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Regulation of Hepatic Long Non-coding RNAs by PXR and CAR Agonists in Mouse Liver

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LncRNAs regulated by PXR and CAR agonists in mouse liver

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Abbreviations:

5MeC, DNA methylation and cytosine 5th position; AhR, aryl hydrocarbon receptor; AS, anti-sense; BAM, binary alignment/map; CAR, constitutive androstane receptor; ChIP-Seq, chromatin immunoprecipitation coupled with high throughput sequencing; CYP, cytochrome P450; FPKM, fragments per kilobase of exon per million reads mapped; H3K27me3, H3 lysine 27 tri-methylation; H3K4me2, H3 lysine 4 di-methylation; HISAT, Hierarchical Indexing for Spliced Alignment of Transcripts; HNF, hepatocyte nuclear factor; lncRNA, long noncoding RNA; PCG, protein-coding gene; PCN, pregnenolone-16α-carbonitrile; PXR, pregnane X receptor; SAM, sequencing alignment/map; siRNA, small interfering RNA; TCPOBOP, 1, 4-bis[2-(3, 5-dichloropyridyloxy)]benzene; TSS, transcription start site; TTS, transcriptional termination site; UTR, untranslated region

ABSTRACT

Altered expression of lncRNAs by environmental chemicals modulates the expression of xenobiotic biotransformation related genes and may serve as therapeutic targets and novel biomarkers of exposure. The pregnane X receptor (PXR/NR1I2) is a critical xenobiotic-sensing nuclear receptor that regulates the expression of many drug-processing genes, and it has similar target gene profiles and DNA binding motifs with another xenobiotic-sensing nuclear receptor, namely constitutive andronstrane receptor (CAR/Nr1i3). To test our hypothesis that lncRNAs are regulated by PXR in concert with protein-coding genes (PCGs) and to compare the PXR-targeted lncRNAs with CAR-targeted lncRNAs, RNA-Seq was performed from livers of adult male C57BL/6 mice treated with corn oil, the PXR agonist PCN, or the CAR agonist TCPOBOP. Among 125,680 known lncRNAs, 3,843 were expressed in liver and 193 were differentially regulated by PXR (among which 40% were also regulated by CAR). The majority of PXR- or CAR-regulated lncRNAs were mapped to the introns and 3'-UTRs of PCGs as well as intergenic regions. Combining the RNA-Seq data with a published PXR ChIP-Seq dataset (Cui et al., 2010b), we identified 774 expressed lncRNAs with direct PXR-DNA binding sites, and 26.8% of differentially expressed lncRNAs had changes in PXR-DNA binding following PCN exposure. *De novo* motif analysis identified co-localization of PXR with LRH-1, which regulates bile acid synthesis, following PCN exposure. There was limited overlap of PXR binding with an epigenetic mark for transcriptional activation (histone-H3K4-di-methylation, H3K4me2), but no overlap with

epigenetic marks for transcriptional silencing (H3K27me3 and DNA methylation). Among differentially expressed lncRNAs, 264 were in proximity of PCGs, and the lncRNA-PCG pairs displayed a high co-regulatory pattern by PXR and CAR activation. This study was among the first to demonstrate that lncRNAs are regulated by PXR and CAR activation and that they may be important regulators of PCGs involved xenobiotic metabolism.

INTRODUCTION

Long non-coding RNAs (lncRNAs) are functional transcripts over 200 nucleotides in length whose genes are estimated to comprise at least 62-75% of the human genome (Djebali et al., 2012; St Laurent et al., 2015). According to the ENCODE Project, lncRNAs represent approximately 80% of functional sequences in the human DNA and are the predominant non-ribosomal and non-mitochondrial RNA species in human cells (Kapranov et al., 2010; Consortium, 2012). LncRNAs transcribed proximally or distally to protein-coding genes (PCGs) can modulate a wide spectrum of biological events, including chromatin epigenetic remodeling, transcription factor assembly, alternative splicing, mRNA stability, and protein translation efficiency (Geisler and Coller, 2013; Karlsson and Baccarelli, 2016; Dempsey and Cui, 2017). Growing evidence in the literature suggests that lncRNAs are novel biomarkers and/or key contributors during physiological, pharmacological, and toxicological responses, including complex human diseases, developmental disorders, as well as xenobiotic-induced adverse outcomes (Dempsey and Cui, 2017).

Many lncRNAs are highly expressed in liver, which is a major organ for xenobiotic biotransformation and nutrient homeostasis. A stringent computational pipeline identified 15,558 lncRNAs that are expressed in mouse liver, based on an analysis of 186 mouse liver RNA-Seq datasets ranging over 30 biological conditions (Yu et al., 2017). Interestingly, Yu et al. also demonstrated greater inter-species conservations within DNA sequences and higher frequency of proximal binding by hepatic transcription factors in the liver-

expressed lncRNA gene promoters as compared to protein-coding gene (PCG) promoters (Yu et al., 2017). The liver-enriched lncRNAs were predicted to be metabolically sensitive regulators with diverse functions in physiological homeostasis, especially energy metabolism (Yang et al., 2016). Regulation of genes involved in energy metabolism and nutrient homeostasis by lncRNAs found in the literature include gluconeogenesis (Goyal et al., 2017), cholesterol and bile acid homeostasis (Ananthanarayanan, 2016; Lan et al., 2016; Yu et al., 2017; Zhang et al., 2017), lipid metabolism (Li et al., 2015; Chen, 2016; Yang et al., 2016; Li et al., 2017; Zhao et al., 2017), and non-alcoholic fatty liver disease (Chen et al., 2017).

In addition to modulating intermediary metabolism in liver, lncRNAs are suggested to modulate hepatic xenobiotic biotransformation. For example, two lncRNAs that are transcribed from the anti-sense strands of the DNA encoding the hepatocyte nuclear factor 1α (HNF1α) and HNF4α are important in regulating the major drug-metabolizing cytochrome P450s (Cyps) in human liver cancer-derived HepaRG cells (Chen et al., 2018). Specifically, short hairpin RNA (shRNA) knockdown of the lncRNA gene *HNF1α* antisense 1 (*AS-1*) decreased the mRNA expression of PXR and CAR, as well as the expression of seven major P450s (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, and CYP3A4) in hepaRG cells. In addition, small interfering RNA (siRNA) knockdown of the lncRNA gene *HNF4α-AS1* increased the mRNA expression of PXR as well as the expression of six P450s (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2E1, and CYP3A4). This indicated that there is a complex

network between transcription factors and lncRNAs that regulate the expression of P450s (Chen et al., 2018).

During postnatal liver maturation, the developmental expression patterns of various lncRNAs have been unveiled at 12 developmental ages in mice (Peng et al., 2014), and the potential role of lncRNAs in regulating the ontogenic expression of the xenobiotic-metabolizing Cyps was proposed (Ingelman-Sundberg et al., 2013). LncRNAs have also been suggested to confer drug resistance through modulating the stability and translation of mRNAs that produce proteins involved in cell survival, proliferation, and drug metabolism (Pan et al., 2015). However, very little is known regarding to what extent the hepatic lncRNAs are regulated by exposure to drugs and other xenobiotics.

Regarding xenobiotic biotransformation, in liver, the nuclear receptors pregnane X receptor (PXR/Nr1i2) and constitutive androstane receptor (CAR/Nr1i3) are major xenobiotic-sensing nuclear receptors that can be activated by a wide spectrum of therapeutic drugs, environmental toxicants, dietary factors, and endogenous chemicals (Kliewer et al., 2002; Willson and Kliewer, 2002; Moore et al., 2003; Pacyniak et al., 2007). Upon activation, PXR and CAR play critical roles in xenobiotic bio-activation and detoxification (Handschin and Meyer, 2003). In addition, recent studies have unveiled novel functions of these drug receptors in various intermediary metabolism pathways, such as lipid and glucose metabolism (Poulin-Dubois and Shultz, 1990; Wada et al., 2009; Gao and Xie, 2010; Mackowiak et al., 2018; Pu et al., 2018). Our research group and others have extensively characterized the effect of

pharmacological activation of PXR and CAR on the regulation of various protein-coding genes, especially the drug-processing genes in liver (Cheng et al., 2005b; Maher et al., 2005; Kiyosawa et al., 2008; Pratt-Hyatt et al., 2013; Oshida et al., 2015; Cui and Klaassen, 2016).

