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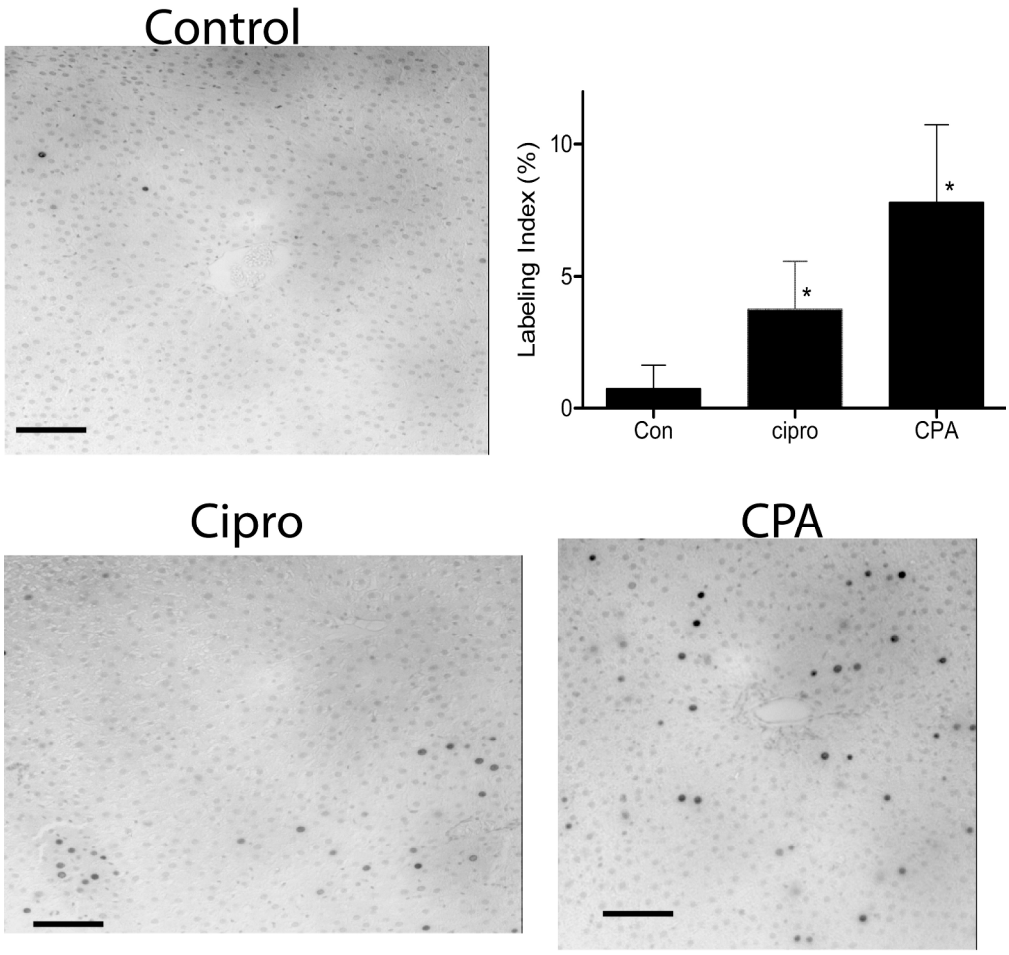

DNA synthesis in rat liver

132x140mm (600 x 600 DPI)

Genes involved in the induction of liver growth by peroxisome proliferators

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Abstract

The mechanisms regulating the induction of hepatic DNA synthesis by PPARα agonists are currently incompletely understood, and we set out to determine if there are different mechanisms of induction for PPARα agonists and other hepatic growth agents. High levels of hepatic DNA synthesis (3-7%) were induced by a PPARα agonist, ciprofibrate, and by a PXRα agonist, cyproterone acetate, and liver samples were taken for transcriptomic analysis in a contemporaneous experiment. Microarray analysis of tissue RNAs detected gene induction at 24 hours after dosing, but failed to detect any biologically plausible response at 1-5 hours after dosing. RNA sequencing of control and ciprofibrate samples at 3 hours after dosing revealed 527 perturbed genes, including known PPARα target genes. Seven candidate genes of interest in regulating cell growth and apoptosis were examined by RT-PCR, and were confirmed to be induced by ciprofibrate treatment. Cyproterone acetate, TCPOBOP and partial hepatectomy induced a distinct spectrum of gene induction to ciprofibrate, demonstrating that ciprofibrate induces DNA synthesis through a unique mechanism. These data show that RNA sequencing is a powerful tool for analysis of differentially induced genes in rat liver, and identify candidate genes that mediate the induction of DNA synthesis by PPARα agonists.

Introduction

The peroxisome proliferators were discovered as a class of structurally diverse compounds that caused liver cancer in rodents [1], and are now known to act through activation of the Peroxisome Proliferator-Activated Receptor α (PPARα) [2]. The potent carcinogenicity of these agents is exemplified by the fact that Wy-14,643 can induce 100% multifocal liver cancer in rodents after one year [3]. Ciprofibrate and methylclofenapate are potent PPARα ligands [4] that are also potent hepatocarcinogens [5, 6]. This class of carcinogen has a non-genotoxic mode of action [7, 8], and serve as an informative prototype for dissecting non-genotoxic mechanisms of chemical carcinogenesis.

The mechanism whereby PPARα ligands cause cancer remains largely unclear [7, 9, 10]. DNA synthesis plays an important role in carcinogenesis, and peroxisome proliferators induce hepatic DNA synthesis which is related to carcinogenesis [11]. It is therefore desirable to understand the mechanisms controlling the induction of DNA synthesis by ligands of the PPARα, particularly since these ligands augment normal liver size, in contrast to the regenerative growth pathways induced by partial hepatectomy [12]. The mechanisms underlying PPARα ligand-induced augmentative liver growth are poorly understood [10, 13, 14], beyond noting that DNA synthesis is dependent upon the PPARα [8].

Some previous work has examined the induction of genes by PPARα ligands, *e.g.* [15-17]. However, these studies lack the high level of induction of hepatic DNA synthesis seen *in vivo*, or are not aimed at detecting genes involved in the induction of DNA synthesis. We have previously characterised an *in vivo* system where high-level induction of DNA synthesis by PPARα ligands is observed [18], and leverage this system to identify genes that are induced as an early response to PPARα ligands in the liver response. Previous studies have shown that genes induced in an immediate/ early response to a proliferative stimulus in the liver can have a causal role in the induction of DNA synthesis [19, 20]. We characterise the induction of early genes induced in the hepatic response to PPARα ligands, and compare these results with the induction of specific genes by a distinct class of liver growth agents, ligands of PXR. The results identify candidate genes that are involved in the induction of DNA synthesis by PPARα ligands.

