Glucocorticoid-induced pancreatic-hepatic trans-differentiation in a human cell line in vitro

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ABSTRACT

The rodent pancreatic AR42J-B13 (B-13) cell line differentiates into non-replicative hepatocyte-like cells in response to glucocorticoid mediated via the glucocorticoid receptor (GR). The aims of this study were to identify a human cell line that responds similarly and investigate the mechanisms underpinning any alteration in differentiation. Exposing the human pancreatic adenocarcinoma (HPAC) cell line to 1 - 10μM concentrations of dexamethasone (DEX) resulted an inhibition of proliferation, suppressed carcinoembryonic antigen expression, limited expression of pancreatic acinar and hepatic gene expression and significant induction of the constitutively-expressed hepatic CYP3A5 mRNA transcript. These changes were associated with a pulse of genomic DNA methylation and suppressed notch signalling activity. HPAC cells expressed high levels of GR transcript in contrast to other nuclear receptors - such as the glucocorticoid-activated pregnane X receptor (PXR) - and GR transcriptional function was activated by DEX in HPAC cells. Expression of selected hepatocyte transcripts in response to DEX was blocked by co-treatment with the GR antagonist RU486. These data indicate that the HPAC response to glucocorticoid exposure includes an inhibition in proliferation, alterations in notch signaling and a limited change in the expression of genes associated with an acinar and hepatic phenotype. This is the first demonstration of a human cell responding to similarly to the rodent B-13 cell regarding formation of hepatocyte-like cells in response to glucocorticoid. Identifying and modulating the ablating factor(s) may enhance the hepatocyte-like forming capacity of HPAC cells after exposure to glucocorticoid and generate an unlimited in vitro supply of human hepatocytes for toxicology studies and a variety of clinical applications.

1. Introduction

The pancreas and liver are embryonic endoderm-derived tissues with a close developmental relationship (in that hepatocytes and biliary epithelial cells are derived from a population of embryonic pancreas cells (Deutsch et al., 2001)). Both pancreas and liver tissues have endocrine and exocrine functions with ductal progenitor cells in each tissue generating the endocrine (e.g. islet cells in pancreas) and exocrine (i.e. acinar cells in pancreas) tissue. Hepatocytes in the liver perform both endocrine (e.g. albumin secretion) and exocrine (bile acid excretion) functions (Wallace et al., 2008).

At present, limited supplies of human liver, the costs of hepatocyte isolation and difficulties in maintenance of function in vitro limit use of hepatocytes in cell-based studies (Wallace et al., 2010a). Considerable effort has been directed towards generating hepatocytes from either embryonic stem cells or induced pluripotent stem cells (Sullivan et al., 2010; Rashid et al., 2010; Hannan et al., 2013). Improvements in protocols have increased the proportion of cells differentiating toward hepatocytes but there remain a number of key hurdles to overcome before they will have significant basic or clinical utility. These include a lack of maturity, such that the cells remain at best, functionally equivalent to foetal hepatocytes and that they de-differentiate in vitro from a difficult-to-predict peak in function. There is also currently a requirement to follow complex differentiation protocols and the high costs associated with their production limit uptake and use (Wallace et al., 2010a; Szkolnicka et al., 2013).

An expandable rat pancreatic exocrine-like and/or ductal progenitor cell line - AR42J-B13 (henceforth referred to as B-13 cells) – to our knowledge, is unique in its quantitative ability to differentiate into hepatocyte-like cells that remain stably differentiated for many weeks on simple culture substrata. B-13 cells differentiate into non-replicative functional hepatocyte-like (B-13/H) cells after exposure to a single simple hormone (glucocorticoid), with many functions at levels quantitatively similar to the levels in freshly isolated rat hepatocytes (Shen et al., 2000; Marek et al., 2003; Wallace et al., 2010a; Probert et al., 2015).