There has been some investigation on effect of the CAR ligand 1, 4-bis[2-(3, 5-dichloropyridyloxy)]benzene (TCPOBOP) on the hepatic chromatin assembly and certain lncRNAs that contribute to liver tumor promotion (Lempiainen et al., 2013; Lodato et al., 2018). TCPOBOP differentially regulated 166 lncRNAs in liver that are produced from intragenic or anti-sense strand relative to PCGs that encode CAR-regulated drug-metabolizing enzymes, suggesting that an efficient co-regulatory mechanism may exist (Yu et al., 2017). In addition, two studies have investigated the effect of activating the xenobiotic-sensing transcription factor aryl hydrocarbon receptor (AhR) on the hepatic expression of lncRNAs (Recio et al., 2013; Grimaldi et al., 2018). However, there have been no systematic studies characterizing the effect of PXR activation on the regulation of liver-enriched lncRNAs and how PXR activation differs from CAR activation regarding lncRNA expression profiles.

Therefore, the purpose of the present study was to 1) determine the effect of pharmacological activation of PXR on the hepatic expression of lncRNAs; 2) to identify direct PXR-binding sites to the lncRNA gene loci and associated epigenetic signatures under basal and PXR activated conditions; 3) to compare the similarities and differences between PXR and CAR targeted lncRNAs; and 4)

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to predict the lncRNA-regulated protein-coding gene networks following PXR/CAR activation in mouse liver.

MATERIALS AND METHODS

Chemicals

Corn oil (vehicle), the mouse PXR ligand pregnenolone-16α-carbonitrile (PCN; ≥ 97% purity; CAS Number 1434-54-4), and the mouse CAR ligand 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene,3,3′,5,5′-tetrachloro-1,4-*bis*(pyridyloxy)benzene (TCPOBOP; ≥ 98% purity; CAS Number 76150-91-9) were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and procedures

As described previously (Cui and Klaassen, 2016), 12-week-old adult male C57BL/6 wild-type mice were purchased from Charles River Laboratories (Wilmington, MA) and were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility (Animal Care and Use Program number 2011-1969) at the University of Kansas Medical Center (KUMC) with *ad libitum* access to the Laboratory Rodent Chow 8604 (Harlan, Madison, WI) and drinking water. The housing conditions were temperature- and humidity-controlled with a 14-hour light and 10-hour dark cycle in a temperature- and humidity-controlled environment. Mice were acclimated for at least one week within the animal facilities prior to experiments. Mice were administered PCN (200 mg/kg, i.p.), TCPOBOP (3 mg/kg, i.p.), or vehicle (corn

oil, 5 ml/kg, i.p.) once daily for four consecutive days (n=5 per group). Twenty-four hours after the final dose, livers were collected, immediately frozen in liquid nitrogen, and stored in a -80 °C freezer. All animal experiments were approved by the IACUC at KUMC.

RNA isolation

Total RNA was isolated from mouse liver using RNA-Bee reagent (Tel-Test Inc., Friendswood, TX) per the manufacturer's protocol and quantified using a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Wilminton, DE). RNA integrity was confirmed using gel electrophoresis and a dual Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). RNA samples used for cDNA library construction and RNA sequencing had RNA integrity (RIN) values between 7.0 and 10.0. .

cDNA library construction and RNA-Seq data analysis

The cDNA library preparation from poly-A selection was performed in the KUMC Genome Sequencing Facility, as described previously (Cui and Klaassen, 2016). Samples for sequencing were randomly selected (n=3 per group) from all biological replicates (n=5) for all studies. Sequencing was performed on an Illumina HiSeq2000 sequencer using 100 bp paired-end multiplexing strategy. FASTQ files and analyzed data are available at NCBI GEO database (GSE104734), and data were re-analyzed for the present study. Briefly, FASTQ files containing paired-end sequence reads were mapped to the mouse reference genome (GRCm38/mm10) using HISAT2 (Hierarchical Indexing for Spliced

Alignment of Transcripts) (version 2.0.5). The output SAM (sequencing alignment/map) files were converted to BAM (binary alignment/map) files and sorted using SAMtools (version 1.3.1). The transcript abundance for lncRNAs and PCGs was estimated by Cufflinks (version 2.2.1) using the NONCODE 2016 lncRNA and UCSC mm10 PCG reference databases, respectively. The mRNA abundance was expressed as fragments per kilobase of exon per million reads mapped (FPKM). LncRNAs and PCGs with an average FPKM above 1 in at least one sample were considered expressed. Differential analysis was performed using Cuffdiff, and transcripts with $p < 0.05$ were considered differentially regulated by chemical exposure. Data were expressed as mean FPKM ± S.E., and asterisks (*) represent statistically significant differences between vehicle and chemical exposure. Two-way hierarchical clustering dendrograms of differentially regulated lncRNAs were generated using the native function of R.

Genomic annotation of lncRNAs and proximal lncRNA-PCG pair identification.

To annotate and visualize the genomic location of lncRNAs relative to the closest PCGs, the web-based tool peak annotation and visualization (PAVIS, https://manticore.niehs.nih.gov/pavis2/) was used to identify lncRNAs proximal to PCGs, including 5 kb upstream of the transcription start site (TSS), intronic, exonic, 5'-untranslated region (UTR), 3'-UTR, and up to 1 kb downstream of the transcriptional termination site (TTS). A lncRNA and PCG are considered paired if 1) the lncRNA overlaps with or is within 5 kb upstream of the TSS or 1 kb

downstream of TTS of any PCG and 2) both the lncRNA and the proximal PCG were differentially expressed between vehicle and chemical exposed mice (FPKM > 1 at least one sample and $p < 0.05$). Gene structure and relative genomic location of the lncRNA-PCG pairs were visualized using Integrated Genome Viewer (Broad Institute, Cambridge, MA). Pathways that are associated with PCGs paired with lncRNAs were shown using Ingenuity Pathway Analysis (IPA) software.

PXR ChIP-Seq and motif analysis for PXR-DNA binding near lncRNA gene loci.

The PXR ChIP-Seq was performed in livers of corn oil or PCN exposed adult male C57BL/6 mice as described previously (Cui et al., 2010b), and data were re-analyzed for the present study. The chromosome coordinates of positive PXR DNA binding peaks in either corn oil or PCN exposed conditions were retrieved. The chromosome coordinates for the enrichment locations were adjusted to match mm10 using Galaxy to be consistent with the reference genome used in the RNA-Seq dataset. The lncRNA genes differentially regulated by PCN were examined for positive enrichment of PXR-DNA binding peaks within 10 kb upstream of the TSS and 10 kb downstream of the TTS. The Motif analysis of PXR binding sites near lncRNA gene loci was performed using Homer (findMotifsGenome.pl). DNA sequences associated with the PXR-DNA binding sites were retrieved using the mouse mm10 genome. The size of the analyzed DNA regions was set as 200 bp, and the motif lengths for *de novo* motif search were set at 8, 10, and 12bp.

Epigenetic marks near PXR-regulated lncRNAs.

The positive enrichment of the active gene transcription mark histone H3 lysine 4 di-methylation (H3K4me2) as well as the gene silencing marks H3K27me3 and DNA methylation (5MeC) on chromosomes 5, 12, and 15 in livers of adult C57BL/6 male mice was determined by ChIP-on-chip as described before (Cui et al., 2009; Li et al., 2009; Choudhuri et al., 2010; Cui et al., 2010a). The chromosome coordinates for the epigenetic mark locations that were originally generated were lifted-over to mm10 using Galaxy to be consistent with the reference genomes used in the PXR ChIP-Seq and RNA-Seq datasets. The potential co-localizations of PXR and the three epigenetic marks within ±10 kb of the lncRNA gene loci were determined.

RESULTS

Overall comparison of PCN- and TCPOBOP-regulated liver-expressed lncRNAs

As shown in Figure 1, the majority (>120,000) of the lncRNAs were below the detection limit in mouse livers under any exposure condition, whereas approximately 4,000 lncRNAs were expressed (average FPKM > 1 in at least one group). Among these liver-expressed lncRNAs, most of them showed stable expression and were not altered following PCN (96%) or TCPOBOP (86%) exposure. PCN up-regulated approximately 2% and down-regulated 2% of the liver-expressed lncRNAs, whereas TCPOBOP up-regulated 7% and down-regulated approximately 7% of the liver-expressed lncRNAs.

As shown in Figure 2A, TCPOBOP in general had a more prominent effect than PCN in differentially regulating the liver expressed lncRNAs in that 193 lncRNAs were altered by PCN exposure as compared to 625 that were altered by TCPOBOP exposure. There were 81 lncRNAs that were commonly regulated by both PCN and TCPOBOP, suggesting that PXR and CAR have unique lncRNA gene targets in liver. A two-way hierarchical clustering dendrogram showed that biological replicates from the same exposure groups clustered together for the lncRNA gene expression (Figure 2B) and confirmed that TCPOBOP had a more profound effect in both up- and down-regulation of lncRNAs. This may be due to TCPOBOP being a highly potent activator of CAR, whereas the potency of PCN is less towards PXR activation.