Materials and Methods

Materials were of the highest quality available. Ciprofibrate was a generous gift from Dr. T. J. B. Gray, Sanofi- Aventis (Alnwick, UK), Cyproterone acetate CPA was bought from Sigma-Aldrich®, amino allyl cDNA labelling kit from Ambion the RNA Company, Alexa Fluor® 555 and Alexa Fluor® 647 reactive dyes from Invitrogen, High Capacity RNA-to cDNA kit, and TaqMan® Gene Expression Master Mix were from Applied Biosystems. The oligonucleotide probes and primers were synthesized by Eurofins, MWG Operon.

Animal treatment

Male F-344/NHsd (Fisher) rats (14-15 weeks, 260±20g) were bought from Harlan Laboratories, Inc. UK. Animals were matched for sex, strain, supplier and age, and were randomized on arrival on the basis of body weight in to the appropriate number of groups. Animals were maintained under specific pathogen-free conditions, in plastic cages and were kept at 24±4^{o}C. The humidity was 70±5%, with a 12 h day/night light cycle, with food and water available ad labium throughout the experimental period. The animals were humanely killed with a single overdose of pentobarbital 200 mg ml^{-1}. Animal experiments were performed in accordance with the Animals and Scientific Procedures Act 1986. The ciprofibrate was dosed at 50mg kg^{-1} ciprofibrate by gavage, followed by sacrifice at 1, 3, 5, 16, 20, 24, or 30 hours after treatment in this study. Cyproterone acetate CPA was dosed at 100mg kg^{-1}, followed by sacrifice of animals at 24 hours. The drugs were administrated to the animals in corn oil as a vehicle by gavage. 100 mg kg^{-1} 5-bromo-2'-deoxyuridine (BrdU) was given to the animals by intra peritoneal (IP) injection, in a volume of 5 ml kg^{-1}, two hours prior to killing the animals for animals at 16 hours and later after treatment with xenobiotic. For timepoints with DNA synthesis analysis, there was an n of 6 per group, and for RNA analysis, an n of 4 per group. Analysis of DNA synthesis in hepatocytes by immunohistochemical detection of BrdU, and analysis of zonal distribution of DNA synthesis, was as described by Al-Kholaifi et al [18].

Transcriptomic analysis

RNA was isolated with Trizol reagent, and cDNA synthesis and labelling was with an amino allyl cDNA labelling kit, using Alexa Fluor® 555 and Alexa Fluor® 647 reactive dyes. Whole genome mouse Mouse Exonic Evidence Based Oligonucleotide (MEEBO) array slides

were used. The arrays were printed over 2 slides (A+B), and were accompanied with a specific GenePix Array List (GAL) file. The array slides were Gentix Aldehyde Plus arraying slides. For the microarray hybridization of the cDNA, the tRNA used was from Invitrogen and 2X-enhanced cDNA hybridization buffer from Genisphere. An Axon 4200 scanner and GenePixPro(6) program was used to scan and analyse the slides. The false discovery rate was controlled according to Benjamini and Hochberg [21]. The microarray experimental information [(in accordance with Minimum Information About a Microarray Experiment (MIAME)] was stored in ArrayTrack. R software version 2.9.0 (R foundation for statistical computing) was used to analyse the final results for the microarrays.

10µg of Total RNA was enriched for protein coding mRNA using the PolyA purist kit (Ambion, Cat. No. AM1919) for the control and ciprofibrate-treated 3 hour timepoint (Figure 2A) for four biological replicates per group. Solid whole transcriptome libraries were made according to the Solid whole transcriptome protocol (Applied Biosystems, Cat. No. 4425680). Quant-it HS dsDNA assay kit (Invitrogen, Cat. No. Q32851) was used to measure the concentration of libraries and make an equimolar pool of the libraries. ePCR containing a final concentration of 0.5pM pooled library followed by templated bead enrichment was carried out according to Solid 3 templated bead preparation guide. Enriched beads were sequenced on a SOLiD 3 ABi sequencer according to the manufacturer instructions to generate 50 bp reads in colour space. All the sequenced reads for this study has been submitted to the EBI short read archive and is available under study accession no. ERP001082. RNA sequencing was done with the AB SOLiD 3 platform, using short reads. The data from the RNA sequencing facilities (University of Nottingham Next Generation Sequencing Facility) were read-mapped with LifeScope Whole Transcriptome Pipeline. Reads were first filtered against a set of sequencing Library adaptors. Reads that passed the filter were mapped against the reference genome in the context of known gene exon coordinates. The samples had 8-16x10^6 uniquely aligned reads with Mapping Quality Score (MAPQ) above 20, and these reads were used to calculate the read count and the RPKM (Reads Per Kilobase per Million) value for each gene [22], as implemented in HTSeq (http://www-huber.embl.de/users/anders/HTSeq/doc/index.html). Gene expression was analysed using both DESeq [23] and Partek® Genomics Suite™ [Partek® software, version 6.3 Copyright © 2008 Partek Inc., St. Louis, MO, USA. http://www.partek.com/]. DESeq detected 1,302 genes with significantly differential expressions, while Partek found 527 genes, suggesting that Partek was much stricter than DESeq on

the differential expression analysis. The total 527 genes verified by Partek were all included in the results by DESeq, and the genes from Partek analysis suite were used for further analysis. GO enrichment analysis and pathway analysis has been performed by Partek using KEGG database. Interactions of induced genes with miRNA were evaluated by searching miRTarBase [24].

Quantitative RT-PCR (RT-PCR)

RNA was isolated using TRI reagent® solution from frozen cells, and converted into cDNA using a High Capacity RNA-to-cDNA kit. A no reverse-transcriptase (No RT) and a no RNA control were also run alongside each experiment to confirm there was no DNA contamination. Quantitative RT-PCR was used to measure the RNA of CYP4A1, CYP3A1 G0s2, Ccnd1, and Scd1, in a multiplex reaction containing AhR, β-actin and the gene of interest. The primer and probe nucleotide sequences can be found in RT-PCR oligonucleotides primers and probes for rat genes.. The PCR reaction contained: 20 μl TaqMan® gene expression master mix (Applied Biosystems #4369016), β-actin, AhR and the gene of interest primers (10μM) and probes (5 μM), ~150 ng of cDNA and DEPC treated water (made up to 40 μl), and was performed in an Applied Biosystems 7500fast RT-PCR machine with the following protocol - 1 cycle: 2min at 50°C; 10 min at 95°C / 40 cycles: 20 sec at 95°C; 90 sec at 58°C. RT-PCR measurement of Mycl1, Ppm1k, Tbx3 and Etv6 were measured with a master mix containing 20 ul Brilliant SYBR Green QPCR master mix, gene of interest primers (10 μM), ROX internal dye (30 nM), ~150 ng cDNA and DEPC treated water (made up to 40 μl). This was then split between two wells of a 96-well RT-PCR plate. RT-PCR was conducted using the following times and temperatures - 1 cycle: 10 min at 95°C / 40 cycles: 30 sec at 95°C; 1 min at 59°C; 1 min at 72°C. The normalisation genes, AhR and β-actin were run as a duplex using both probes, separately from the genes of interest, using the method described above. The No RT and No RNA controls were run in parallel as negative controls, with all samples done in replicate. A No template control (NTC) was included in the reaction which confirmed there was no RNA contamination. Biogazelle qBasePluse software was used to normalise all of the mRNA levels of the genes of interest from the C_t values [25]. The copy number of the samples was normalised to that of the endogenous controls AhR and β-actin.

