Time and dose-dependent effects of Phenobarbital on the rat liver miRNAome

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Abbreviations: miRNA microRNA, PB phenobarbital, CAR Constitutive Androstane Receptor, ppm parts per million, pparα peroxisome proliferator-activated receptors

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Abstract

In a previous study we had shown that treatment of male Fischer rats with exogenous chemicals for three months resulted in prominent, mode-of-action dependent effects on liver microRNA (miRNA) (Koufaris *et al*., 2012). Here we investigated how the effects of chemicals on liver miRNA in male Fischer rats relate to the length and dose of exposure to Phenobarbital (PB), a drug with multiple established hepatic effects. Importantly, although acute PB treatment (1-7 days) had significant effects on liver mRNA and the expected effects on the liver phenotype (transient hyperplasia, hepatomegaly, cytochrome P450 induction), limited effects on liver miRNA were observed. However, at 14 days of PB treatment clear dose-dependent effects on miRNA were observed. The main effect of PB treatment from days 1-90 on liver miRNA was found to be the persistent, progressive, and highly correlated induction of the miR-200a/200b/429 and miR-96/182 clusters, occurring after the termination of the xenobiotic-induced transient hyperplasia. Moreover, in agreement with their reported functions in the literature we found associations between perturbations of miR-29b and miR-200a/200b by PB with global DNA methylation and zeb1/zeb2 proteins respectively. Our data suggest that miRNA are unlikely to play an important role in the acute responses of the adult rodent liver to PB treatment. However, the miRNA responses to longer PB exposures suggest a potential role for maintaining liver homeostasis in response to sub-chronic and chronic xenobiotic-induced perturbations. Similar studies for more chemicals are needed to clarify whether the temporal and dose pattern of miRNA-toxicant interaction identified here for PB are widely applicable to other xenobiotics.

1.Introduction

Rodents are routinely used to evaluate the safety of pharmaceutical, industrial, and environmental agents. A substantial fraction of the tested chemicals target the liver due to the metabolic and detoxifying functions of this organ. Such biologically effective agents elicit hepatic responses, which maintain homeostasis (adaptive) or those that disrupt homeostasis (adverse) (Williams and Iatropoulos 2002). Hepatic responses to xenobiotic exposure include the induction of biotransformation enzymes, activation of anti-oxidative stress response, liver enlargement, increased hepatic necrosis, and altered apoptosis or proliferation (Williams and Iatropoulos 2002). However, the underlying molecular mechanisms driving hepatic responses to xenobiotics are often not well defined. An improved understanding of these molecular mechanisms would facilitate the extrapolation of exposure outcomes from rodents to humans and improve risk assessment.

It has been suggested that miRNA, small non-coding genes which regulate protein levels at the post-transcriptional stage, could contribute to the cellular responses to toxicant exposures (Taylor and Gant, 2008). Examining miRNA profiles could therefore enhance mechanistic understanding of how xenobiotics elicit their diverse effects on their target cells and tissues. Significantly, regulation by miRNA has been demonstrated to be crucial for the proper functioning of the liver, a complex organ with multiple key functions in physiology and disease. For example liver miRNAs are involved in the regulation of liver regeneration after hepatectomy (Chen *et al*., 2011; Song *et al*., 2010), in hepatocyte differentiation and development (Gailhouste *et al*., 2013), lipid and glucose metabolism (Esau at al., 2006; Trajkovski *et al*., 2013), and bile acid homeostasis (Li *et al*., 2013). Recently we reported for the first time an investigation into the effects of multiple chemicals with different modes of action on the liver miRNAome after three months of exposure (Koufaris *et al*., 2012). Importantly, all the tested chemicals affected the liver miRNAome, with the hepatic miRNA profiles being associated with their mode of action. However, reported studies so far have been limited to examining effects of a single dose of xenobiotics on the liver miRNAome at one or few time-points, which limits the ability to interpret the biological significance of observed effects. Additionally, there exists little

rationalization regarding the dosage levels and time-points to be used in studies investigating the contribution of miRNA to toxicological responses.

For this study we evaluated the expression of hepatic miRNAs of male Fischer rats dietary exposed to a control diet; a diet supplemented with a PB dose that induced centrilobular hypertrophy and weak CYP450 induction, but does not induce hepatomegaly or proliferation (50 parts per million (ppm)); or with two PB doses that cause strong CYP450 induction, hepatomegaly, and transient proliferation (500 and 1000 ppm). For each group miRNA profiles were assessed at 1, 3, 7 and 14 days. The effects of these PB treatments on liver growth, morphology, pathology, clinical parameters, metabolism, and expression of liver mRNAs in these animals were reported previously (Waterman *et al*.,2010). Importantly, by the end of the 14 days alterations on liver growth and proliferation due to PB treatment were complete (Waterman *et al*.,2010). Our analysis indicates that initially liver miRNA are relatively resistant to acute PB treatment, but more prominent, dose-dependent effects are observed following long-term treatments.