Genomic annotation of PCN- and TCPOBOP-regulated liver-expressed lncRNAs relative to PCGs and predicted gene networks

As a first step to predict the function of lncRNAs with regards to influencing the transcriptional output of PCGs, the genomic locations of PCN- and TCPOBOP-regulated lncRNAs relative to the PCGs were determined using PAVIS, and the lncRNA-PCG gene pairs were defined if they met all of the following criteria: 1) the lncRNA gene overlaps with or is within 5 kb upstream of TSS or 1 kb downstream of TTS of any PCG and 2) both the lncRNA and the proximal PCG were differentially expressed by PCN or TCPOBOP exposure (average FPKM > 1 and $p < 0.05$). These criteria were set based on the assumption that although exceptions may exist, lncRNAs produced locally around the neighboring PCGs have a more spatial advantage in influencing the

transcriptional output of these PCGs as compared to lncRNAs produced in distal regions (Orom et al., 2010; Kim et al., 2012; Villegas and Zaphiropoulos, 2015; Engreitz et al., 2016; Chen et al., 2018).

As shown in Figure 3A and Supplemental Table 1, following PCN exposure, 141 (73.1%) of 193 lncRNAs differentially regulated by PCN paired with distinct PCGs. The remaining 52 (26.9%) were produced from the intergenic regions and did not pair with any PCGs. As shown in Figure 3B, among the lncRNAs that paired with PCGs, the majority of them were produced from the intronic regions of PCGs (43%), followed by the 3'-untranslated regions (3'-UTRs) (14.5%), exonic regions (6.7%), downstream of TTS (5.2%), and upstream of TSS (3.6%). There were no lncRNAs that were co-differentially regulated by PCN and were produced within the 5'-UTR of PCGs that were differentially regulated by PCN. The PCGs that paired with lncRNAs formed distinct signaling networks including lipid metabolism, molecular transport, and small molecular biochemistry centering around ERK1/2, which is a MAP kinase that catalyzes the phosphorylation of many cytoplasmic and nuclear substrates (Supplemental Figure 1), as well as cellular growth and proliferation (Supplemental Figure 2). For example, HDL as well as its regulator serum amyloid A1 (SAA1) was down-regulated, whereas AMPK, which activates glucose and fatty acid uptake and oxidation, was up-regulated.

Following TCPOBOP exposure, additional lncRNA-PCG pairs were discovered. As shown in Figure 3C and Supplemental Table 2, 359 (73%) lncRNAs were paired with PCGs, whereas 134 were not paired (27.1%). Similar

to the PCN exposure conditions, the majority of the paired lncRNAs were produced from the intronic (34%) and 3'-UTR (21.5%) of the PCGs, followed by downstream (6.4%), upstream (6.1%), and exonic (5.3%) regions, whereas the mapping to 5'-UTR was minimal (0.2%) (Figure 3D). Also similar to PCN exposure, the PCGs that paired with lncRNAs following TCPOBOP exposure were also important for lipid metabolism and molecular transport (Supplemental Figure 3). For example, Apoliprotein A4 (ApoA4), which is a lipid transporter, was up-regulated, whereas SAA1, which plays an important role in HDL metabolism and cholesterol homeostasis, was down-regulated (Supplemental Figure 3). In addition, the carcinogenesis network was enriched in TCPOBOP-regulated PCGs that paired with lncRNAs (Supplemental Figure 4).

PXR-DNA binding to PCN-regulated hepatic lncRNAs and motif analysis

As shown in Figure 4, both the total numbers of PXR-DNA binding sites and the cumulative PXR-DNA binding fold enrichment near the lncRNA gene loci (±10 kb) increased following PCN exposure. Approximately 70% of the differentially regulated lncRNAs had no PXR binding. Interestingly, among the direct PXR-targeted lncRNA genes, both the up-regulated and the down-regulated lncRNAs had increased PXR-DNA binding. This indicates that PXR has dual functions in the transcription of these lncRNAs—cis-activation and cis-suppression (Figure 4C and 4D)—likely due to a functional switch of other transcription factors that co-localize with PXR at these sites. To address this, *de novo* motif analysis was performed in lncRNA gene-associated PXR-DNA binding peaks in corn oil and PCN exposed conditions independently (Figure 4E).

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Interestingly, in control conditions, HNNRNPA2B1 and RARα were significantly enriched in PXR-DNA binding sites of the lncRNA genes, whereas Nr5a2/LRH-1 was enriched by PCN. The molecular switches of these transcription factors may alter the fate of transcriptional output of PXR-targeted lncRNA genes. Other transcription factors associated with known DNA binding motifs within the PXR-DNA binding intervals around the differentially regulated lncRNA gene loci by PCN are shown in Supplemental Figure 5A.

Determining the potential co-localization of epigenetic factors and PXR near lncRNA gene loci.

Distinct PCN exposure associated permissive and repressive epigenetic marks have previously been identified on mouse chromosomes 5, 12, and 15 [see Materials and Methods]. In a preliminary investigation into the role of epigenetic marks and PXR binding on lncRNA expression, we searched for co-localization of PXR-DNA binding, specific epigenetic marks (H3K4me2, H3K27me3, and 5MeC) near lncRNA loci on the same chromosomes. The majority of the lncRNAs associated with these epigenetic marks or PXR were not expressed or not detected on the three chromosomes, likely due to the presence of other suppressive marks that were not investigated in the present study, or were not detected due to the method of RNA selection in the cDNA library preparation procedure (i.e. only poly-A tailed lncRNAs were captured) (Supplemental Table 3). Among the liver-expressed lncRNAs on these three chromosomes, the majority of stably expressed lncRNAs had positive enrichment of the active chromatin epigenetic mark H3K4me2 and PXR. The overlap

between H3K4me2 and PXR was also the most in the stably expressed lncRNA gene category. This indicates that H3K4me2 and PXR may act in concert to maintain the constitutive expression of liver-enriched lncRNAs. Interestingly, DNA methylation (5MeC), commonly thought to silence gene expression, was another epigenetic mark that co-localized with stably expressed lncRNA gene loci and overlapped a moderately with PXR binding sites. Paradoxically, although 5MeC is considered a gene silencing mark, our observation is consistent with the more recent literature report that many actively transcribed gene bodies are marked with 5MeC. This correlates with transcriptional activity rather than repression, that the basal function of gene body methylation facilitates the establishment of their constitutive expression, and that cytosine-specific methylation may regulate gene expression (Coleman-Derr and Zilberman, 2012; Vyhlidal et al., 2016). Following PXR activation, a moderate number of PCN-regulated lncRNAs (either up- or down-regulated) had positive enrichment of H3K4me2, and most of these lncRNAs had direct PXR-DNA binding sites. In contrast, almost none of the PCN-regulated lncRNAs had positive enrichment of H3K27me3 or 5MeC, and there was also no overlap between these epigenetic marks and PXR at these lncRNA gene loci (Supplemental Table 3).

Two examples of lncRNAs that had co-localization of PXR and H3K4me2 are shown in Figure 5A (a lncRNA that paired with a PCG encoding P450 reductase [Por] that is important for cytochrome P450-mediated hepatic drug metabolism) and Figure 5B (an intergenic lncRNA) with the gene annotation

shown in Supplemental Figure 6. As shown in Figure 5A, the lncRNA NONMMUG034025.2 and the neighboring PCG Por were co-up-regulated by PCN, and this was associated with increased PXR-DNA binding fold-enrichment around the lncRNA-PGC loci. In addition, this region was marked with the active gene transcription mark H3K4me2, but not H3K27me3 or 5MeC. As shown in Figure 5B, the PCN-mediated up-regulation of the lncRNA NONMMUG014541.1 was also positively associated with increased PXR-DNA binding and positive enrichment of H3K4me2, but was independent of PCGs.