Statistics

All data is represented as mean ± standard deviation. Statistical significance was tested by Un-paired Student's t- test to compare between two data sets with two tailed distributions and a two sample equal variance. Dunnett's Multiple Comparison Test with a one way analysis of variance (ANOVA) was used for multiple comparisons. This was done with GraphPad prism 5.0 software (Inc, SanDiego, CA).

Results

Induction of hepatic DNA synthesis

In order to identify genes that are induced as part of the immediate/ early response leading to DNA synthesis in the liver after induction by the PPARα ligand, ciprofibrate, it was necessary to investigate whether the dose of ciprofibrate induces DNA synthesis in the experimental system. Additionally, the use of a ligand for the PXR receptor was investigated to determine if there is specificity in the induction of immediate/ early genes in response to hepatomitogens. Cyproterone acetate was investigated as a PXR ligand which might induce hepatic DNA synthesis. ~15 week old Fisher 344 rats were dosed with 50 mg/kg ciprofibrate, or 100 mg/kg cyproterone acetate, and hepatic DNA synthesis was measured with an i.p. administration of BrdU at 22 hours after dosing, prior to killing at 24 hours after dosing. As shown in Figure 1A, C-E, the labelling index in hepatocytes from male rats was significantly increased after treatment with either ciprofibrate or cyproterone acetate. In agreement with previous results, ciprofibrate treatment induced DNA synthesis in the periportal space, by comparison with the centrilobular area [18] (data not shown). It is also evident that cyproterone acetate also induces DNA synthesis with a preferentially periportal distribution, as shown in Figure 1B, which is statistically significant. In contrast to ciprofibrate which shows no marked sex difference in induction of DNA synthesis in mice [18] or rats (unpublished data), cyproterone acetate shows a marked sex difference in induction of DNA synthesis, with female rats showing a labelling index in excess of 33% (Figure 1 F-I). Thus these data show that the induction regime for these two ligands induces statistically-significant increases in hepatocyte DNA synthesis at 24 hours after dosing.

Identification of transcripts induced during the induction of DNA sythesis

We sought to investigate whether there is a link between the induction of immediate or early-induced genes, and subsequent DNA synthesis in the rat liver *in vivo*. Figure 2A shows a schematic of the experimental design. Animals were dosed at zero time, with either vehicle, ciprofibrate or cyproterone acetate. At 1, 3 and 5 hours, animals were killed and organs harvested for analysis of RNA, while at subsequent time points, six animals per group were dosed with BrdU 2 hours before killing, and taking of tissue for immunohistochemistry and RNA analysis. This experimental design allows the analysis of RNA signals in an experiment

which is contemporaneous with measurements of hepatic DNA synthesis, thereby investigating the relationship between induction of DNA synthesis and immediate/ early genes. Figure 2B shows that liver to body weight ratio was not significantly affected by treatment during the course of the experiment, consistent with previous data [18]. However, both CPA and Ciprofibrate significantly induced DNA synthesis in hepatocytes at 24 hours after dosing. The control animals showed low levels of hepatocyte DNA synthesis throughout the period of examination (<0.35%), whereas the induced levels of DNA synthesis in hepatocytes at 24 hours post-dosing were statistically-significantly higher for both ciprofibrate (3%) and cyproterone acetate (7%). The time course of the induction response is a key variable, with no significant induction of DNA synthesis in hepatocytes at 16 or 20 hours after treatment, but significant induction of hepatocyte DNA synthesis at 24 and 30 hours after treatment with ciprofibrate. These results demonstrate that there is a synchronised induction of DNA synthesis which starts approximately 24 hours after dosing with either ciprofibrate or cyproterone acetate and demonstrate that the induction of hepatocyte DNA synthesis has occurred in this experiment, thereby linking the analysis of RNA in samples from earlier time-points with the subsequent DNA synthesis response in hepatocytes.

Microarray analysis

In order to identify if there were genes upregulated during an early induction response, microarray analysis was undertaken. The microarray technique was validated to have good reproducibility in analysis of replicated samples (data not shown), and analysis of the 24 hour control, ciprofibrate and CPA samples was undertaken to determine if the technique was capable of detecting changes in gene expression. Samples from n=4 control and treated rats were analysed, detecting 1597 genes that were statistically-significantly induced or repressed by xenobiotic treatment (false discovery rate of $P<0.05$). For ciprofibrate, these included known markers of treatment with PPARα agonists, including CYP4A1 (3-fold induction) and genes of the peroxisomal β-oxidation pathway (*e.g.* ketoacyl-CoA thiolase 3.5-fold induction), whereas for CPA these included detoxification enzymes GSTA1 (2-fold) and ugt1a5 (1.6-fold induction). Thus the ability of the technique to detect genes that are known to be induced by the relevant ligands provides evidence that the technique is working correctly, although it is notable that the fold-induction of these genes as detected by the microarray tech-

nique is lower than that reported by alternative methods (*e.g.* with RNAase protection assay [26]).

The microarray technique was then used to determine if it was possible to measure the induction of RNAs in samples at 1, 3 and 5 hours after treatment with ciprofibrate. In view of the large number of genes being sampled, there was a likelihood of false positive results being generated as a result of Type I errors, based on an α of 0.05. In order to minimise the number of false positive results, we established a prior hypothesis that the technique would be able to detect genes that are known to be induced by PPARα ligands at an early time point [26], and that there would be some commonality between the genes induced at 1, 3 and 5 hours after dosing. After analysis of the data, there were 473, 24 and 80 genes that were statistically-significantly induced at 1, 3 and 5 hours after dosing, respectively (Table 2). However, the known markers of induction, such as CYP4A1 and ketoacyl-CoA thiolase, were not found to be induced, and there was low concordance between the results of analysis at 1, 3 and 5 hours after dosing. For example, of the 473 genes that were found to be induced at 1 hour after dosing, only 8 and 13 were found to be significantly induced at 3 and 5 hours after dosing, and there were none in common between all three timepoints. Finally, the genes that were induced in common between two time points showed low fold-induction, and did not have a biological role consistent with induction of DNA synthesis. Similar results were obtained for CPA treated samples (data not shown). Given that the technique had failed to detect genes that are known to be induced at an early time point, and that there was no consistency amongst the genes putatively induced at 1, 3 and 5 hours after dosing, it was necessary to conclude that the technique was not capable of detecting a meaningful transcriptional signal in these early time point samples.