2.Materials and methods

2.1 Animal study

All animal procedures conformed to the Home Office (UK) guidelines for experimentation with animals and were approved by local ethics committee. Groups of six-week old male Fischer (F344) rats were obtained from Harlan Olac (Netherlands), randomly assigned to cages and treatment, and left to acclimatise for seven days prior to commencement of PB treatments. Three animals were examined per group. The rats were kept under controlled lighting (12h light cycles), humidity (30-70%), air flow (15 changes per hour), and temperature (22 ± 3^{o} C) conditions. Each group was exposed to different concentrations (0, 50, 500, 1000 ppm in the diet) of the sodium salt of phenobarbital which was added to the standard laboratory diet and milled to homogeneity. Rats were given access to mains water and powdered diet *ad libitum*. At the end of the study animals were killed midway through the light cycle to minimise circadian effects by an overdose of anaesthetic (halothane Ph.Eur.Vapour) followed by exsanguination after 1, 3, 7, 14 days of phenobarbital exposure. Control rats (0 ppm PB diet) were killed at each time point. Liver tissue

(left lateral lobe) was obtained from the animals immediately upon sacrifice, weighed, snap frozen and stored at -80C until needed. For some analyses, samples were also examined from rats treated in a separate study. In this additional study, groups of male Fischer (F344) rats (Harlan Olac) treated with PB (0, 50, 500 and 1000 ppm in the diet) for 28 and 90 days were used.

2.2 RNA extraction

Total RNA was extracted using Trizol (Invitrogen), following the manufacturer's instructions. RNA was used for subsequent analysis only if it had an RNA integrity number, as determined using an Agilent 2100 Bioanalyser and the RNA 6000 nano kit (Agilent), greater than eight and a 260:280 ratio greater than 2.0, as determined with a ND-1000 nanodrop spectrophotometer.

2.3 miRNA microarrays

The hepatic miRNAome was profiled at the MRC Genomics laboratory at Imperial College London using the Agilent microRNA microarray platform as previously described (Koufaris *et al*., 2012). Livers from three animals were examined per group. The hybridisation data were extracted using the Agilent feature extraction software and normalised to the 75th percentile using Genespring GX (Agilent). For subsequent analysis we retained miRNAs which were flagged as present in at least two out of the three examined animals in any group. Box plots of normalized microarray data for individual samples displayed similar distributions supporting the quality of the hybridization data. The miRNA microarray data is available at Gene Expression Omnibus (GSE48489). The miRNA profiling data from male Fischer rats treated with 1000 ppm PB for 90 days were generated previously (Koufaris *et al*., 2012) and is available at Gene Expression Omnibus (GSE48492).

2.4 Analysis of mRNA microarray data

The mRNA microarray data (GSE18753) was processed in BRB-Array tools by MAS5 summarization, averaging the replicate spots in each array and setting the threshold for each spot to 10, baseline transforming each gene to the median. Genes failing to appear in at least 25% of the samples were excluded for further analysis. Where a gene was represented by more than one probe, the average expression for the probes was used.

2.5 Hierarchical clustering analysis

Hierarchical clustering analysis for mRNAs and miRNAs was performed in Cluster 3.0 using genes that had passed our filtering criteria. The probes were median centered and normalized and the tree was constructed using correlation centered as the similarity metric and average linkage.

2.6 Polymerase chain reaction (PCR)

For quantitative reverse-transcription real time PCR (qRT-PCR), total RNA was reverse transcribed using the miRNA reverse transcription kit (Agilent) and then amplified using the Taqman 2X Universal PCR master mix, No AmpErase UNG (Applied Biosystems), with each PCR reaction performed in triplicate. Mature miRNA Taqman assays were purchased from Applied Biosystems. The qRT-PCR data were analysed in the ABI 7500 Sequence Detection System (Applied Biosystems) using the comparative Ct Method (ΔΔCT Method) to quantify miRNA expression using snoRNA as the endogenous control. For semi-quantitative RT-PCR total RNA was reverse transcribed using MMLV reverse transcriptase (Promega). The cDNA was then amplified using Tfi polymerase (Invitrogen). The primers used were: e-cadherin For ACAGCAAGCATGCCAGTGAA; e-cadherin Rev GCACCAACACACCCAGCATA; cyp2b1 For GGA GAG CGC TTT GAC TAC; cyp2b1 Rev CTC GTG GAT AAC TGC ATC; gapdh For CAT GGA CTG TGG TCA TGAG; gapdh Rev TTC AAC GGC ACA GTC AAGG

2.7 Immunoblotting

Livers were homogenised with a polytron machine (Labortechnik) in 500 µl of lysis buffer (50 mM Tris–HCl, pH 7.4; 1% IGEPAL (Sigma-Aldrich); 0.25% sodium deoxycholate (Sigma-Aldrich); 150 mM NaCl (Sigma-Aldrich); 1 mM EDTA (SIGMA-Aldrich); 100 X Halt Protease inhibitor (Pierce)). Homogenisation was followed by sonication and incubation at 4 °C for 30 min. The solutions were then centrifuged for 20 minutes to remove insoluble debris. The protein extracts were quantified using the BCA (bicinchoninic acid) protein assay (Pierce). Primary antibodies were purchased from Santa Cruz (Heidelberg) (zeb1 1:100, zeb2 1:100) or Sigma-Aldrich (Beta-actin; 1:10000).

2.8 Global DNA methylation analysis

DNA was extracted from liver samples using the DNeasy Blood & Tissue kit (Qiagen) following the manufacturer's protocol, and using the reagents and buffers included in the kit. Extracted DNA was quantified using the Nanodrop ND-1000 and used for subsequent experiments if its 260:280 ratio was higher than 1.8. The levels of global DNA methylation for extracted DNA were determined using the Methylamp Global DNA Methylation Quantification Ultra Kit (Epigentek) following the manufacturer's instructions. This kit is based on the recognition of the methylated fraction of DNA by a labelled 5-methylcytosine which is subsequently quantified through an ELISA-like reaction.