Confirmation of the literature-reported CAR-targeted lncRNAs in liver following TCPOBOP exposure and comparison with the effect of PCN exposure

Consistent with the recently published study regarding the effect of the CAR ligand TCPOBOP on the hepatic lncRNA gene expression (Lodato et al., 2018), the present study confirmed the TCPOBOP-mediated increase in 6 lncRNAs (NONMMUG002974.2, NONMMUG017205.2, NONMMUG020358.2, NONMMUG021206.2, NONMMUG026099.2, and NONMMUG036870.2) (Figure 6A), as well as the TCPOBOP-mediated decrease in 5 lncRNAs (NONMMUG005073.2, NONMMUG009893.2, NONMMUG015071.2, NONMMUG028068.2, and NONMMUG041315.2) (Figure 6B). In comparison, at the given dose, the PXR ligand PCN up-regulated 3 of the same lncRNAs (NONMMUG021206.2, NONMMUG026099.2, and NONMMUG036870.2) as TCPOBOP, albeit to a lesser extent. PCN had only minimal effects on the other TCPOBOP up-regulated lncRNAs.

DISCUSSION

The key findings of the present study are 1) at the given doses for the nuclear receptor agonists, CAR activation affects the expression of more lncRNAs than PXR activation in liver, and both commonly and uniquely regulated lncRNAs are observed for the two receptors; 2) the majority of the differentially regulated lncRNAs are present in introns, intergenic, and 3'-UTRs relative to PCGs; 3) for both receptors, the top differentially regulated PCGs paired with lncRNAs are involved in lipid metabolism and molecular transport, highlighting the potential importance of lncRNAs in fine-tuning this signaling pathway in liver; 4) increased PXR-binding by PCN exposure may lead to either trans-activation or trans-suppression of direct lncRNA targets, and motif analysis suggests that this context-specific duality may be due to a molecular switch of co-localized transcription factors; 5) the epigenetic activation mark H3K4me2 may facilitate the PXR-recruitment to the genomic regions proximal to lncRNA gene loci for activation of gene transcription.

One technical limitation of this study was the inability to detect non-polyadenylated lncRNAs, which may also be important for liver functions, due to the use of poly-A tail selection in RNA-Seq library construction. This likely explains why a previous study identified more liver-enriched lncRNAs than the present study in mice (Figure 1) (Yu et al., 2017). It has been shown that the non-polyadenylated lncRNAs are generally expressed at lower levels than the non-polyadenylated mRNAs and are prevalent in the nucleus, suggesting that they may be involved in the transcriptional regulation of target genes (Cheng et

al., 2005a; Furuno et al., 2006). At this time, the ratio between polyadenylated lncRNAs and non-polyadenylated lncRNAs in liver is not known and most of the well characterized lncRNAs are produced using the same machinery as the PCGs (i.e. transcribed by RNA Pol-II, polyadenylated, and spliced) (Kung et al., 2013). Future studies using whole transcriptome analysis could use ribosomal depletion as an alternative library construction option, although the trade-off appears to be lower signal per transcript at the same read depth.

An experimental limitation of the present study is the lack of validations of the findings using PXR and CAR knockout mice. Using these knockout mice would demonstrate the extent which the regulation of lncRNAs are dependent on PXR and CAR under basal conditions. Previously, one study examined the species differences in gene regulation using human CAR transgenic mice as well as CAR-null mice (Cheng et al., 2017). This study showed that expression of Cyp3a11 and Cyp2b10 was up-regulated by TCPOBOP only in livers of WT mice but not in CAR-null mice. In a separate study, α-tocopherol was shown to be a PXR activator using wild-type and PXR-null mice. PXR-null compared to wild-type mice did not differ greatly in the regulated gene expression of the major P450s (Johnson et al., 2013). The purpose of the current study is to investigate those lncRNAs that are regulated by the pharmacological activation of PXR or CAR (similar to a toxicological response) as a first step. In future directions it will be important to determine the necessity of the nuclear receptors in modulating the constitutive lncRNA gene expression using knockout mice.

Another interesting finding is that the majority of the differentially regulated lncRNAs are produced within the intronic region of the PCGs (43%), followed by the intergenic regions (26.9%) and 3'-UTRs (14.5%) (Fig. 3B). Because only the mature mRNAs that are poly-A tailed are enriched, the intronic transcripts should be separate lncRNA fragments instead of nascent mRNA transcripts. This suggests that the mammalian transcription machinery is highly efficient in that both lncRNAs and PCGs may share the same transcription machinery due to the relative genomic distributions. Intronic lncRNAs are known to initiate their transcription inside introns of PCGs in either direction and terminate without overlapping exons (Rinn and Chang, 2012). Little is known regarding the specific functions of intronic lncRNAs, although their potential involvement in cancer has been suggested (Tahira et al., 2011). Intergenic lncRNAs are encoded completely within intergenic regions between PCG loci and have been suggested to be more stable than intronic lncRNAs (Rinn and Chang, 2012). The differential regulation of intergenic lncRNAs by PXR and CAR ligands indicates the importance of PXR and CAR genomic binding to the intergenic regions. Indeed, we have previously demonstrated that approximately 30% of PXR-genomic DNA binding are present in the intergenic regions (Cui et al., 2010b). CAR ChIP-Seq experiments are challenging due to lack of a good antibody targeting endogenous CAR protein, although one could overcome this technical challenge using an adenovirus-based system to direct the expression of YFP-CAR fusion constructs in transgenic mice (Niu et al., 2018). However, it seems reasonable to speculate that a substantial number of differentially regulated

intergenic lncRNAs by TCPOBOP may be a result of direct CAR-binding to the intergenic regions. Last, lncRNAs produced from the 3'-UTR regions relative to PCGs may be important in protecting the mRNAs from miRNA-mediated degradation and/or inhibition of protein synthesis (Karapetyan et al., 2013; Dempsey and Cui, 2017). Although lncRNAs produced in distal regions may migrate to the 3'-UTRs of PCGs and perform similar functions, the local production of lncRNAs may have a special advantage in this process.

Regarding the potency of the two nuclear receptors, at the given dose, CAR activation by TCPOBOP differentially regulated more lncRNAs in liver than did PXR activation by PCN (Figure 1). Similarly, as we observed before, CAR activation also differentially regulated more PCGs than PXR activation (Cui and Klaassen, 2016). Therefore, it appears that TCPOBOP-mediated CAR activation is a highly potent stressor leading to an overall greater change in the mouse transcriptome. Among all of the differentially regulated PCGs that paired with lncRNAs, two pathways appeared to be affected the most by PXR and CAR activation, namely lipid metabolism (Figures 3C and 4C) and cell proliferation/cancer (Supplemental Figures 1 and 2). Both nuclear receptors and certain lncRNAs have been implicated in these two pathways independently (Mo et al., 2016; Schmitt and Chang, 2016; Kong and Guo, 2018), whereas the present study is among the first to show that the biological outcomes (changes in lipid metabolism and cell proliferation status) mediated by PXR/CAR may be co-regulated by lncRNAs. The PXR-mediated change in the lipid metabolism pathway is further supported by the motif analysis in that the PXR-targeted ChIP

DNA from PCN-treated mouse livers had enrichment in the LRH-1 DNA binding motifs (Figure 4E). LRH-1 is an important orphan nuclear receptor that regulates cholesterol, bile acid, and steroid hormone synthesis (Lee et al., 2008). The PXR activation by PCN has also been shown to be important for regulating bile acid synthesis (Staudinger et al., 2001). Under basal conditions, there was no enrichment in LRH-1 DNA binding motifs; instead, the DNA-binding motifs for hnRNP2B1, which is involved in alternative splicing of pre-mRNAs, as well as RARα, which is a receptor for retinoic acid, were enriched (Fig. 5E).

Traditionally, PXR is considered a transcriptional activator for various drug metabolizing enzymes and efflux transporters. However, recent studies using ChIP-Seq and microarrays have suggested that increased PXR binding may result in either trans-activation or trans-suppression of *bona fide* PXR-targeted protein coding genes (Cui et al., 2010). The present study adds to the existing literature showing that the PXR-targeted lncRNA genes can also be further divided into inducible vs. suppressive gene batteries upon increased PXR binding. We propose two potential mechanisms for this phenomenon: 1) the co-regulators that interact with PXR may ultimately determine the fate of PXR-target gene transcription. There is evidence in the literature showing that human PXR may interact with either a co-activator (e.g. SRC-1) or a co-repressor (e.g. SMRT or NCoR) (Navaratnarajah et al., 2012). Human PXR activity is repressed by the co-repressor silencing mediator of retinoid and thyroid hormone receptors (SMRT) (Johnson et al., 2006). 2) Another possibility is that PXR may compete with other trans-activators that are more important in the transcription of the

target genes. Previous studies showed that PXR activation inhibits cAMP responsive element binding protein (CREB) and subsequently down-regulates the expression of rate-limiting enzymes for glucose homeostasis, such as glucose-6-phosphatase catalytic subunit (G6Pase) and phosphoenolpyruvate carboxykinase 1 (PEPCK1) (Kodama et al., 2007; Oh et al., 2013). In the present study*, in silico* analysis suggests that pharmacological activation of PXR is associated with increased nuclear occupancy of LRH-1 but decreased nuclear occupancy of RARα (Fig. 4E). This molecular switch may also contribute to the transcriptional silencing of a subset of PXR-targeted lncRNA genes. Additional studies using GST pull down assays as well as overlay between PXR cistrome and ChIP-Seq data of other transcription factors will verify our hypotheses.

