association between PFOA and kidney dysfunction is revealed through several recent studies [67,35], which also includes evidence for abnormal histological changes and renal hypertrophy [20]. In chronic kidney disease, activation of fibrotic fibroblast is directly related to

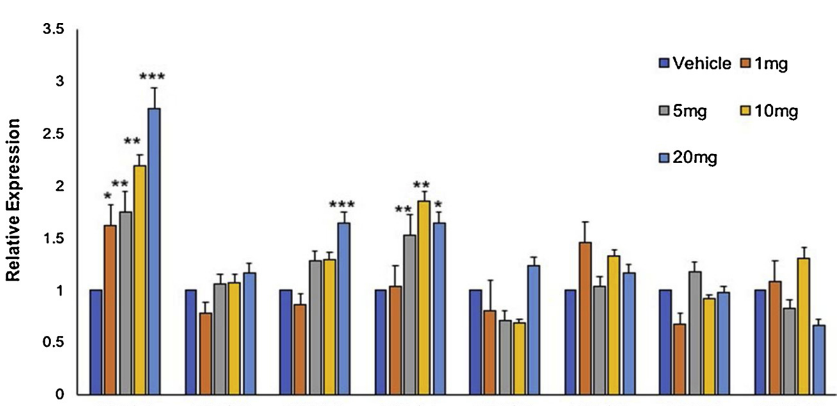

Fig. 3. Oral exposure of PFOA induces significant changes in *Hdac* expression levels. Histograms quantifying mRNA expression levels of different *Hdacs*. PFOA exposure significantly increased *Hdac1* and *Hdac4* levels with no or minimal changes in other *Hdacs*. (n = 3 for each concentration, *** $P < 0.0001$, ** $P < 0.001$, * $P < 0.05$).

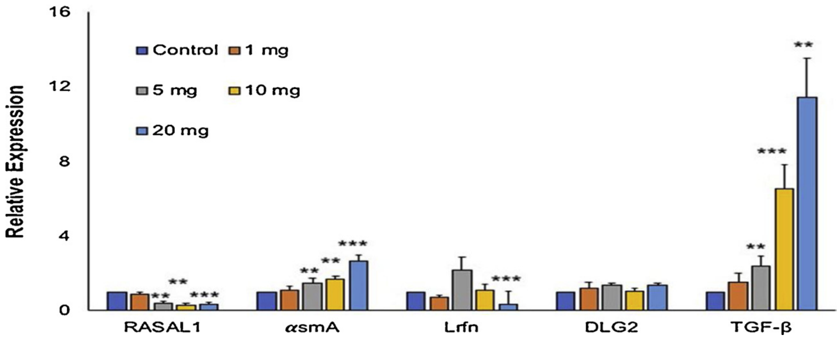

Fig. 4. PFOA exposure significantly changes fibroblast activation genes inducing fibrosis in kidney. Histogram quantifying mRNA expression levels of *Rasal1*, *α smA, Lrfn2, Dlg2* and *TGF-β*. The expression levels were normalized with GAPDH/actin b. *Rasal1* expression levels were significantly reduced with increased concentration of PFOA whereas *αSma* and *TGF-β* expression levels significantly increased with PFOA concentration (n = 3 for each concentration, *** $P < 0.0001$, **$P < 0.001$).

development of fibrosis [68,69]. Several recent reports have implicated epigenetic regulation in the development of fibrotic pathways, thus opening new avenues in the identification of biomarkers and therapeutic strategies [70–72].

DNA methylation is one of the well-studied epigenetic modification playing predominant role in development of embryo and cellular differentiation [73]. The gene expression is regulated by the addition of a methyl group to the cytosine [74]. Epidemiological studies in humans have indicated that environmental pollutants or toxicants lead to changes in DNA methylation patterns within children when exposed prenatally [73]. A few *in vivo* and *in vitro* studies have identified the association between PFOA exposure and methylation level changes either at the genome levels or at a specific-loci [75,76]. For example, reduced IGF2 methylation in cord blood was observed in prenatal PFOA exposure population [77]. Other studies have reported that PFOA exposures were associated with higher Long Interspersed Nuclear Element-1 (LINE-1) methylation and alterations within the genes involved in cholesterol metabolism. In neonates PFOA exposure lower global DNA cytosine methylation and decreased insulin-like growth factor-2 methylation [77–80]. However, to the best of our knowledge past studies have not explored PFOA induced epigenetic changes in the kidney. This is important to investigate since understanding the perturbation of epigenome upon exposure to PFOA will provide valuable insights on epigenetic regulatory mechanisms that lead to kidney dysfunction. The RRBS analysis also shows altered methylation profile in the PFOA exposed kidney tissues compared to control. In our current study, we noted that methylation predominated at CpG sites, along with differential methylation levels between the CGI versus promoter sites. Although differential methylation was predominant in the gene body, we found significant changes in the hyper and hypomethylation at the