2.9 Statistical analysis

Differentially expressed mRNAs and miRNAs were identified by one-way fixed-model ANOVA with False Discovery Rate (FDR), the expected percentage of false positives within a given set of predicted differentially regulated genes, set to <0.1 in BRB-Array tools. The identified genes were then examined to determine whether they are over-represented for gene sets defined to be members of common biological pathways in the KEGG database using the ConsensuspathDB tool (Kamburov *et al*., 2009). Similarly, enriched KEGG pathways for predicted and known targets of miRNAs were identified using miRSystem (Lu *et al*., 2012) and setting the threshold for a true target as being predicted by at least three software packages or being a verified target, an observed/expressed ratio above or equal to 2, and using pathways containing 25-500 genes. The student's T-test or Analysis of variance (ANOVA) was used to test for significance of all other analysis.

3.Results

3.1 Hierarchical clustering analysis of liver mRNA and miRNA profiles

As a first step towards evaluating the temporal effects of PB treatment we performed hierarchical clustering analysis on the liver miRNA and mRNA profiles, normalized to their respective time-point controls (**Fig.1A-B;** heatmap for miRNA clustering can be seen at **Suppl.Fig.1**). This analysis revealed a highly similar pattern of PB-induced perturbations on the liver miRNA and mRNA profiles, with a clear demarcation between acute (days 1-3) and sub-chronic (days 7-14 treatment). Importantly, the top

pathway enriched for mRNA differentially expressed between livers exposed to acute (1-3 days) and sub-chronic (7-14 days) PB treatments were the cell cycle and DNA replication ($p<0.01$) (**Fig.1C**). Consequently, the dichotomy in mRNA and miRNA profiles between 1-3 days and 7-14 days of PB exposure relate to the liver switching from a proliferative to a non-proliferative state.

Moreover, hierarchical clustering analysis on individual days revealed a clear separation of the effect of PB doses on the liver miRNAome on the 14th day of treatment (**Fig.1D**), but was essentially random on the previous time-points (**Suppl.Fig.2**), indicating that the distinct effects of PB treatments on the liver miRNAome become more prominent with longer exposures. In contrast, clustering analysis based on hepatic mRNA profiles could discriminate the different PB doses already from day 3 of PB treatment (**Fig.1E**). Therefore hierarchical clustering analysis supports a time-and-dose dependent pattern of effects on the liver miRNAome following PB treatment. Similarly, dosing with the drug induced time and dose-dependent effects on liver mRNAs, although prominent dose effects appeared to occur earlier for liver mRNAs compared to miRNAs.

3.2 Temporal analysis of mRNA expression compared to miRNA expression

We next aimed to identify significantly differentially expressed miRNA at each of the four time-points of the study. Our expectations were that we would detect both transient and persistent effects of PB on liver miRNA through the 14 days of the study. Surprisingly, using one-way ANOVA with FDR set at <10% no miRNAs were significant at days 1, 3, and 7, while there were 11 significant miRNAs at day 14. This was in contrast to the effects of the drug on liver mRNAs, were with the same analysis significantly deregulated genes could be identified from day 1 onwards (day 1 34 probes; day 3 156 probes; day 7 45 probes; day 14 195 probes). The relative expression of the significantly differentially expressed miRNAs at day 14 in the livers of animals treated with each PB dose can be seen in **Table 1**.

In a previous study we had generated miRNA data from animals treated with 1000 ppm PB for a period of 90 days (Koufaris et al., 2012). We therefore examined that dataset to determine whether the miRNA that we identified to be significantly differentially expressed at 14 days were also affected at that later time-point. In fact,

the majority of miRNAs identified as significantly deregulated at 14 days displayed normal expression at 90 days of PB treatment (**Table 2**), indicating that their deregulation is a transient event. Intriguingly, for miR-200a, miR-200b, and miR-96 we identified a progressive increase in expression from day 7 to day 90 of PB treatment (**Table 2**). Additionally, these miRNAs display a dose-dependent response to PB-treatment (**Fig.2A**) and a highly correlated induction in response to PB treatment from day 7 to three months of PB treatment (**Fig.2B**). Evaluation of hepatic miR-200b levels by qRT-PCR following PB treatment displayed highly similar levels to those determined by the miRNA microarray data, thus confirming the validity of the observed effects of PB treatment (**Fig.2C**). The miRNAs miR-200a, miR-200b, and miR-429 are arranged in a chromosomal cluster with the mature miRNAs being processed from a single pri-miRNA regulated by a common promoter (Bracken *et al*.,2008). Consequently the expressions of miRNA members of this chromosomal clusters miRNAs are highly correlated to each other. A similar arrangement into a common transcriptional unit also exists for miR-96 and miR-182 (Xu *et al*.,2007). In this microarray dataset we did not detect miR-182 or miR-429, due to their lower expression and technical variability, although they are expected to be highly correlated with their co-clustered miRNAs. To prove the point we demonstrated by qRT-PCR the same dose-and-time dependent effects of PB treatment on miR-182 (**Fig.2D**). It should be noted that the analysis of miR-182 in the samples at the 28 and 90 day time points were conducted on livers obtained from a different animal study to those used for the 1-14 day time points (**Fig 2D**). Nevertheless the trend for an increase in the expression of miR-182 with increasing time of exposure to PB and the dose-dependency of the effect is maintained. The highly correlated induction of the miR-200a/200b/429 and miR-96/182 miRNA clusters suggests that these miRNAs may have synergistic activities in regulating key signalling pathways and, subsequently, hepatic phenotypes. To investigate this possibility we used miRSystem, a tool that combines prediction from seven different target-prediction algorithms and two validated target databases to identify enriched functions and pathways for individual or group of miRNAs (Lu *et al*., 2012). Intriguingly, this analysis for the five PB-induced members of the two miRNA clusters identifies the mapk and the erbb signalling pathways, known drivers of cell proliferation, among the top KEGG pathways jointly regulated by these selected miRNAs (**Suppl. Table 1**).