Regarding the human relevance, it is known that both PXR and CAR both have species differences between mice and humans, especially their contribution in regulating cell proliferation (Kong and Guo, 2018; Niu et al., 2018). However, the regulation of lncRNAs may be more conserved between the two species, because it has been shown that lncRNA promoters are more conserved than lncRNA exons and almost as conserved as those of PCGs (Carninci et al., 2005; Guttman et al., 2009). In addition, there are greater species conservations and higher frequency of proximal binding by hepatic transcription factors in the liver-enriched lncRNA gene promoters than PCG promoters (Yu et al., 2017). Therefore, it is reasonable to speculate that similar regulatory patterns of the lncRNAs may also be present in human livers following PXR and CAR activation.

The present study unveiled PCG-lncRNA pairs based on the positive associations of the neighboring PCG and lncRNAs following chemical exposure. Further mechanistic investigations are needed using lncRNA knockdown approach to validate the dependency of lncRNAs in the transcriptional/translational output of the paired PCGs. In humans, a recent study demonstrated that knocking down the neighboring lncRNAs produced from the antisense strand of the transcription factors HNF1α and HNF4α affected the expression of P450s in HepaRG cells (Chen et al., 2018). These observations have demonstrated the critical role of lncRNAs may play in modulating the PCG expression.

The rationale for the selection of H3K4me2, H3K27me3, and 5MeC over other epigenetic marks was that these marks were shown to associate with the regulation of important drug-processing genes in liver, as well as the focus of the present study on xenobiotic-sensing nuclear receptors (PXR and CAR). For example, the age-specific enrichment in H3K4me2 around the *Cyp3a* gene loci positively associates with the age-specific expression of the Cyp3a gene isoforms in mouse liver (Li et al., 2009). In addition, adult-specific enrichment of H3K4me2 positively associated with the adult-specific mRNA expression of other drug-processing genes including *glutathione S-transferase zeta 1* (*Gstz1*) (Cui et al., 2010a), *UDP glucuronosyltransferase 2 and 3* (*Ugt2* and *Ugt3*) (Choudhuri et al., 2010), as well as *Ahr* gene locus (Cui et al., 2009). Conversely, the presence of H3K27me3 was associated with the down-regulation of CYP1A2 mRNA in human embryonic stem cell-derived hepatocytes (hESC-Hep) and primary

human hepatocytes (Park et al., 2015). Regarding 5MeC, an investigation of DNA methylation in human liver samples demonstrated variable CpG hyper-methylation of the *CYP3A4* promoter region in adults, as well as in other CCAAT-enhancer-binding proteins (C/EBP) and HNF4α binding sites (Kacevska et al., 2012), indicating DNA methylation contributes to the regulation of CYP3A4 expression and the subsequent modifications in xenobiotic metabolism. It has also been suggested that methylation of gene bodies can serve as a novel therapeutic target for cancer treatment (Yang et al., 2014). Because many drug-processing genes are known PXR and CAR targets, and the present study identified that many lncRNAs are also regulated by these drug receptors, the primary goal of the present study was to determine the interactions between these liver genes and the enrichment of these three epigenetic marks related to xenobiotic biotransformation. Many other epigenetic marks have been identified in the literature (Tan et al., 2011; Rivera and Ren, 2013), and it is important to investigate the involvement of these other marks in PXR- and CAR-mediated regulation of PCGs and lncRNAs in future studies.

Previously, it was demonstrated that the majority of lncRNAs share similar epigenetic marks at the promoter regions, such as H3K4me3 and RNA Pol-II binding sites as PCGs. However, a certain fraction of lncRNAs display a high prevalence of H3K4me1, which marks the enhancer region (Kashi et al., 2016). The present study adds to the evidence showing that a subset of mouse chromosomes (5, 12, and 15), H3K4me2, which marks the enhancers and actively transcribed gene bodies, is co-localized with PXR-DNA binding near the

PCG-lncRNA gene loci, leading to active gene transcription. H3K4me2 may provide a permissive chromatin environment for PXR-binding and the subsequent gene transcription, and may serve as an important mechanism for the co-expression of the PCG-lncRNA pairs.

Taken together, the present study is among the first to systemically compare the PXR- and CAR-targeted lncRNA profiles in liver, and has provided novel insights into the molecular mechanisms underlying the lncRNA gene transcriptional regulation and potential biological outcomes in liver, and lay the foundation for further decoding the mechanism of the regulation of the gene transcription and the lncRNA-PCG networks *in vivo*.

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AUTHORSHIP CONTRIBUTIONS

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FOOTNOTE

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FIGURE LEGENDS

Figure 1. Number of lncRNAs that were not expressed (or not detected using the poly-A selection method) and expressed in liver. A lncRNA is considered to be expressed in liver if the average FPKM is above 1 in at least one exposure groups (corn oil, PCN, or TCPOBOP). Among the liver-expressed lncRNAs, the percentages of lncRNAs that were not differentially regulated, increased, or decreased by chemical exposure were calculated and displayed as two pie charts (PCN and TCPOBOP). Differential expression was considered at $p < 0.05$ (Cuffdiff).

Figure 2. A. Common and unique lncRNA targets following PCN and TCPOBOP exposure in mouse liver ($p < 0.05$ as determined by Cuffdiff). **B.** A hierarchical clustering dendrogram showing the relative expression patterns of lncRNAs in liver in corn oil, PCN, and TCPOBOP exposed conditions. Data were standardized and are expressed as z-scores.

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Figure 3. A. Number of lncRNAs that paired with PCGs or not paired with PCGs following PCN exposure. A lncRNA-PCG gene pair is defined as: 1) the lncRNA gene overlaps with or is within 5 kb upstream of transcription start sites (TSS) or 1 kb downstream of transcription termination sites (TTS) of any PCG and 2) both the lncRNA and the proximal PCG were differentially expressed by PCN exposure (average FPKM > 1 and $p < 0.05$). **B.** Genomic annotation of PCN-regulated lncRNAs ($p < 0.05$) relative to the PCGs. Data were analyzed using PAVIS. **C.** Number of lncRNAs that paired with PCGs or not paired with PCGs following TCPOBOP exposure. A lncRNA-PCG gene pair is defined as: 1) the lncRNA gene overlaps with or is within 5 kb upstream of transcription start sites (TSS) or 1 kb downstream of transcription termination sites (TTS) of any PCG and 2) both the lncRNA and the proximal PCG were differentially expressed by PCN exposure (average FPKM > 1 and $p < 0.05$). **D.** Genomic annotation of TCPOBOP-regulated lncRNAs ($p < 0.05$) relative to the PCGs. Data were analyzed using PAVIS.

Figure 4. A. Number of PXR-DNA binding sites within ±10 kb of lncRNA gene loci in corn oil and PCN exposed conditions. **B.** Cumulatively PXR-DNA binding fold enrichment within ±10 kb of lncRNA gene loci in corn oil and PCN exposed conditions. **C.** Percentages of induced lncRNA gene battery by PCN that had no PXR binding, no change in PXR binding, increase in PXR binding, and decreased PXR binding following PCN exposure. **D.** Percentages of decreased lncRNA gene battery by PCN that had no PXR binding, no change in PXR

binding, increase in PXR binding, and decreased PXR binding following PCN exposure. **E.** Homer *de novo* motif analysis of PXR-DNA binding intervals in corn oil and PCN exposed groups using findMotifsGenome.pl. Data were re-analyzed from the PXR ChIP-Seq dataset as described in MATERIALS AND METHODS.