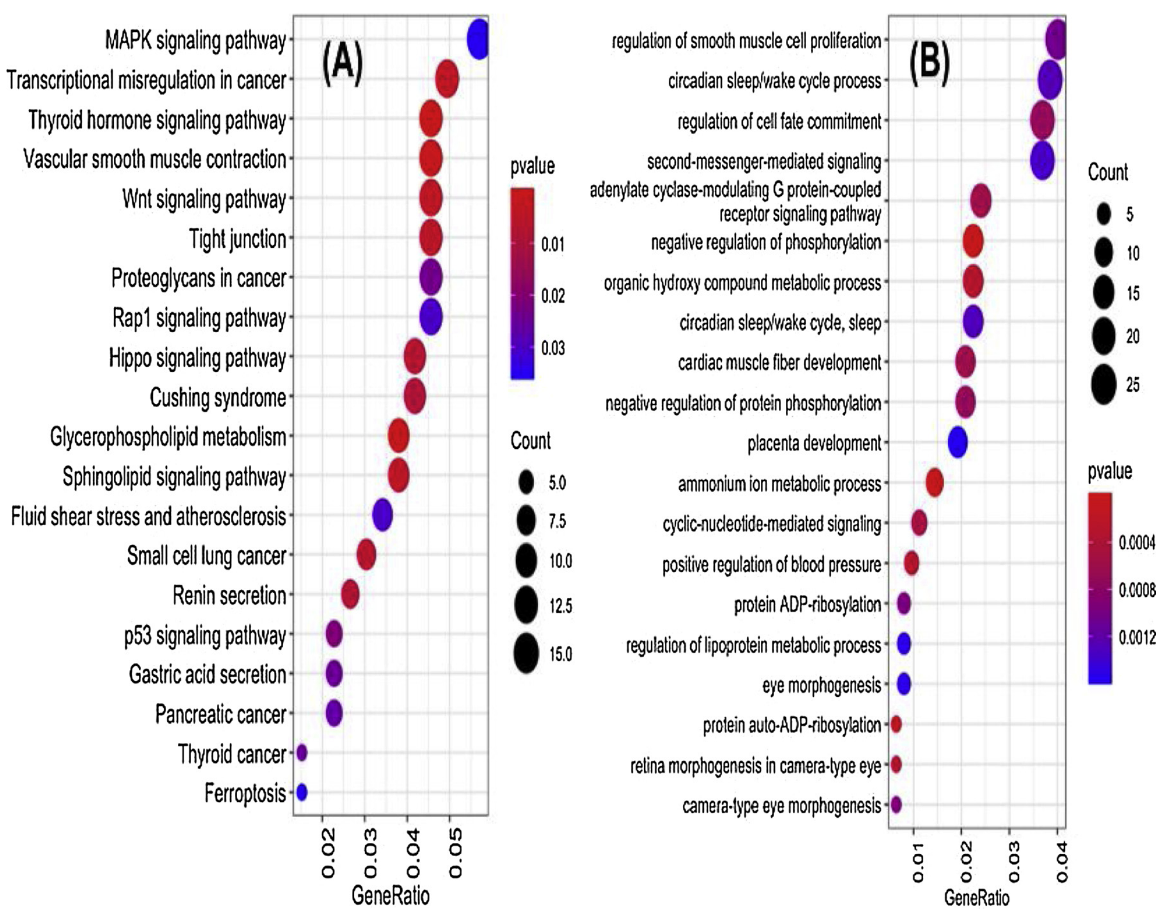

Fig. 5. Enrichment pathway analysis of differentially methylated genes show major kidney fibrosis pathway. (A) Over representation analysis of top 30 KEGG terms with p-value ≤ 0.05, DMR TSS region Gene IDS (20 % difference, q-value ≤ 0.05). (B) Over representation analysis of top 30 Gene Ontology functional analysis terms with p-value ≤ 0.05.

promoter sites of the PFOA exposed kidney compared to control, suggestive of altered gene regulation.