3.3 Effect of PB on miRNA associated with hepatic proliferation

The two highest PB doses (500 and 1000ppm) caused a transient increase in hepatic proliferation that peaked on the third day of treatment (39% and 61% increase in Ki67 labelling index) (Waterman *et al*.,2010). However, our previous analysis did not identify any differentially regulated miRNA at the early time-points (days 1-3). We then decided to inspect the effect of the proliferation-inducing PB doses on selected miRNA that had been previously associated with hepatic proliferation following peroxisome proliferator-activated receptors (pparα) activation or liver regeneration after hepatectomy. However, none of these miRNA were affected in our dataset by the proliferation-inducing PB treatments (**Suppl**.**Table 2**), indicating that they are not involved in this phenotype.

3.4 Association between miR-29b with global hepatic DNA methylation and miR-200a/200b with zeb1/zeb2 transcription factors

In the microarray dataset hepatic miR-29b was transiently repressed by PB treatment at 14 days of treatment (**Fig 3A**). Importantly, miR-29b has been reported to be master regulator of global DNA methylation (Fabbri *et al*., 2007; Garzon *et al*., 2009). Interestingly, PB treatment is known to affect DNA methylation patterns in the liver (Counts *et al*.,1996), leading us to enquire whether there existed an association between miR-29b and global DNA methylation. Indeed, in agreement with the reported activity of miR-29b to antagonise global DNA methylation the transient repression of this miRNA at day 14 of PB treatment was associated with a transient increase in global DNA methylation (**Fig. 3B**).

Epithelial to mesenchymal transition (EMT) is a developmental program that facilitates the acquisition of invasive and metastatic capabilities by cells. It has been reported that genes involved in EMT are affected by PB treatment (Phillips et al.,2009). Interestingly, members of the miR-200 family that are upregulated by PB treatment are known to antagonize EMT by repressing the zeb1 and zeb2 transcription factors (Bracken *et al*., 2008). It is therefore possible that the observed upregulation of miR-200a/200b/429 could be involved in the regulation of EMT. Indeed, we found a dose-dependent decrease in the levels of zeb1 and zeb2 proteins at day 14 of PB

treatment (**Fig. 4A-B**), consistent with an inverse association to the dose-dependent induction of the miR-200 family at that timepoint (**Fig. 2A**). However there was no associated change in the expression levels of hepatic *e-cadherin*, a key epithelial marker that is downstream of zeb1 and zeb2 (**Fig. 4C-D**).

4. Discussion

To the best of our knowledge this is the first study to investigate both the temporal and dose effects of xenobiotic treatments on the liver miRNAome. Significantly, in agreement with other studies examining the effects of acute dioxin, benzo(a)pyrene, and N-ethyl-N-nitrosourea exposures (Moffat *et al*., 2007; Li *et al*., 2010; Yauk *et al*., 2010) liver miRNA were initially relatively unresponsive to chemical treatment compared to liver mRNA (**Fig.1**). This observation suggests that liver miRNA networks are under particularly stringent regulation, perhaps due to their important master-regulatory functions. However, after sub-chronic (>one week) PB-treatment, liver miRNA deregulation clearly becomes more prominent and dose-dependent (**Fig. 1-2; Table 1-2**). Interestingly, the initiation of more prominent changes in hepatic miRNA coincides temporally with the achievement of equilibrium (termination of the proliferation and achievement of maximum hepatomegaly) in the liver of animals being continuously exposed to PB. This observation raises the interesting possibility that the predominant role of miRNA in response to xenobiotics in the adult liver may be to direct homeostatic adaptation to sub-chronic and chronic exposures. Consequently, our PB data suggest that studies interested in investigating the role of miRNA in liver response to xenobiotics should focus more on the effects of high dose/long term exposures. However, more studies are needed to determine whether the time and dose dynamics of PB-miRNA interaction identified here reflect the majority of xenobiotic agents targeting the liver.

We had previously identified the co-ordinated increased expression of the hepatic miR-200a/200b/429 and miR-96/182 miRNA clusters specifically in the livers of animals treated for 90 days with PB, di(2-ethylhexyl)phthalate, and benzophenone (Koufaris *et al*.,2012). That observation led us to hypothesize that the co-ordinated upregulation of these miRNA clusters could be characteristic of nuclear-receptor agonists. Another commonality of these chemicals is that they induce a transient