Figure 5. A. Co-localization of PXR and H3K4me2 around the lncRNA NONMMUG034025.2 and the paired PCG Por gene loci. Integrated Genome Viewer (IGV) is a high performance visualization tool for displaying and exploring large datasets including RNA-Seq data, and can be accessed at http://software.broadinstitute.org/software/igv/. The genomic locations of lncRNA and PCG are visualized by IGV. Asterisks represent statistically significant differences as compared to corn oil control group ($p < 0.05$, Cuffdiff). FPKM: Fragments Per Kilobase of transcript per Million mapped reads, which is a unit to express RNA abundance from the RNA-Seq data.Data from RNA-Seq (corn oil and PCN exposed groups), ChIP-Seq (for PXR-DNA binding in corn oil and PCN exposed conditions), and ChIP-on-chip (for H3K4me2, H3K27me3, and 5MeC on mouse chormosomes 5, 12, and 15) were integrated as described in MATERIALS AND METHODS. **B.** Co-localization of PXR and H3K4me2 around the intergenic lncRNA NONMMUG014541.1 gene locus. The genomic location of lncRNA and PCG is visualized by IGV. Asterisks represent statistically significant differences as compared to corn oil control group ($p < 0.05$, Cuffdiff). Data from RNA-Seq (corn oil and PCN exposed groups), ChIP-Seq (for PXR-

DNA binding in corn oil and PCN exposed conditions), and ChIP-on-chip (for H3K4me2, H3K27me3, and 5MeC on mouse chormosomes 5, 12, and 15) were integrated as described in MATERIALS AND METHODS.

Figure 6. Examples of hepatic lncRNAs that were up-regulated (**A**) or down-regulated (**B**) by TCPOBOP (which are consistent with the literature report (Lodato et al., 2018). The effect of PCN on these CAR-targeted lncRNAs is also shown. Asterisks represent statistically significant differences as compared to the corn oil group ($p < 0.05$, Cufdiff). FPKM: Fragments Per Kilobase of transcript per Million mapped reads, which is a unit to express RNA abundance from the RNA-Seq data.

Figure 1

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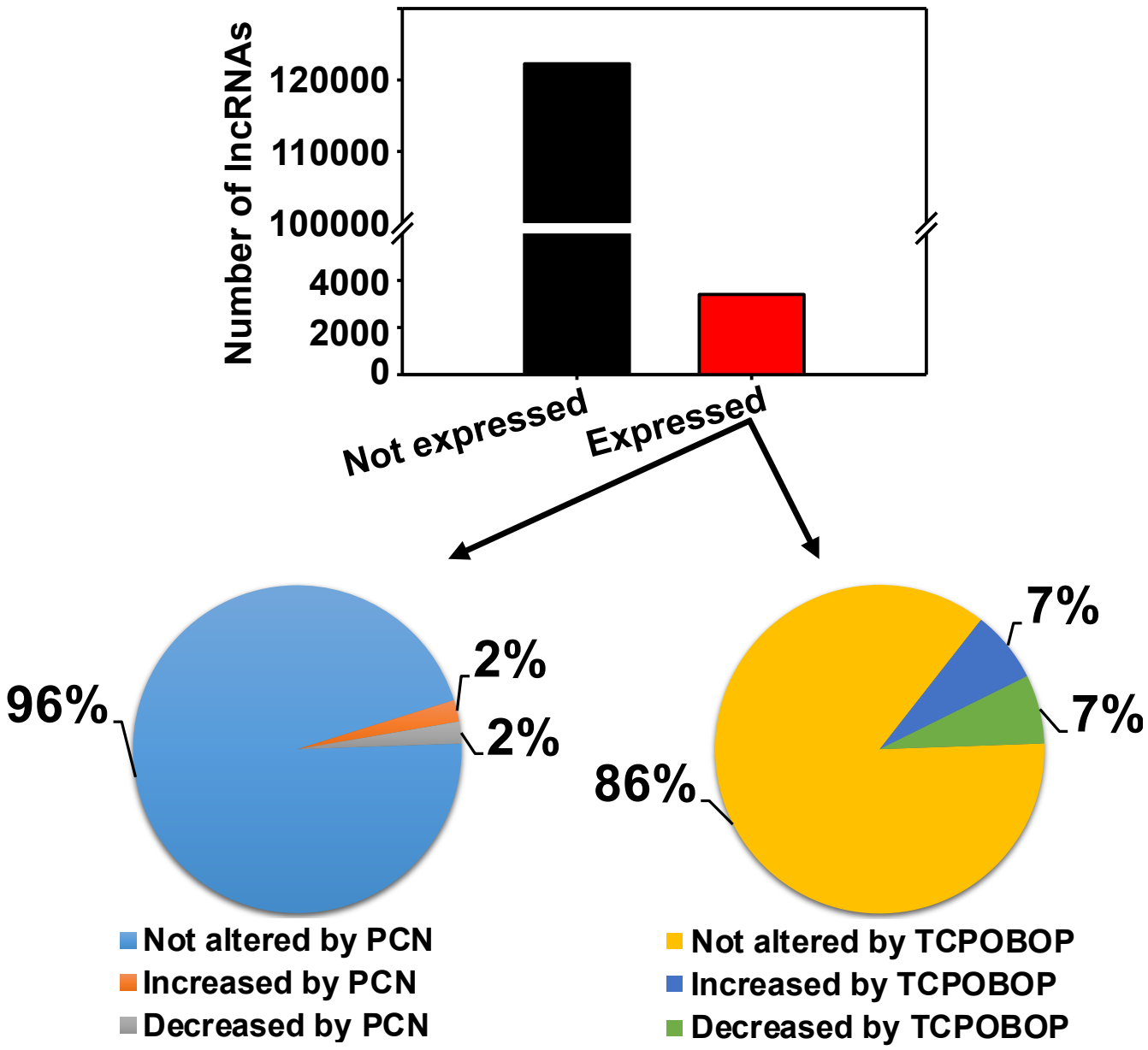

Figure 2

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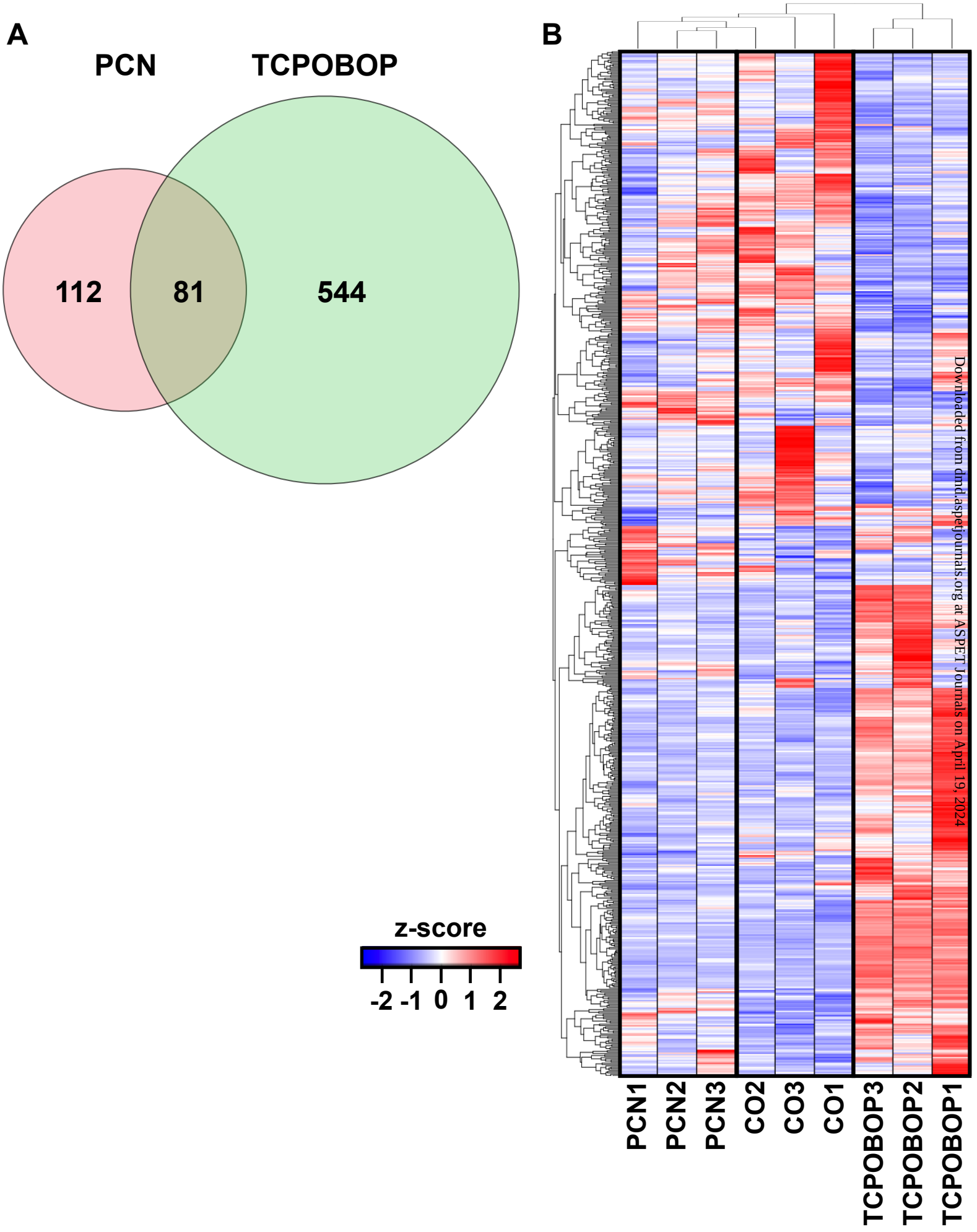