RNA sequencing to identify induced transcripts

'RNA sequencing' is a technique for sequencing of segments from large numbers of cDNAs, enabling a quantitative analysis of the transcriptome. RNA sequencing analysis was undertaken on the control and ciprofibrate treated RNA samples from the 3 hour timepoint, to determine if this technique could detect genes induced by ciprofibrate. The three hour timepoint was chosen since this has previously been shown to identify immediate-early genes successfully [27]. 8-16x10^6 uniquely aligned sequence reads with Mapping Quality Score (MAPQ)

above 20 were generated for the samples, mapped against the rat genome, with a correction applied for the length of transcript (kilobase of exon), and normalised per million mapped reads (RPKM). 527 genes were found to be statistically-significantly changed (increased or decreased), in ciprofibrate-treated liver RNA, as compared to control liver RNA, using the Partek analysis. These included genes that are known to be induced by PPARα ligands, such as CYP4A1 and genes in the peroxisomal β-oxidation pathway (*e.g.* acyl-CoA thioesterase, carnitine palmitoyltransferase) [26], demonstrating that the technique has the ability to detect genes that are known to be induced by ciprofibrate treatment.

Validation of identification of induced genes

However, it was important to establish if the technique can reliably detect induced genes, and to test this, a selection of candidate induced genes was analysed by quantitative RT-PCR to determine if the results of 'deep-sequencing' analysis are reproducible. The amplification efficiency of RT-PCR primer and probe sets were determined before use (~98±6%). AhR and β-actin were both used as control genes, with samples normalised according to the method of [25]. The positive control genes, CYP4A1 and Cpt1a were used as a marker for the ciprofibrate-induced response, and CYP3A1 was used as a marker for the response to CPA (Figures 3A, B and C, respectively). As expected, CYP4A1 and Cpt1a RNAs were significantly induced in ciprofibrate-treated rat liver as early as 3 hours after dosing, rising to ~10-30-fold above control levels at 24 hours, while CPA caused only minimal perturbation in CYP4A1 or Cpt1a RNA. Likewise, CYP3A1 RNA showed elevation at 5 hours (P~0.059) after CPA treatment, attaining statistical significance at 24 hours at ~30-fold above control levels, with no statistically significant change in CYP3A1 RNA after ciprofibrate treatment. Seven additional genes of interest (G0S2, Ccnd1, Scd1, Etv6, Mycl1, Ppm1k and Tbx3) were examined by quantitative PCR, in order to determine if the results of the RNA sequencing analysis were reproducible. In all seven, there was a statistically-significant elevation of transcripts in the ciprofibrate-treated samples, as compared to the control samples. Examples of response are shown in Figure 3D-H. Thus the 'RNA sequencing' methodology successfully identified novel genes that are perturbed by xenobiotic treatment.

Inspection of the profile of induced genes revealed different responses with time after treatment with ciprofibrate. CYP4A1 and Ppm1k showed more elevated levels at 24 hours,

whereas G0S2, Ccnd1, Mycl1 showed a peak at 3-5 hours after dosing, and Tbx3, Scd1 and Etv6 showed peak induction at 1 hour after dosing. These distinct induction profiles suggest that there are distinct phases to the gene induction response. The data also allow for investigating the hypothesis that the xenobiotic hepatomitogens, ciprofibrate and CPA, induce hepatic DNA synthesis through a common mechanism. Indeed, Ccnd1, and Mycl1 are induced by both CPA and ciprofibrate, consistent with a common mechanism. However G0S2, Tbx3, Etv6, Scd1 and Ppm1k show marked induction with ciprofibrate, but not with CPA. This is especially marked for the genes that have maximal induction at one hour after treatment (Etv6 and Tbx3), and suggests a different mechanism at the early stages of induction of hepatic DNA synthesis between the two xenobiotics.

Analysis of induced genes

GO enrichment analysis and pathway analysis has been performed using the KEGG database for genes found to be significantly induced. Biological processes which are significantly enriched are summarised in Figure 4A, and growth processes are one of the processes which are significantly induced. A number of genes are identified which are associated with regulation of DNA synthesis, cell death or cell cycle (Figure 4B). Several of these are candidates for further investigation to determine their role in the induction of DNA synthesis.

In order to determine if there is a common profile of genes involved in the induction of DNA synthesis, genes that were significantly affected by ciprofibrate in this study were compared against 82 genes affected by 1,4-bis[2-(3,5-dichoropyridyloxy)]benzene (TCPOBOP) and 116 genes affected by partial hepatectomy at 1 and 3 hours after treatment of CD-1 mice [27]. Four genes were affected in common between ciprofibrate and TCPOBOP, and 6 between ciprofibrate and partial hepatectomy; however, only three of these genes showed a consistent direction of effect with ciprofibrate (Tumor necrosis factor receptor superfamily member 1B (TNFRSF1B), Ribosome biogenesis protein NSA2 homolog (NSA2) and Solute Carrier Family 16, Member 6 (SLC16A6)). However, the 527 rat genes affected by ciprofibrate represent ~2% of known rat genes, and screening against 100 random genes would typically result in 2 genes in common. Hence the results are not significantly different from what would be expected by chance, and hence, in common with results for the PXR agonist cyproterone acetate (Figure 3), there is little evidence for a common series of genes induced by ciprofibrate at

three hours after treatment with the other known hepatic DNA synthesis agents, cyproterone acetate, TCPOBOP and partial hepatectomy.

In contrast with earlier reports of induction of c-myc in a mouse model [28], our data show that c-myc is not induced. Additionally, using a search for genes that are known to be regulated by miRNAs only revealed two candidates, Ccnd1 which is known to be regulated by rno-miR-322-5p, and Ogt, which is known to be regulated by rno-miR-290. This analysis does not provide direct support for a role of microRNA function in the mechanism of immediate early gene induction in ciprofibrate-mediated induction of DNA synthesis.

Discussion

A previously validated model system was used for examining the induction of immediate early genes in response to peroxisome proliferators in rat liver. In a contemporaneous experiment, animals were treated with the PPARα agonist, ciprofibrate, the PXRα agonist, cyproterone acetate, or vehicle control, and tissues harvested at various times after treatment for determination of transcriptome profile and hepatocyte DNA synthesis. This work shows that there is a high level of induction of hepatocyte DNA synthesis at 24 hours after treatment of Fisher 344 rats with both agonists, and analysis of liver samples taken at early time points after treatment reveals the identity of genes that are induced by ciprofibrate, and are linked to the in vivo induction of the DNA synthesis response in hepatocytes. The design of the experiment in looking in a system which is known to be responsive to DNA synthesis is crucial for the interpretation of the results, for example in being able to establish a link to induction of DNA synthesis in a system where this is not characterised [19, 20]. Moreover, it is also essential to be able to look at immediate early genes; it has previously been shown that there are multiple, distinct kinetic patterns in the transcriptional induction response to the model xenobiotic, 2,3,7,8-tetrachlorobenzo-*p*-dioxin [29], and this is confirmed by data in Figure 3. For example, in the time-point samples examined, the marker genes acyl-CoA oxidase and CYP4A1 rise to peak expression at 24 hours after dosing, whereas many of the immediate early genes identified (e.g. Mycl1, Tbx3) fail to show significant induction at 24 hours after dosing.