hepatic proliferative response. Interestingly, from this study that this miRNA response (i) is temporally associated with the termination of the PB-induced proliferation (initiated after 7-14 days) (ii) is persistant and progressive (iii) occurs only in response to proliferation-inducing 500 and 1000 ppm PB doses (**Fig.2**). The co-ordinated upregulation of this miRNA clusters could therefore be a molecular signature of the liver adapting to xenobiotic-induced transient hyperplasia. However, it is unclear whether this miRNA response is causally involved in this hepatic response. We have also associated PB-induced deregulation of miR-29b and miR-200a/200b/429 with altered global DNA methylation and repression of the zeb1/zeb2 transcription factors (**Fig.3-4**). The functional importance of these miRNA deregulations is not clear at present. However, it is important to note that both DNA methylation and EMT have been associated with PB-induced carcinogenicity. Global hypomethylation is more pronounced in PB-treated livers of mice strains susceptible to PB-treated carcinogenesis (Counts *et al*., 1996). Similarly, EMT has been reported to be strongly activated in mice susceptible to PB-induced hepatocarcinogenicity (Phillips *et al*.,2009). It should be noted that PB is an established non-genotoxic hepatocarcinogen in some mice strains, but not in rats (Whysner *et al*.,1996). Recently we have argued that altered miRNA regulation could be involved in differential species responses to equivalent toxic exposures (Koufaris and Gooderham, 2013). Interestingly, a recent study of the effects of a carcinogenic dose of PB on mice liver miRNAome over similar time-points as this study (1-90 days) did not identify the miR-200a/200b/429 cluster, miR-96/182 cluster or miR-29b to be among the affected miRNA (Lempiäinen et al.,2013). The repression of miR-29b in PB-treated rats, and the resultant effects on global methylation may act to protect the animal from excessive genomic demethylation. The induction of miR-200b in the liver of PB-treated animals was inversely associated with the expression of zeb1/zeb2 transcription factors. Hence, one consequence of the upregulation of miR-200a/200b (and the subsequent repression of zeb1/zeb2) may be to contribute to the protection of the epithelial nature of hepatocytes exposed to biologically effective levels of xenobiotics. However, it is not clear if that is the main effect of miR-200 in PB treated livers, as we did not observe here any associated changes in the expression of the epithelial gene *e-cadherin*, It will be an interesting avenue for future work to investigate whether miRNA are involved in the different susceptibilities of mice and rats to PB-induced carcinogenicity.

A better comprehension of the molecular mechanisms driving liver responses to exogenous agents will be highly important for predicting the toxic side-effects of xenobiotic exposures, for extrapolating the effects of the exposures across species, and for enhancing understanding of fundamental liver biology. In this study we have identified a complex pattern of PB-treatment related effects on the liver miRNAome. Given the key regulatory role of miRNAs, alterations in the expression of these genes could be an important molecular mechanism by which the liver initiates and sustains responses to the drug. More generally, we have now performed two studies, here and Koufaris *et al*., (2012), which enhance our understanding of the effects of xenobiotic treatments on liver miRNAs. In unison our data demonstrate that hepatic miRNA expression changes are affected by xenobiotics; are dependent on the dose; are temporally regulated; indicate the mode of action of chemicals; and have important phenotypic effects. These fundamentally important observations support the need for further investigation into the regulatory functions of hepatic miRNAs that will enhance our understanding of the adaptive and adverse hepatic responses to xenobiotic exposures.

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Figure legends

Figure 1 Hierarchical clustering analysis of hepatic miRNA and mRNA profiles (**A**) Hierarchical clustering analysis of animal treatment groups based on expression of hepatic miRNAs. For each treatment group the fold change of each miRNA from the day control were calculated; (**B**) Hierarchical clustering analysis of animal treatment groups based on expression of hepatic mRNAs. For each treatment group the fold change of each miRNA from the day control were calculated; (**C**) Enriched KEGG pathways ($p<0.05$) for mRNA genes differentially expressed between acute (days 1-3) and sub-chronic (days 7-14) treatments; (**D**) Clustering analysis of samples after 14 days of treatment using hepatic miRNA profiles; (**E**) Clustering analysis of samples after 3 days of treatment using hepatic mRNA profiles.

Figure 2 Dose-dependent and correlated induction of hepatic miR-200b and miR-96 clusters following PB treatment. (**A**) Expression of miR-200b and miR-96 in animals treated with different PB-doses at day 14; (**B**) Correlation of miR-200b and miR-96 in PB-treated samples from day 3 onwards, Pearson's correlation is shown; (**C**) Correlation between miR-200b expressions in PB-treated samples, calculated by microarrays and qRT-PCR (qRT-PCR was not performed for every dose at every timepoint), Pearson's correlation is shown; (**D**) Quantitation of miR-182 by qRT-PCR in control, 50ppm, and 1000 ppm animals after treatment for 3, 7, 14, 28 and 90 days. N=3, error bars indicate s.e.m, Student's t-test was performed * $p<0.05$.

Figure 3 Association between miR-29b expression and hepatic global DNA methylation (**A**) Hepatic miR-29b expression in animals treated with 1000 ppm PB relative to untreated animals at 7, 14, and 90 days; (**B**) quantitation of global DNA methylation levels in animals treated with 1000 ppm PB relative to untreated animals at 7, 14, and 90 days. N=3, error bars indicate s.e.m, Student's t-test was performed * $p<0.05$.

Figure 4 Association between miR-200a/200b expression and zeb1/zeb2 transcription factors (A) Immunoblot of zeb1/zeb2 in the livers of PB-treated animals at 14 days, each lane is a lysate from a different rat; (**B**) Quantification of zeb1 and zeb2 proteins ;(**C**) PCR amplifications of cyp2b1/2, e-cadherin, and gapdh in control and 1000 ppm PB-treated samples at 90 days, cyp2b1 was amplified and quantified as a positive control; (**D**) Quantification of PCR bands. Values are mean ± s.d, n=3 * $p<0.05$ Student's T-test.

Tables

Table 1 Relative expression levels of miRNAs that were significantly differentially expressed at 14 days of PB treatment

MiRNA	50 ppm PB	500 ppm PB	1000 ppm PB
miR-200b	1.3±0.3	1.7±0.3	2.7±0.2
miR-200a	1.2±0.1	1.2±0.4	1.6±0.2
miR-96	0.8±0.1	1.3±0.3	2.1±0.1
miR-494	0.7±0.2	1.1±0.3	1.7±0.1
miR-324-3p	0.7±0.1	1.1±0.3	1.5±0.2
miR-93	0.9±0.3	1.2±0.1	1.4±0.1
miR-29b	0.7±0.2	0.8±0.1	0.7±0.04
miR-99a	0.8±0.2	0.9±0.1	0.8±0.04
miR-19b	0.7±0.3	0.7±0.1	0.7±0.1
miR-195	0.8±0.1	0.9±0.0	0.9±0.0
miR-199a-5p	0.9±0.4	0.7±0.1	0.7±0.1

Expressions relative to control group and s.d. are shown.