Figure 3

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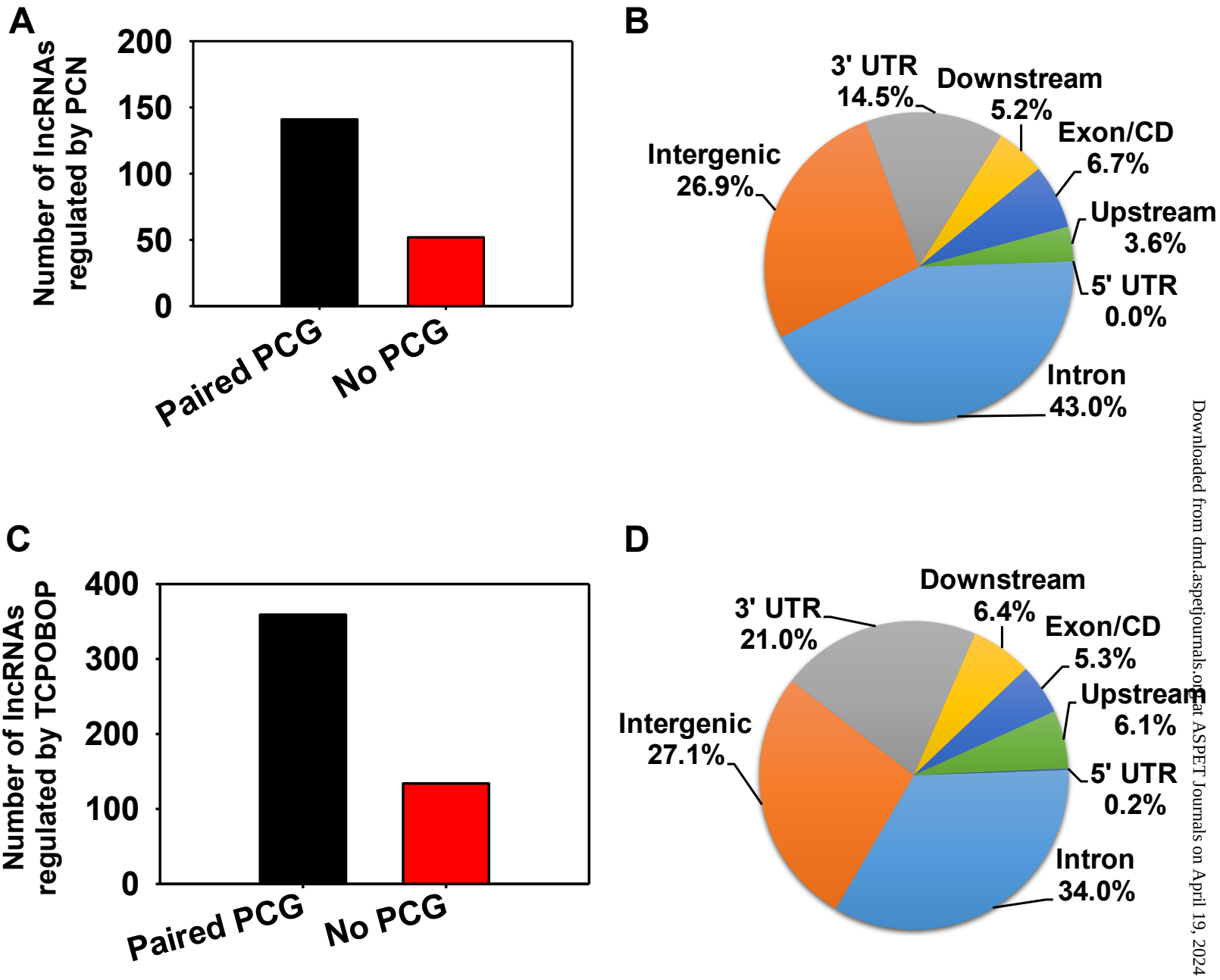

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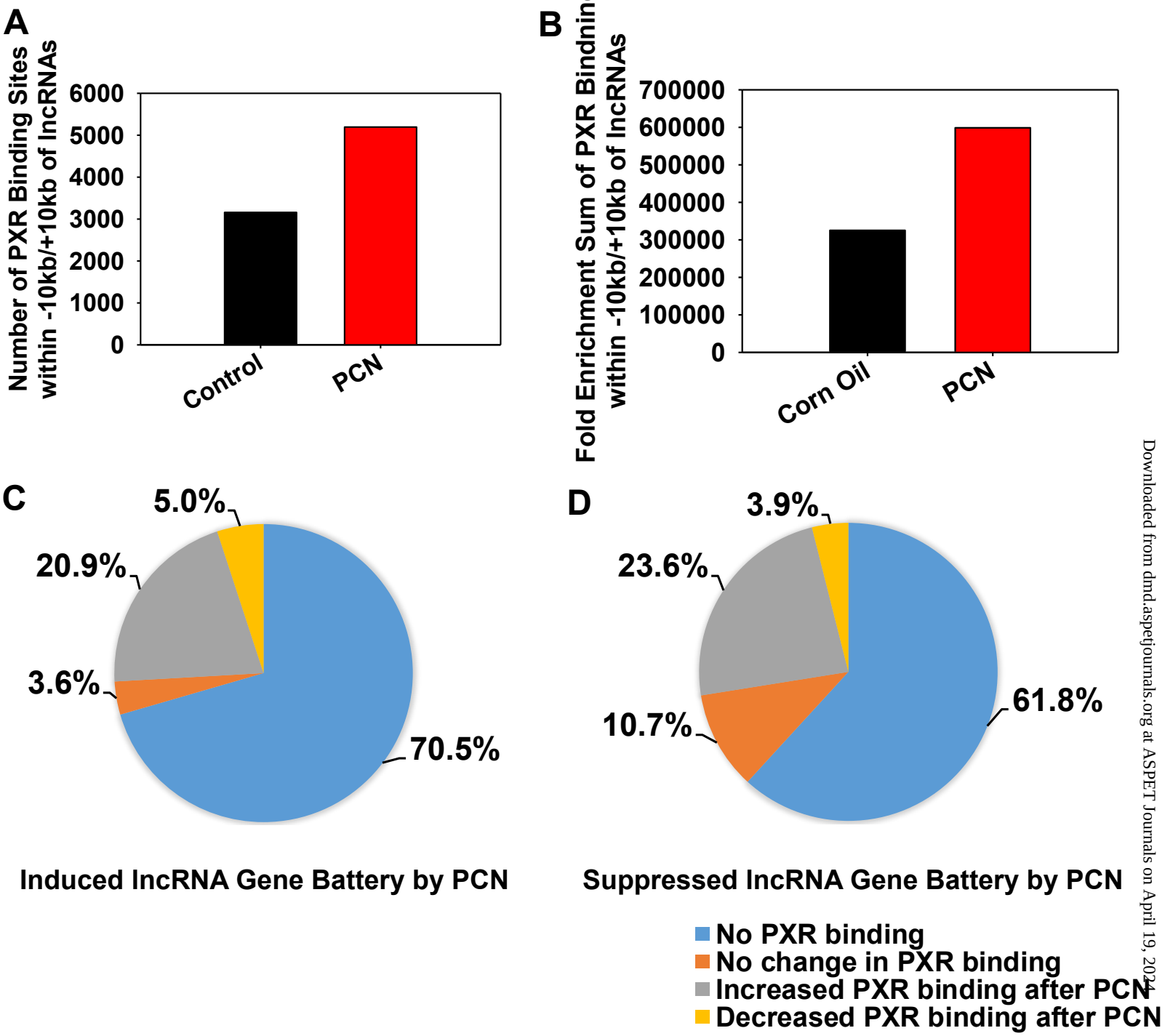
Figure 4