In analysing the gene induction response, the results obtained with microarray and deep-sequencing analysis was compared. The microarray analysis was able to successfully identify known agonist-induced genes at 24 hours after dosing, when there is a comparatively high level of induction of these transcripts [26]. The microarray analysis used rigorous statistical procedures to identify genes that were induced at early time points, but failed to satisfy several predetermined quality criteria for identifying biological relevance. Specifically, there was a failure to identify known induced genes (with comparatively low fold-induction [26], Figure 3A, B), and there was no overlap between the induction of genes at 1, 3 or 5 hours (Table 2). This is consistent with previous studies showing an ability to identify highly induced genes [30], but confirms a difficulty in identifying those genes with a lower fold induction [31]. By contrast, deep-sequencing identified known target genes that are induced, such as CYP4A1, and seven genes identified by deep-sequencing were confirmed as being significantly induced in an in-

dependent analysis using RT-PCR (Figure 3). These data show that the results of the deep-sequencing analysis are robust, and confirm the utility of deep-sequencing in quantitative analysis of gene expression data, even when the signal (fold-induction of induced genes) is comparatively small [22].

The deep-sequencing analysis identified 527 transcripts that were perturbed at three hours after dosing, of which several are involved in DNA synthesis or regulation of apoptosis (Figure 4B). These genes are candidates for further analysis in the DNA synthesis response to determine if they have a causal role in the induction of DNA synthesis, suppression of apoptosis and liver growth [32, 33]. Indeed, in addition to the stereotypical metabolic genes known to be affected by PPARα agonists, GO analysis showed that genes associated with induction of growth and regulation of apoptosis were significantly affected by ciprofibrate treatment (Figure 4A).

The gene induction response to xenobiotics is clearly differentiated, with ciprofibrate and cyproterone acetate inducing a distinct spectrum of genes (Figure 3D-H). This differential response is not due to a higher level of induction of hepatocyte DNA synthesis with ciprofibrate, since the cyproterone acetate induced a higher level of DNA synthesis (7% of hepatocytes) as compared to ciprofibrate (Figure 2C). However, this is consistent with previous reports of distinct responses of liver to the mitogenic effects of xenobiotics [27, 34, 35], and distinct biological responses to treatment with PPARα and PXRα agonists; for example, in the sex difference in induction of hepatocyte DNA synthesis (Figure 1D, H). Further, comparison of the genes perturbed by partial hepatectomy, or the hepatic mitogen TCPOBOP [27] with those induced by ciprofibrate, yielded only three with a common response, which is not significantly different from that expected with a random selection of genes. Thus this data suggests that ciprofibrate induces a PPARα-specific set of early genes which are involved in the induction of DNA synthesis in liver.

The PPARα-agonists clofibric acid, methylclofenapate [36] and ciprofibrate [18] (Figure 1) all induce hepatic DNA synthesis in the periportal region, whereas the induction of peroxisomal or cytochrome P450 proteins is either centrilobular or panlobular [26]. Given this different localisation of DNA synthesis, and more general markers of peroxisome proliferation, it would be important to determine if any of the candidate genes identified in this analysis as important for

induction of DNA synthesis are induced in the periportal region of the liver lobule. Intriguingly, apc and β-catenin signalling [37] have been shown to be important in the zonation of the liver, and CyclinD1 is a known target of the β-catenin pathway, and β-catenin signalling regulates cell growth in the liver [38]; it is tempting to speculate that the β-catenin pathway is involved in DNA synthesis induced by peroxisome proliferators.

Supplementary Data description (if applicable)

An excel file containing a list of genes induced during the microarray experiment is available as supplementary information (1.microarray.xlsx). The raw sequencing data have been deposited in the European Bioinformatics Institute short read archive (http://www.ebi.ac.uk/ena/data/view/ERP001082). Deep sequencing data after statistical analysis by DESeq (2.DESeq.xlsx) and Partek (3.Partek.xlsx) are provided.

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Tables

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TABLE 1: RT-PCR oligonucleotides primers and probes for rat genes.

Primers and probes are designated by letters indicating the forward primer F, the reverse primer R or the probe P. Sequences are given from 5'-->3'. The reporter dye is at the 5' end of the oligonucleotide. At0647N was used as an alternative to Cy5 where indicated. Gene names are Carnitine palmitoyltransferase 1a (Cpt1a), Cyclin D1 (Ccnd1), V-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian) (Mycl1), Protein phosphatase 1K (PP2C domain containing) (Ppm1k), T-box 3 (Tbx3), Ets variant 6 (Etv6), Cyclin D1 (Ccnd1), G0/G1 switch regulatory protein 2 (G0S2), stearoyl-CoA desaturase 1 (Scd1).

Rat gene	Oligonucleotide sequence	Genebank accession no.	Reporter dye
AhR F R P	 GCA GCT TAT TCT GGG CTA CA CAT GCC ACT TTC TCC AGT CTT A TAT CAG TTT ATC CAC GCC GCT GAC ATG	Af082124	HEX- BHQ1
β-actin F R P	 CTG ACA GGA TGC AGA AGG AG GAT AGA GCC ACC AAT CCA CA CAA GAT CAT TGC TCC TCC TGA GCG	V01217	Cy5-BHQ2
Ccnd1 F R P	 GCG TAC CCT GAC ACC AAT CT GGC TCC AGA GAC AAG AAA CG CTG GAT GCT AGA GGT CTG CGA	NM_171992	HEX-BHQ1
Cpt1a F R	 CCG GAC GAG TCC CGA TGC CT GCT CTG CCG TTG ACT TGG GGTC	NM_031559.2	N/A
CYP3A1 F R P	 AGT GGG GAT TAT GGG GAA AG CAG GTT TGC CTT TCT CTT GC TAG AGC CTT GCT GTC ACC CA	NM_013105	FAM-BHQ1

CYP4A1 F R P	 TCA TGA AGT GTG CCT TCA GC TGT GTG ATC ATG GGC AAG TT ATC CAG GCC ATT GGG AAC TT	NM_175837	FAM-BHQ1
G0s2 F R P	 GGT GTG GTG CTC GGT CTA GT ACA AAG TCG CCT CCT GTG TC CAG GCC CTG ATA GCA GAA GG	NM_001009632	At0647N-BHQ2
Scd1 F R P	 TCC TGC TCA TGT GCT TCA TC GGA TGT TCT CCC GAG ATT GA TAC TGC TGG GGC GAA ACT TT	NM_139192	HEX-BHQ1
Mycl1 F R	 GAG CCC CAG CGA TTC CGA AGG TCC AGG GGG TCT GCT CGC AC	NM_001191763.1	N/A
Ppm1k F R	 TGT CTG CTG ATG CAA GCC TCC TG TGG GGC TGT CCC AGG CTA TTC C	NM_001107863.1	N/A
Tbx3 F R	 CGT TCT GGC CTC CCA GGG TTT G TGG ACG GTA ACG CAG TGG CC	NM_181638.1	N/A
Etv6 F R	 CCA GTG GCG AGC TAC GGT CC GGC TGC AAA CGC AGG TGT GCT	NM_001037353.1	N/A

TABLE 2: Microarray analysis of 1, 3 and 5 hour time points

Animals were treated with vehicle or ciprofibrate as described for Figure 2, and tissue samples taken at 1, 3 or 5 hours. RNA was prepared, and subjected to microarray analysis as described in the materials and methods. RNAs that were induced by ciprofibrate treatment, relative to control, at a $P<0.05$, controlling for the false discovery rate [21], were identified at each time point. The number of

induced genes in common between each time point are shown below.

