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Transdifferentiated rat pancreatic progenitor cells (AR42J-B13/H) respond to phenobarbital in a rat hepatocyte-specific manner.

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ABSTRACT

Phenobarbital (PB) is known to produce species-specific effects in the rat and mouse, being carcinogenic in certain mouse strains, but only in rats if treated after a DNA damaging event. PB treatment in the rat and mouse also produces disparate effects on cell signalling and miRNA expression profiles. These responses are induced by short term and prolonged PB exposure, respectively, with the latter treatments being difficult to examine mechanistically in primary hepatocytes due to rapid loss of the original hepatic phenotype and limited sustainability in culture. Here we explore the rat hepatocyte-like B13/H cell line as a model for hepatic response to PB exposure in both short-term and longer duration treatments. We demonstrate that PB with Egf treatment in the B13/H cells resulted in a significant increase in Erk activation, as determined by the ratio of phospho-Erk to total Erk, compared to Egf alone. We also show that an extended treatment with PB in the B13/H cells produces a miRNA response similar to that seen in the rat *in vivo, via* the time-dependent induction of miR-182/96. Additionally, we confirm that B13/H cells respond to Car activators in a typical rat-specific manner. These data suggest that the B13/H cells produce temporal responses to PB that are comparable to those reported in short-term primary rat hepatocyte cultures and in the longer term are similar to those in the rat *in vivo*. Finally, we also show that Car-associated miR-122 expression is decreased by PB treatment in B13/H cells, a PB-induced response that is common to the rat, mouse and human. We conclude that the B13/H cell system produces a qualitative response comparable to the rat, which is different to the response in the mouse, and that this model could be a useful tool for exploring the functional consequences of PB-sensitive miRNA changes and resistance to PB-mediated tumours in the rat.

1. Introduction

Phenobarbital (PB) is a model rodent drug metabolising enzyme inducer and can cause hepatic hyperplasia and neoplasia (Butler 1978; Becker 1982; Hagiwara et al., 1999). PB acts through the constitutive androstane receptor (Car/Nr1i3), and pregnane x receptor (Pxr/Nr1i2) to induce genes involved in drug metabolism, energy metabolism and cell proliferation (Yang and Wang 2014), such as the prototypical PB/Car marker, Cyp2b (Honkakoski et al., 1998). PB indirectly activates Car through a signalling pathway that dephosphorylates Car, causing its translocation into the nucleus (Kawamoto et al., 1999; Yoshinari et al., 2003; Kobayashi et al., 2003). PB is an hepatocarcinogen in certain strains of mice that are predisposed to liver tumours (reviewed in (Elcombe et al., 2014), and it has been demonstrated that activation of Car is essential to PB's tumour promoting abilities (Yamamoto et al., 2004; Huang et al., 2005). In the mouse, Mutoh et al. (Mutoh et al., 2013) suggest that PB-mediated indirect activation of Car is through antagonism of the Egf receptor (Egfr).

Unlike the mouse, in the rat PB only acts as a liver tumour promoter when given after a DNA damaging compound such as diethylnitrosamine (Kolaja et al., 1996). PB also appears to exhibit disparate effects on cell signalling pathways in the mouse compared to the rat. In mouse primary hepatocytes, it has been reported (Osabe and Negishi 2011) that 1 hour PB treatment was able to reduce Erk activity, which has been linked to activation of Car, in agreement with the reported inhibition of Egfr

Abbreviations: B13/H, AR42J-B13/H cells; Cyp, Cytochrome P450; PB, phenobarbital; PROD, pentoxyresorufin-O-dealkylase; qRT-PCR, quantitative reverse transcription-PCR. BSA bovine serum albumin; Egf, epidermal growth factor; Erl., erlotinib; Veh, vehicle. DMSO dimethyl sulphoxide; TCPOBOP, 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene.

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phosphorylation (Mutoh et al., 2013). However, in rat primary hepatocytes, PB with Egf treatment has been shown to increase Erk activation (Joannard et al., 2006). This opposite effect in the rat may imply a negative feedback mechanism to modulate Car activation. This suggestion is supported by the potentiation of PB-induced Cyp2b mRNA, when also treating with an inhibitor of the Erk upstream kinase, Mek1/2, compared to PB alone (Joannard et al., 2006). The reason for this species difference remains unclear.

Furthermore, PB treatment results in species-specific effects on the rat and mouse hepatic miRNAome. MicroRNAs are regulatory noncoding RNAs that control gene expression by targeted mRNA degradation or translation inhibition (reviewed in Carroll et al., 2013). Lempiäinen *et al.* (Lempiäinen et al., 2013a) demonstrated that PB (0.05% w/v in drinking water) administered to B6C3F1/Cr1 mice significantly increased the expression of the pluripotent-associated noncoding RNA cluster, *Dlk1-Dio3*, which the authors suggested may be linked to the promotion of PB-mediated tumours in the mouse. However, we have shown in the rat that PB induced a different set of miRNAs, including the miR-200a/b/429 and miR-96/182 clusters, in a time- and dose-dependent fashion (Koufaris et al., 2012). The miR-200a/b/429 family has been implicated in resisting cellular changes towards a neoplastic phenotype by targeting the Zeb1/2 transcription factors (Koufaris et al., 2013).