Table 2 Temporal trends of mean fold change in expression of miRNAs significantly altered between control and 1000ppm at 14 days of PB treatment

MiRNA	Day 3	Day 7	Day 14	Day 90
miR-200b	0.8±0.2	2.0±0.9	2.7±0.2*	4.2±0.4*
miR-200a	0.8±0.2	1.9±0.5	1.6±0.2*	3.8±0.5*
miR-96	1.1±0.2	1.8±0.5	2.1±0.1*	3.6±0.1*
miR-494	0.7±0.1	0.6±0.3	1.7±0.1*	0.6±0.2
miR-324-3p	1.0±.6	0.7±0.6	1.5±0.2*	0.8±0.4
miR-93	0.9±0.1	1.0±0.2	1.4±0.1*	1.1±0.1
miR-29b	1.5±0.4	1.3±0.4	0.7±0.04 *	1.1±0.2
miR-99a	1.1±0.1	0.9±0.2	0.8±0.04*	0.8±0.02*
miR-19b	1.4±0.5	1.2±0.3	0.7±0.1*	0.8±0.2
miR-195	1.0±0.0	1.0±0.3	0.9±0.0*	0.8±0.1
miR-199a-5p	0.8±0.1	1.1±0.4	0.7±0.1*	1.0±0.0

* Significant at FDR<0.1

Figure
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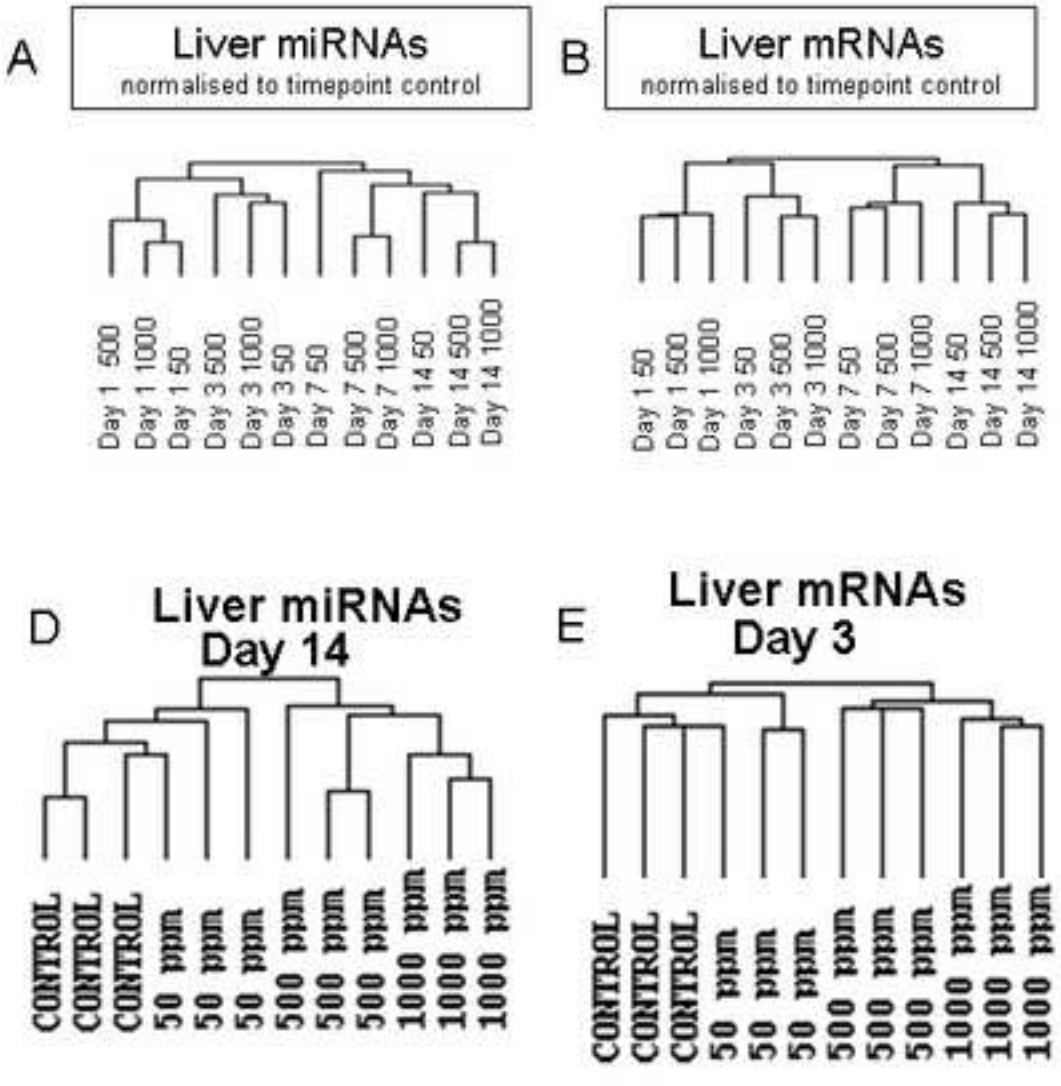

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Pathway	q-value
Cell cycle	>0.0001
DNA replication	>0.0001
Oocyte meiosis	>0.0001
P53 pathway	>0.0001
Mismatch repair	>0.0001
Nucleotide excision repair	>0.0001