Timepoint	1	3	5
1	473	8	13
3		24	5
5			80

Figure Legends

FIG. 1. Ciprofibrate and cyproterone acetate induce DNA synthesis in hepatocytes.

Groups of n=6 male (A-E) or female (F-I) Fisher 344 (NHsd) rats were dosed by oral gavage with vehicle (con), 50 mg kg-1 ciprofibrate (cipro) or 100 mg kg-1 cyproterone acetate (CPA), and subsequently with an i.p. injection of BrdU at 22 hours after dosing. Rats were killed at 24 hours after dosing, and immunohistochemical analysis for detection of DNA synthesis in hepatocytes performed as described in materials and methods. Panels A and F show summary labelling indices in hepatocytes, and C-E and G-I show typical slide sections with Harris' haematoxylin counterstain. Panel B shows the hepatocyte labelling index in CPA-treated rats around the portal space (PS) or central vein (CV) [18]. Panel I shows a section of gut as a positive control for labelling. Results are shown as mean ± standard deviation, and * denotes $P<0.05$.

FIG. 2. Experimental design and induction of DNA synthesis.

Panel A shows the experimental design for examining DNA synthesis and gene induction. Animals were treated with the indicated compound, and n=4 animals per group were sacrificed at timepoints from 1-5 hours. For later timepoints, n=6 animals per group were treated 2 hours before sacrifice with an i.p. dose of BrdU, and sacrificed at the indicated time. Tissues were stored for RNA analysis from all groups, and the later timepoints were subjected to immunohistochemical analysis, as described for Figure 1. Panel B shows the liver: bodyweight ratio for all treatment groups, and Panel C shows the hepatocyte labelling index.

FIG. 3. Analysis of gene induction by RT-PCR.

RNA samples were prepared from n=4 animals per group, and analysed by RT-PCR, with geometric normalisation against Ahr and β-actin control genes, as described in the materials and methods. CYP4A1 (A), CYP3A1 (B), Carnitine palmitoyl transferase 1a (Cpt1a- C), Cyclin D1 (D), L-myc (E), T-box transcription factor 3 (F), G0/G1 switch regulatory protein 2 (G0S2- G), and mitochondrial protein phosphatase 1K (Ppm1k- H) transcript analysis was performed, and results are shown as mean ± standard deviation, and * denotes $P<0.05$.

FIG. 4. Analysis of genes induced by ciprofibrate.

Genes with statistically-significant change in expression, as identified by Partek, were used for GO enrichment analysis and pathway analysis, as performed with Partek using KEGG database. Significantly-induced processes are presented (Fig. 4A). Some induced genes with a GO function including regulation of cell death, cell growth, cell cycle regulation or differentiation, and which may be of interest in mediating the effects of ciprofibrate, are listed in Fig. 4B.

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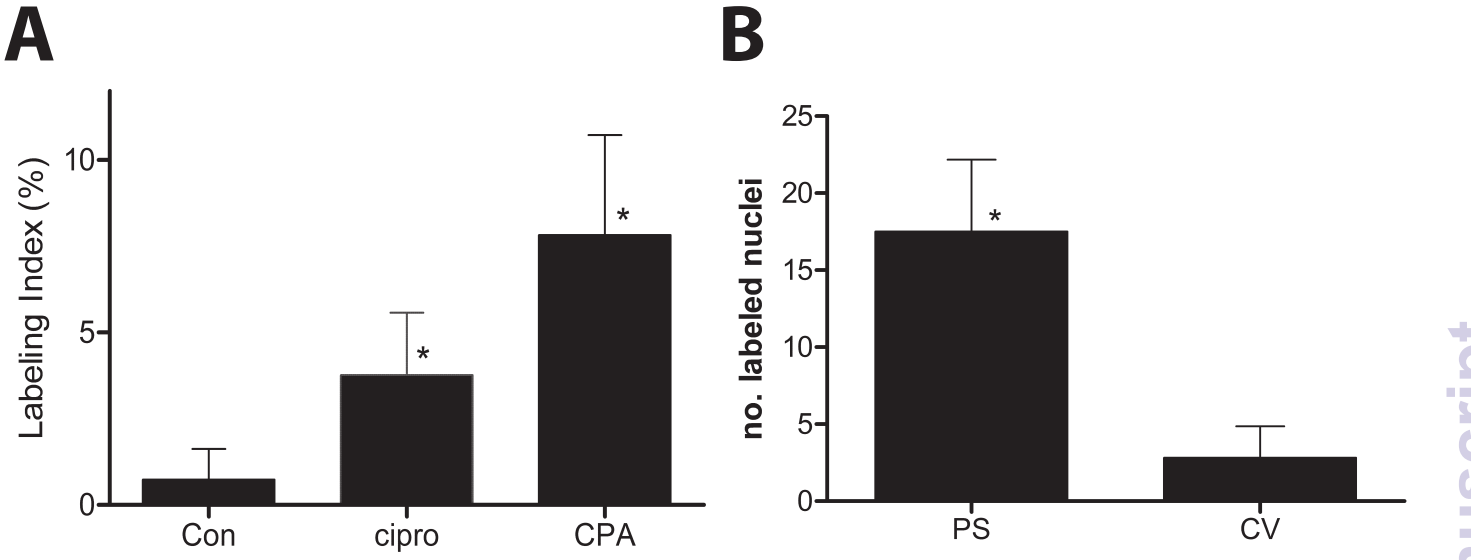
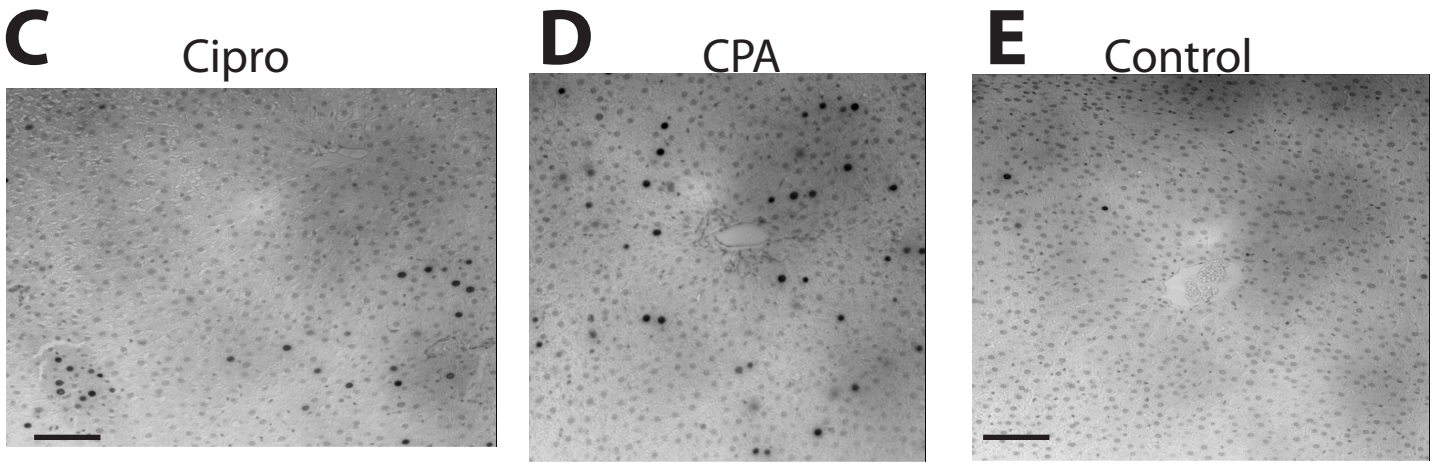
Male