Investigating molecular mechanisms is best accomplished using a suitable *in vitro* based system, and cultured primary hepatocytes are the current gold standard for toxicology testing. However primary hepatocytes rapidly lose their hepatic phenotype, and so the use of alternative models such as stem-cell derived hepatocytes has become the focus of intense research (Hu and Li, 2015). In the rat, it has been proposed that the pancreatic derived progenitor cell line, AR42J-B13 (B13 cells), offers a stable, cost-effective and easy model for generating hepatocyte-like cells known as AR42J-B13/H (B13/H) (Probert et al., 2015). It was reported by Shen *et al.* (Shen et al., 2000) that treatment of the undifferentiated B13 cell with dexamethasone for two weeks stimulated the cell to transdifferentiate into hepatocyte-like B13/H cells. The differentiated cell population display gene expression profiles similar to primary hepatocytes and can be maintained in culture for considerably longer than primary hepatocytes without de-differentiation (Probert et al., 2013; Probert et al., 2015), making them a useful model for the proposed treatments here.

We sought to investigate if the B13/H system was a suitable cell model for temporally extended study following treatment with PB and PB-like compounds. We also interrogated this system for its ability to reliably reproduce PB-induced rat-specific cell signalling and miRNA expression responses.

2. Materials and Methods

2.1. Cell culture

Cell culture consumables were purchased from Life Technologies, Gibco & Invitrogen (Paisley, UK), or Corning (NY, USA). Rat pancreatic AR42J-B13 (B13) cells were a gift from Professor Wright (Newcastle University, UK) and were cultured in GIBCO low glucose (1 g/L) Dulbecco's Modified Eagle Medium supplemented with foetal bovine serum (FBS) (10 v/v%), L-glutamine (233.6 μg/mL), penicillin (80Units/mL) and streptomycin (80 μg/mL). B13 cells were transdifferentiated into hepatocyte-like B13/H cells by treatment with dexamethasone (10 nM) for 13 days (Shen et al., 2000). Unless stated otherwise, B13/H cells were cultured for 24 hours in media lacking dexamethasone prior to further chemical treatments. The duration of chemical treatment is indicated in the text and figures. In experiments using prolonged culture (post transdifferentiation), treatments and media were refreshed every 72 h.

2.2. RNA extraction

MirVana PARIS RNA extraction kits (Life Technologies, Paisley, UK) were used, following the manufacturer's protocol. Samples were lysed directly from cell culture plates after washing with ice cold PBS. Kit disruption buffer was used for lysis, on ice for 10 minutes, with lysate collected and subsequently denatured with 2 x volume of Denaturing Solution. Acid-Phenol:Choloform extraction was used, followed by sample binding to silica filters, washing and elution in DNase/RNase free water. RNA quality and quantity was measured using spectroscopy with the IMPLEN – nanophotometer (Implen, Gmbh, Munich, Germany), and stored at −20 °C until required.

2.3. PCR

Unless otherwise stated, reagents and probes were purchased from Life Technologies (Paisley, UK).

2.3.1. Semi-q PCR: Reverse transcription (RT)

RNA template (500ng–1 μg) was reverse transcribed with random hexamers (300 ng) using the Superscript II kit and deoxynucleotide triphosphates (dNTPs) (425 μM) (Sigma-Aldrich, Dorset, England), following the Superscript II kit protocol. Negative controls were included to check for gDNA contamination. A PTC-200 Peltier Thermal Cycler was used for the RT reaction (Table 1).

2.3.2. Semi-q PCR

The Tfi polymerase assay was used for cDNA (2 μL) amplification, following the manufacturer's instructions. Custom made primers (200 nM) were used for cDNA amplification, with details shown in Table 2 and Table 3. Amplicons were resolved by electrophoresis in an agarose gel (2%) (Sigma-Aldrich, Dorset, UK) made to concentration in a Tris-borate-EDTA (TBE) solution and ethidium bromide (5 μL) (ICN Biomedicals Inc, OH, USA). A 100 bp ladder was used as a size reference (New England Biolabs, MA, USA). Data was analysed using a KODAK image station 4000MM (Carestream molecular imaging, CT, US).

2.3.3. MiRNA RT

RNA template (5 ng) was reverse transcribed using the TaqMan MicroRNA Reverse Transcription assay and miRNA-specific RT primers following the TaqMan MicroRNA Reverse Transcription

Table 1
Thermal cycler and cDNA amplification conditions.

RT/PCR	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
RT (miRNA)	16 °C 30 min	42 °C 30 min	85 °C 5 min	4 °C ∞	
RT (mRNA)	25 °C 10 min	37 °C 120 min	85 °C 5 min	4 °C ∞	
qPCR	95 °C 10 min	95 °C 15sec	60 °C 60sec	Repeat 2–3 × 39	4 °C ∞