It is known that pathological conditions lead to the appearance of hepatocyte-like cells in the pancreas (Zaret et al., 2008). For an extensive review of rodent and human data with regard to this issue, see Probert et al (2015). Since the proliferation of human pancreatic adenocarcinoma (HPAC) cells is inhibited after exposure to glucocorticoid (Gower et al., 1994; Norman et al., 1994), we hypothesised that this cell line could form the basis of a human equivalent to the B-13 cell, capable of conversion into functional hepatocyte-like cells. This paper demonstrates for the first time, that glucocorticoid exposure and the inhibition in proliferation, is also associated with alterations in notch signaling and a change in the expression of genes associated with acinar and hepatic phenotypes.

2. Results

Glucocorticoid exposure inhibits HPAC proliferation and promotes an epithelial phenotype in HPAC cells.

Supplementary Fig 1A demonstrates that exposing HPAC cells to DEX resulted in a dose-dependent reduction in nuclear proliferating cell nuclear antigen (PCNA) expression within 2 days of exposure and an inhibition in proliferation (Supplementary Fig 1B) - as previously reported (Gower et al., 1994; Norman et al., 1994) - with a 50% inhibitory concentration between 1 - 10μM. DEX exposure also resulted in a morphological change to a more epithelial phenotype (Supplementary Fig. 1B) and to a reduction - or complete loss in most cells - in expression of the carcinoembryonic antigen (CEA) (Supplementary Fig. 1C). A similar effect was also seen with the ductal marker cytokeratin 19 (Supplementary Fig. 1D).

Glucocorticoid exposure induces a limited expression of hepatocyte transcripts in HPAC cells.

Fig 1A demonstrates that HPAC expressed undetectable levels of the hepatocyte transcripts CYP2E1 and albumin whereas after treatment with DEX, these transcripts became detectable as determined by semi-quantitative RT-PCR after 7 or 14 days respectively. These data were confirmed by quantitative RT-PCR based on 7 separate assessments over a period of several months (Fig 1B). Fig 1B indicates that CYP2E1 and albumin transcripts were increased to low levels relative to human hepatocytes (approx. 100 fold lower) although responses between cultures were subject to great variability. It has been reported that HPAC cells express CYP3A5 based on Western blotting of cell extracts and metabolism of midazolam to 1′-hydroxymidazolam in cultured cells, although no comparison was made to human hepatocytes (Schmiedlin-Ren et al., 2001). Figure 1B supports the observation that CYP3A5 is constitutively expressed in HPAC cells and has the potential for activity

since HPAC cells also constitutively expressed hepatocyte levels of the P450 reductase (POR) transcript. Fig 1B also indicates that DEX treatment resulted in a statistically significant 2.6 fold increase in CYP3A5 mRNA transcript expression. In contrast, the major hepatocyte expressed CYP3A4 transcript showed a mean fall in expression after DEX treatment. In terms of liver-enriched transcription factor gene expression, Fig 1B indicates that there was a significant 4.8 fold increase in CEBPβ transcript expression after DEX treatment whereas the both HNF1α and HNF4α expressions were suppressed. Given that HPAC constitutive CEBPβ expression was in the range seen in human hepatocytes, this suggests CEBPβ induction is a candidate driver for some of the changes seen in response to DEX. Fig. 1C demonstrates however, that expression of CYP2E1 and albumin proteins were low to undetectable in HPAC cells after treatment with DEX and this was confirmed on examination of cytochrome P450 enzyme activities, including after exposure to a cocktail of compounds that induce these enzymes (Fig. 1D).

The mechanism of acinar and hepatocyte-like formation in HPAC cells is associated with a pulse in DNA methylation and a suppression of Notch signalling.