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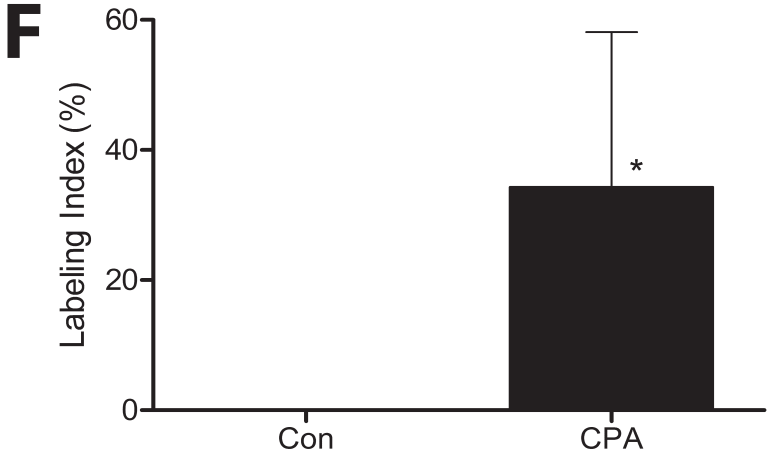

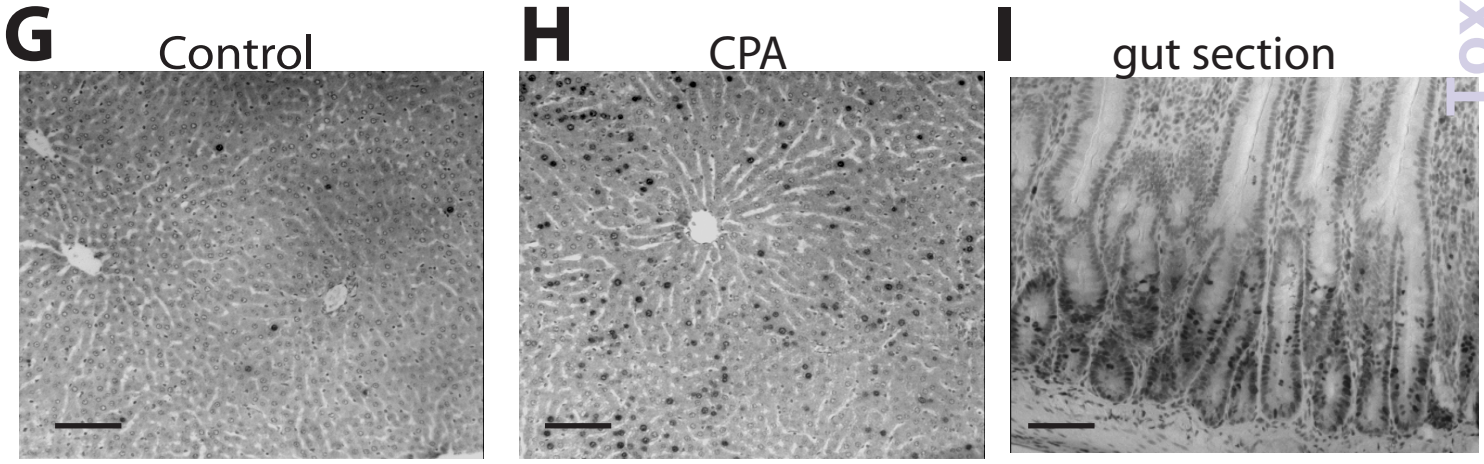

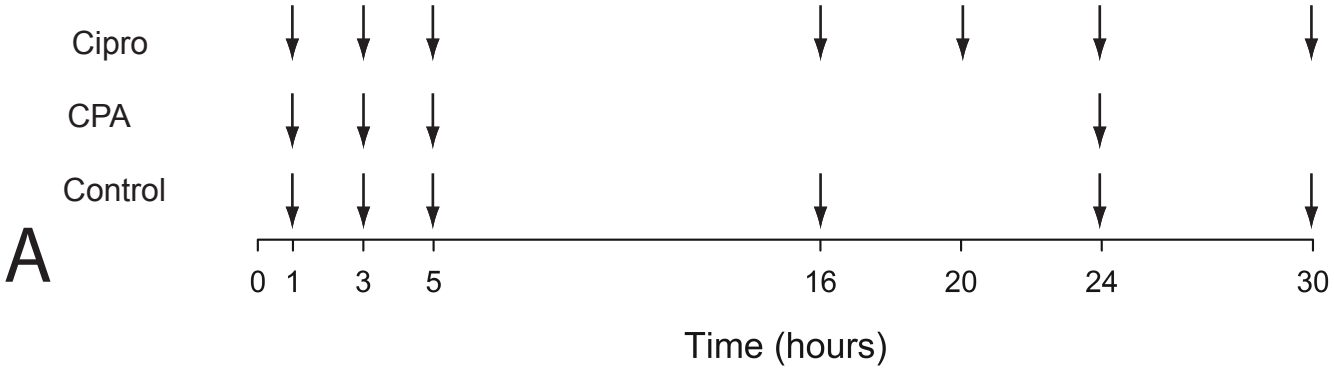

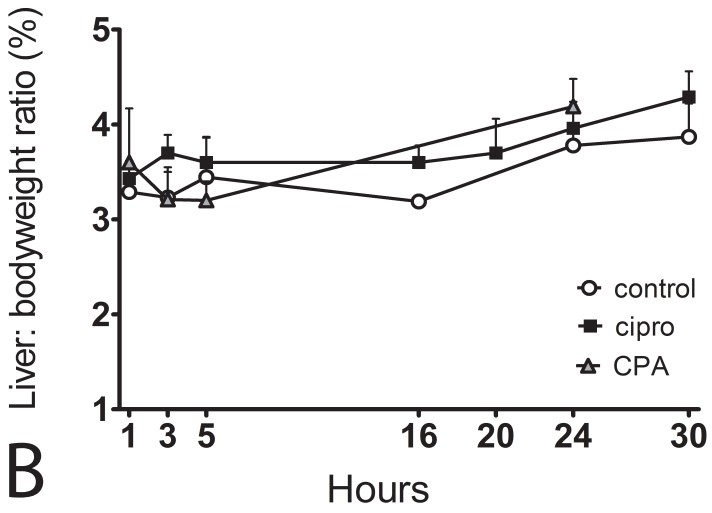

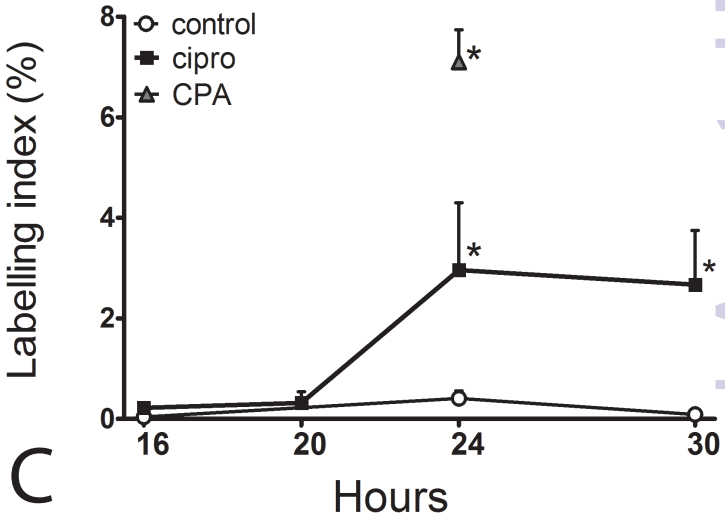

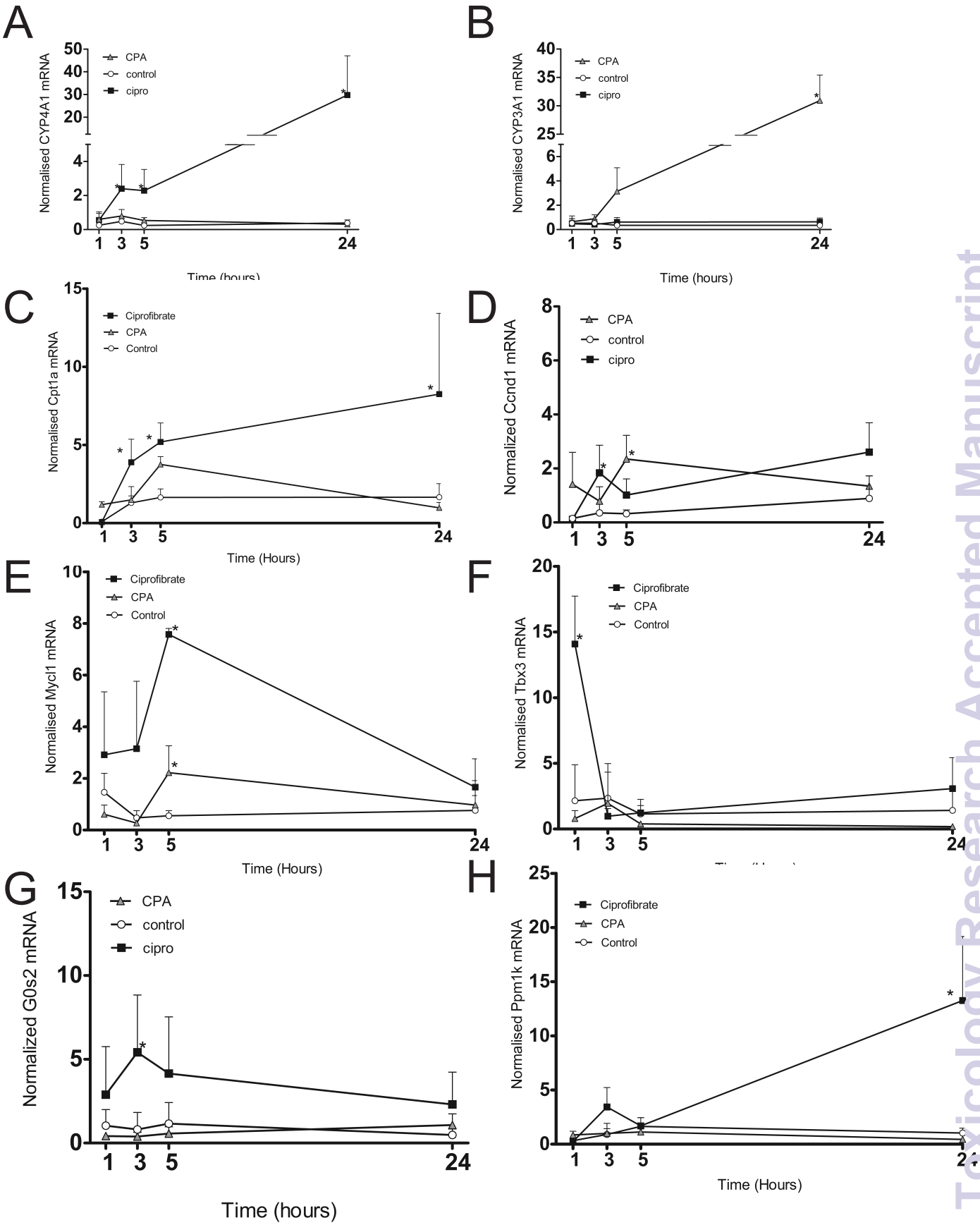