To gain further insights into the differential methylation changes in PFOA exposed mice, we also investigated the expression levels of DNMTs and TETs, as methylation and demethylation are regulated by these group of proteins respectively. Previous studies have shown that hypermethylation of the *Rasal1* promoter and the resulting gene silencing of *Rasal1* to be a key component for activation of experimental fibrotic fibroblast [71,72]. *Rasal1* hypermethylation has been implicated in different types of experimental renal fibrosis conditions, regardless of the underlying disease model. In PFOA exposed mice, we observed reduced expression of *Rasal1* with increasing exposure levels of PFOA. Reduced Rasal1 expression suggests possible changes in the methylation status of the Rasal1 promoter, however RRBS analyses was unable to directly detect a significant shift in methylation. It should be noted that RRBS is a targeted approach and will likely miss consistent methylation changes in uncaptured or low-coverage regions, suggesting a more comprehensive approach in future studies when using such analysis. These include whole-genome bisulfite sequencing so that methylation status can be more selectively evaluated in this region. This also does not rule out the possibility that the expression shift may be indirect, for example a change in an upstream element or regulatory gene. Previous studies have established the fact that in kidney fibroblast activation, DNMT1 hypermethylates *Rasl1* promoter thus silencing Rasal1 transcription [71,72]. We saw similar effect in our studies where *Dnmt1* levels were significantly higher with PFOA exposure. We observed an inverse correlation with increased *Dnmt1* expression levels and decreased *Rasal1* expression suggesting *Rasal1* silencing by *Dnmt1*.

The hydroxymethylation leading to demethylation of CpG is facilitated by Tet1, Tet2, and Tet3. Previous studies have investigated their gene expression in various disease models and have observed reduced expression of Tet3, whereas, Tet1 or Tet2 were not reduced. These changes were found to be associated with experimental fibrosis conditions [72]. This study also shows that Rasal1 promoter hypermethylation and decreased Tet3 expression are primary features of renal fibroblast activation. However, in our studies we observed increased *Tet3* expression levels at higher concentration of the PFOA exposure. This increased expression of *Tet3* could be the consequence of increased *Dnmt1* expression to balance methylation levels within the system. It would be intriguing to evaluate the DNMT1 and TET3 regulation of hypermethylation in PFAS exposed mouse models though it is beyond the scope of the current study.

It is well established that various HDACs are significantly associated with the fibroblast activation and increased expression of specific HDACs that stimulate differentiation of fibroblasts into myofibroblasts [70]. Even though the specific mechanism of HDAC in fibroblast differentiation is somewhat different, cumulative evidence suggests that HDACs accelerate fibrogenesis in a redundant manner and that HDAC inhibitors successfully regulate kidney fibrosis. Therefore, we investigated the *Hdac* expression following PFOA exposure and observed a significant increase in Class I *Hdacs* that are crucially involved in fibroblast activation in PFOA exposed mice.

One of the characteristic features of kidney fibroblast activation is increased expression of *TGF-β* and *α-Sma*. Considerable evidence from both patient and animal models of disease, points to the fact that TGF-β is significantly increased in the affected kidney [81,82]. Moreover, the importance of TGF-β1 in renal fibrosis is further supported by animal studies, whereas increased expression of active TGF-β1 in rodent liver induces a fibrotic response in the kidney. Other studies have also shown that TGF-β with genetic deletion of receptors, antisense oligonucleotides, neutralizing antibody, or inhibitors can attenuate fibroblast activation *in vitro* and *in vivo* [83–89]. Therefore, we examined the levels of *TGF-β* and found considerable increase in *TGF-β* expression with increased concentration of PFOA exposure. TGF-β is known to trigger the expression of α-SMA [71]. We also found that *α-Sma* expression increased at higher levels of PFOA exposure. The enrichment pathway analysis with KEGG and GO also identified the key pathways known to be involved in chronic kidney disease. Thus, increased *Dnmt1, TGF-β* and *α-Sma* expression with decreased *Rasal1* expression could serve as indicators of the initiation of fibroblast activation in kidney following acute PFOA exposure. RASAL1 hypermethylation or α-SMA expression could serve as potential biomarkers to identify early renal injury in population exposed to PFOA.

5. Conclusion

In our study, we highlight two key findings, a) PFOA induces epigenetic changes in the kidney; and b) PFOA induced epigenetic alterations trigger the genes known to be involved in fibrotic fibroblast activation. Though acute PFOA exposure induced epigenetic changes could potentially involve the genes involved in fibroblast activation, long-term studies are required to assess whether fibroblast activation could potentially lead to kidney fibrosis. It would also be intriguing to evaluate whether chronic PFOA exposure causes chronic kidney disease. Further experiments could focus on pathway analysis as well as transgenerational effects.

Declaration of Competing Interest

The authors declare that there is no conflict of interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.toxrep.2019.12.010.

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