Previous work has shown that glucocorticoids bind to the glucocorticoid receptor (GR) in B-13 cells, resulting in an induction of serine/threonine-protein kinase - also known as serum and glucocorticoid-regulated kinase 1 (Sgk1) - expression (Wallace et al., 2011). This is followed by a pulse in genomic DNA methylation (Fairhall et al., 2016), phosphorylation of β-catenin leading to transient down-regulation in canonical wnt signaling (Wallace et al., 2010b) and increases in the expression of a variety of transcription factors associated with hepatocyte phenotype such as Cebp-β (Shen etv al., 2000; Fairhall et al., 2016), Cebp-α, Hnf1α and Hnf4α (Fairhall et al., 2016).

Fig 2A demonstrates that exposing HPAC cells to DEX resulted in a rapid pulse in genomic DNA methylation as observed to occur in B-13 cells (Fairhall et al., 2016). DEX treatment in the hepatocellular carcinoma HepG2 cell line resulted in a demethylation. In contrast to B-13 cells

however, the wnt signalling pathway was inactive in HPAC cells (Fig. 2B) and there was no induction of SGK1 after DEX treatment (data not included). Rather, HPAC cells expressed a functional DEX-suppressed notch signalling pathway which was absent in B-13 cells (Fig.2C and Fig. 2D). Recent work in the liver has demonstrated that notch ligand (Jagged 1) from hepatic portal (myo)fibroblasts activates notch signalling and promotes murine hepatic bi-progenitor (oval cell) differentiation into cholangiocytes and that Wnt3a from macrophages promotes hepatocyte formation (Boulter et al., 2012). In order to test whether (myo)fibroblasts inhibit hepatic trans-differentiation of HPAC cells to hepatocyte-like cells, mouse liver (myo)fibroblasts were co-cultured with HPAC cells. Co-culture inhibited the induction of hepatocyte mRNA transcripts in response to DEX (Fig 2E).

HPAC conversion to an hepatocyte-like phenotype is dependent on the GR.

Fig. 3A demonstrates that HPAC cells expressed relatively high levels of GR in contrast to undetectable levels of several nuclear receptor mRNA transcripts (that share overlapping ligand binding characteristics with the GR), as determined by RT-PCR. Expression of GR mRNA (using primers that hybridize to all transcripts) increased (Fig. 3A) and the levels of the dominant-negative (Lewis-Tuffin et al., 2006) truncated variant GRβ mRNA decreased (Fig. 3B) in HPAC cells after treatment with DEX suggesting the potential for a positive feedback for promoting glucocorticoid responsiveness (as observed in B-13 cells (Fairhall et al., 2016)). However, relatively high (~ 10^{-6} – 10^{-5}M) concentrations of DEX, typically necessary for activation of the human pregnane X receptor (PXR) (Harvey et al., 2000), were required to promote hepatic changes in HPAC cells. In contrast, B-13 cells respond to DEX in the ~10^{-8} M range (Shen et al., 2000; Marek et al., 2003; Probert et al., 2015), expected given the k_D of DEX for the GR is ~10^{-9}M (Wright and Paine, 1994). DEX maximally activated the GR at ~ 10^{-6} M in HPAC cells based on transfection of a synthetic GR-responsive reporter gene construct, an effect inhibited by the GR antagonist RU486 (Fig. 3C). Induction of hepatocyte

transcript expression by DEX was also inhibited by co-treatment with RU486 (Fig. 3D) and human PXR activators [phenobarbitone, rifampicin and metyrapone (Goodwin et al., 2001; Harvey et al., 2000) failed to induce hepatocyte transcript expression (Fig. 3E) or PXR-dependent reporter gene induction (Fig. 3F) in HPAC cells. These data therefore support the central role of the GR in mediating the induction of an acinar and hepatocyte-like phenotype by DEX in HPAC cells.

Since several glucocorticoid resistance phenotypes have been documented to be associated with polymorphisms in the human GR gene (Charmandari et al., 2013), the full amino acid sequence-encoding HPAC GR cDNA sequence [encompassing 2395bp containing the coding sequence of the predominant human variant 1 transcript, also known as GRα or GR-A; note, that 5 other variant transcripts encode the same protein isoform] was cloned by RT-PCR from HPAC mRNA using overlapping amplicons (see Fig. 4A and supplementary Fig. 2) and sequenced. The entire amino acid coding sequence of the HPAC GR cDNA sequence was identical to the human variant 1 transcript (see supplementary data 2) except for a synonymous mutation in the C terminus of the protein - AAT to AAC (Fig. 4B).