Fig.1

Figure
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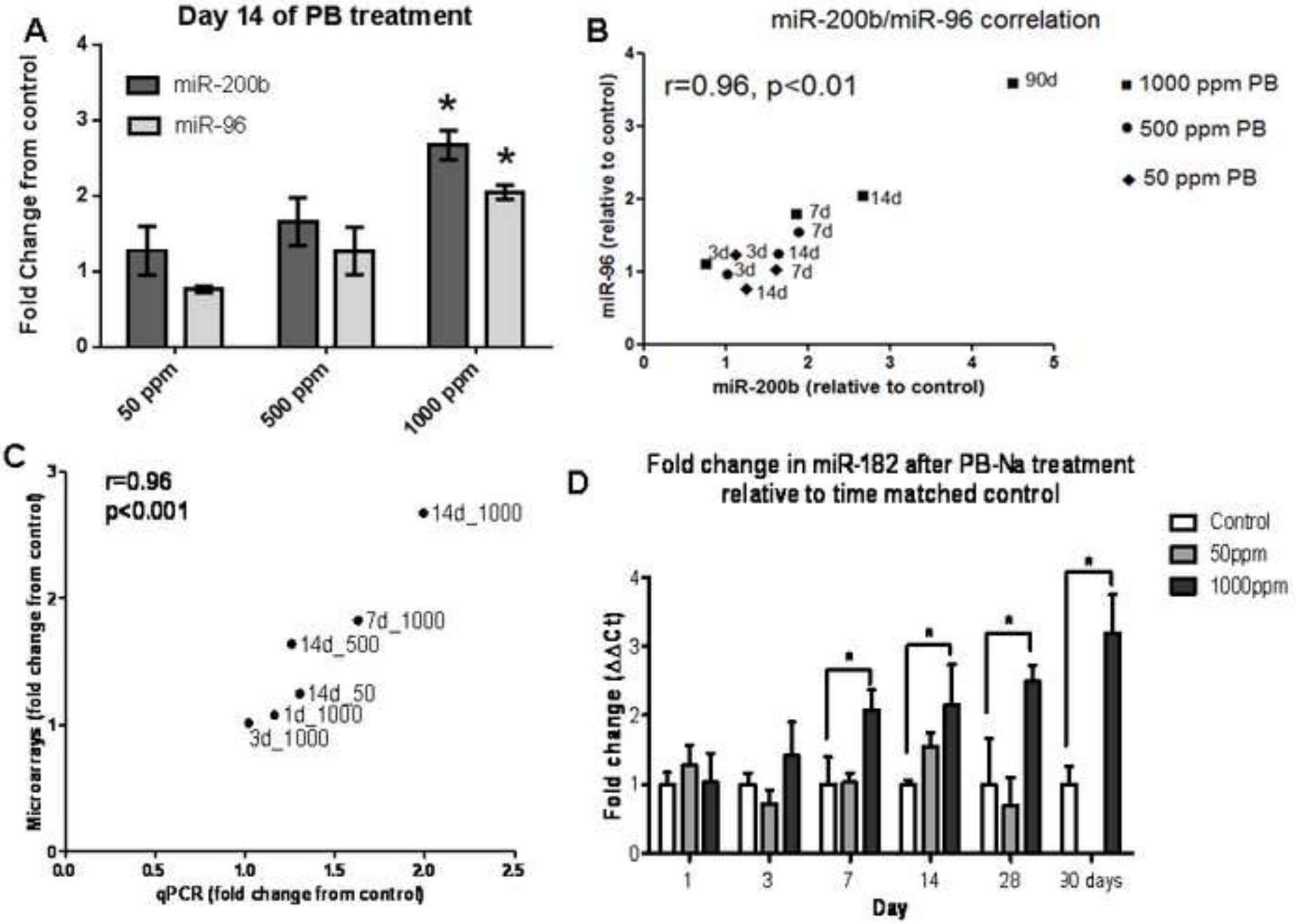