E. Homer *de novo* Motif Analysis

Corn Oil

Rank	Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-13	-3.001e+01	13.04%	0.23%	55.3bp (54.3bp)	HNRNPA2B1(RRM)/Homo_sapiens-RNCMPT00024-PBM/HughesRNA (0.746) More Information | Similar Motifs Found
2		1e-12	-2.801e+01	34.78%	5.97%	50.2bp (64.4bp)	RARa(NR)/K562-RARa-ChIP-Seq(Encode)/Homer(0.662) More Information | Similar Motifs Found

PCN

Rank	Motif	P-value	log P-pvalue	% of Targets	% of Background	STD(Bg STD)	Best Match/Details
1		1e-13	-3.120e+01	10.00%	0.15%	57.5bp (65.4bp)	Nr5a2/MA0505.1/Jaspar(0.811) More Information | Similar Motifs Found

Figure 5

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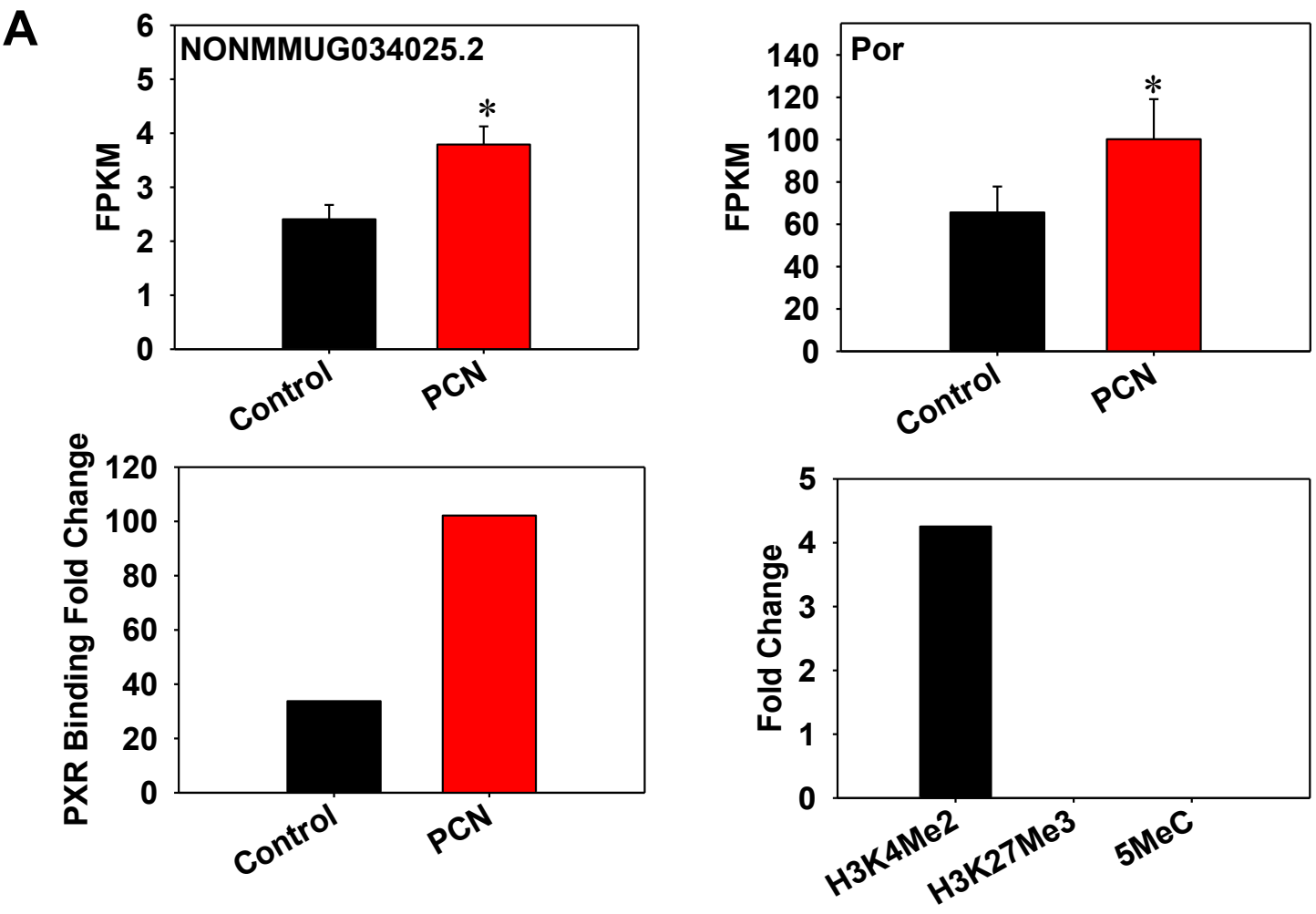

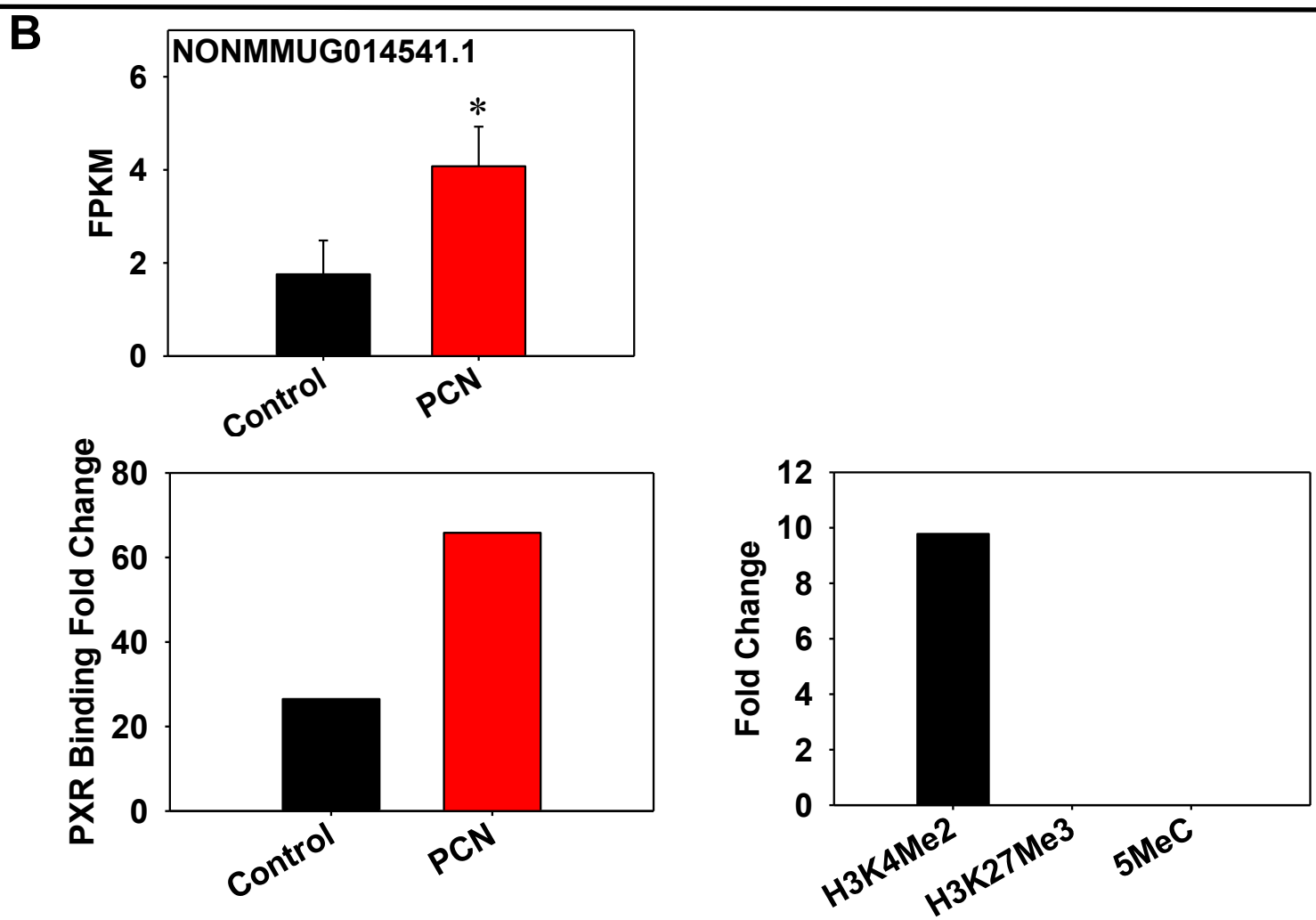

Figure 6

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