A 3 nucleotide (GTA) insertion that predicts an insertion of an arginine amino acid at position 452 in the DNA binding domain of the HPAC GR protein was also detected (Fig 4C). This latter change has been previously reported to occur through alternative splicing whereupon 3 bases are retained from the intron separating exons 3 and 4, leading to an identical variant transcript and an insertion of an arginine (Rivers et al., 1999). Since the HPAC GR US2/DS2 PCR product sequence results in the presence of a single *Acc*I restriction endonuclease site not present in the wild type transcript (Fig. 4D), RFLP was employed to estimate the proportion of HPAC GR variant transcripts encoding a GR protein containing an additional arginine in the DNA binding domain. Fig. 4E demonstrates that variant transcript encoding an insertion of an arginine amino acid at position 452 formed a minor proportion of the total GR transcripts present, a phenomenon also noted in normal human tissues (Rivers et al., 1999).

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3. Discussion

Generating good quality, functional human hepatocytes that can be used as an effective tool to study human hepatic biology and model typical responses to drugs and chemicals remains a significant challenge in Hepatology and Toxicology. Limited access to normal human liver tissue and functionality and cost issues with stem cell-derived hepatocyte-like cells preclude their widespread utility and applicability. Given that a rodent pancreatic progenitor-like B-13 cell line with acinar properties is capable of generating hepatocyte-like cells (Wallace et al., 2010a; Shen et al., 2000; Marek et al., 2003; Wallace et al., 2010b; Wallace et al, 2011; Probert et al., 2014; Probert et al., 2015; Probert et al., 2016; Fairhall et al., 2016), manipulation of a similar human cell may offer the prospect of an unlimited supply of functional human hepatocytes.

Previous recent work has confirmed that the rodent exocrine pancreatic response to elevated glucocorticoid of hepatic differentiation (Wallace et al., 2009; Wallace et al., 2010c) is also observed in man (Fairhall et al., 2013a). A human pancreatic ductal-like cell line that showed a similar behaviour to B-13 cells was therefore sought and HPAC cells were identified. As observed with B-13 cells Fairhall et al., 2013b), although it is considered to be a cancer ductal-like cell line, HPACs demonstrated the property of being unable to proliferate in soft agar (data not included). HPAC cells also shared a number of qualitative properties associated with B-13 cells, including high expression of the GR and a functional response to glucocorticoids (i.e. transcriptional effects). In addition, after exposure to glucocorticoid, both HPAC and B-13 cells undergo a robust pulse in genomic DNA methylation; an induction of selected hepatocyte transcripts (albumin, CYP2E1); a decrease in proliferation and an alteration in morphology (Fairhall et al., 2016). In all cases where it has been investigated, these effects were inhibited by GR antagonists such as RU486, supporting a pivotal role for the GR in mediating these responses.

It remains the case however, that there are differences between the mechanism(s) regulating B-13 and HPAC cell responses to glucocorticoid exposure. Glucocorticoid exposure in HPACs resulted in a quantitatively markedly reduced level of hepatocyte mRNA transcript and protein expression levels than that seen in hepatocyte-like B-13/H cells (which are similar in many cases to rat liver levels (Fairhall et al., 2016)). In addition, for HPAC cells, SGK1 induction did not occur (data not included); higher concentrations of DEX were required; Wnt signalling was not active and the presence of myofibroblasts inhibited hepatic transcript expression (with a more pronounced effect on CYP2E1 than albumin transcript expression), whereas they promote hepatocyte-like B-13/H formation (Fairhall et al., 2014). A possible reason why albumin escaped a robust block in expression in the presence of co-cultured myofibroblasts may be due divergent regulation of these 2 genes, with CYP2E1 expression being directly regulated by the Wnt signalling pathway (Braeuning et al., 2009; Gerbal-Chaloin et al., 2014), whereas no such direct regulatory role has been defined for the albumin gene.