Fig. 2

Figure

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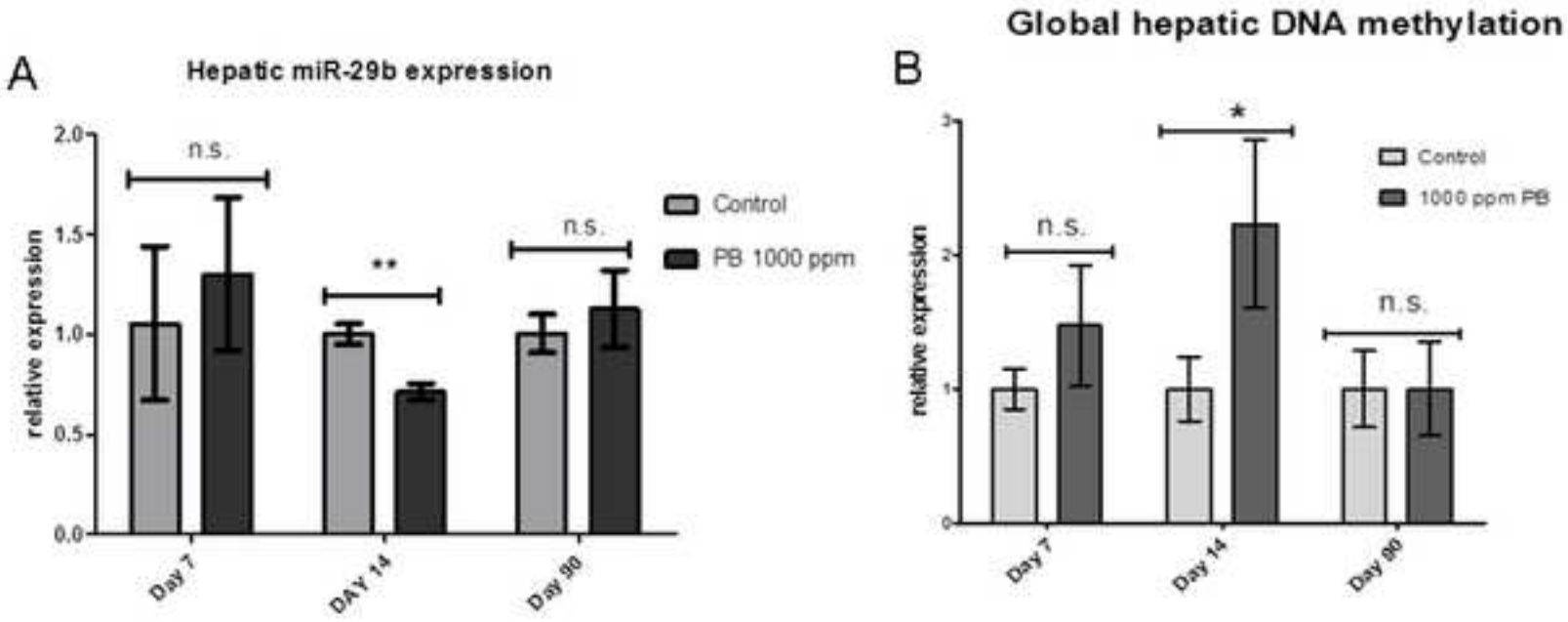

Fig.3

Figure

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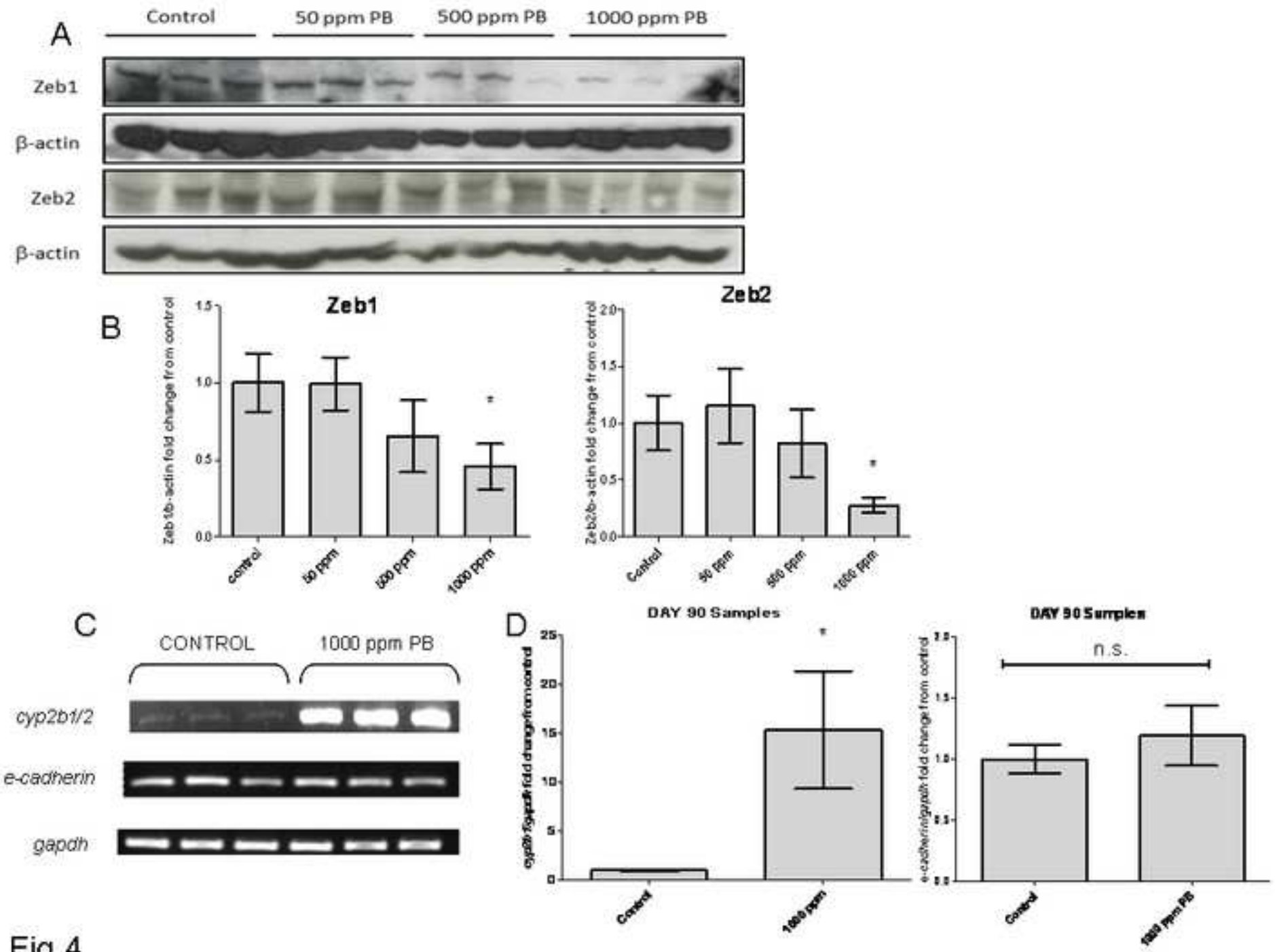

Fig.4