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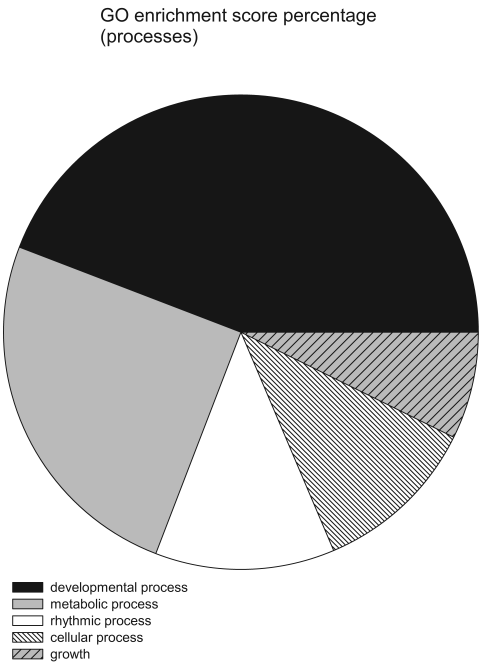

B

Genes involved in regulation of cell death
Ppargc1a
Pdk4
Gata3
Bcap31
Bbc3
Prkci
Gnb2l1
Ahr
Tnfrsf1b

Genes involved in regulation of cell growth
Frzb
Dnajb2
Gnb2l1
Notch2
Igfbp2

Cell cycle regulation/cell differentiation
Ccnd1
Chuk
Foxo4
G0S2
Myd88
Nbn
Ppm1k
Tbx3
Etv6