With respect to higher concentrations of DEX being required for conversion to hepatocyte-like cells compared to B-13 cells, this is unlikely to be due to HPAC GR variant expression, since the only variant GR transcript identified was present at similar levels (between 3.8 and 8.7% of total GR) in normal tissues (Rivers et al., 1999). Several glucocorticoid resistance phenotypes have been documented to be associated with polymorphisms in the human GR gene (Charmandari et al., 2013). However, sequencing and RFLP analysis reported herein suggest that a polymorphic variant is not expressed in HPAC cells and the gene encoding the protein is intact and identical to the human w/t GR. Furthermore, Scatchard analysis of dexamethasone binding data in HPAC extracts identified a single class of high-affinity binding sites with a k_D of 3.8 ± 0.9 nM and maximum binding site concentration of 523 ± 128 fmol/mg protein (Gower et al., 1994) suggestive of normal GR - glucocorticoid ligand interaction. A more likely scenario is that the GR transcriptional response is ablated by a non-GR factor(s). Identifying and modulating the ablating factor(s) may enhance the hepatocyte-like forming capacity of HPAC cells after exposure to glucocorticoid.

Despite clear qualitative similarities to B-13 cells, HPAC cells expressed low levels of hepatocyte transcripts after treatment with glucocorticoid. Therefore, on this basis, although of mechanistic interest, they are of limited practical value at present. However, given their ease and simplicity of propagation, genetic manipulation of HPAC cells though GR-dependent induction of appropriate factors (e.g. liver specific transcription factors) will likely amplify the hepatic phenotype. The HPAC cell line may therefore be a base from which to generate an unlimited supply of donor free human hepatocyte-like cells.

4. Materials and Methods

4.1 Materials.

HPAC (ATCC® CRL-2119™) cells were originally purchased from LGC Standards (Middlesex, UK). HPAC cells used in these studies were confirmed to be of human female origin (data not included) based PCR-RLFP analysis of the mitochondrial genome-encoded cytochrome b according to Losi et al. (2008) using primers L14816 and H15173 for species determination and female using primer sequences given in supplementary data 3. The renilla reporter vector RL-TK was originally purchased from Promega (Southampton, UK). The Notch reporter constructs pCBFRE-luc and pCBFRE-(mut)-luc as described (Yu et al., 2008) were obtained from Addgene. Wnt signalling reporter constructs "Topflash" and "Fopflash" were also obtained from Addgene and used as previously described (Wallace et al., 2010b). All other plasmids and their construction have been previously described - GRE4-pGL4.28 (Fairhall et al., 2016); (ER6)3-pGL3promoter (Haughton et al., 2006). Pancreas tissue was originally obtained with patient consent through the Newcastle Hepatopancreatobiliary Research Tissue Bank, with ethical approval by the Newcastle & North

Tyneside 2 Research Ethics Committee. Pancreas tissue was taken from the margins of fresh tissue removed from donors due to the presence of tumours, but was histologically normal. Human acinar cells were prepared as previously outlined (Fairhall et al., 2013a). Human hepatocytes were isolated from a 42 year old male donor by collagenase perfusion essentially as previously described (Harvey et al., 2000). Cells were washed in ice-cooled PBS and cell pellet aliquots snap frozen in liquid nitrogen prior to long term storage at -80^{o}C for later use in assays. Human liver tissue for hepatocyte isolation was ethically obtained via the Newcastle Biobank (https://www.ncl.ac.uk/biobanks/) with over-arching ethical approval from the Newcastle & North Tyneside 1 Research Ethics Committee.

4.2 Cell culture

All cell lines were routinely cultured in low glucose (1g/L) Dulbecco's Modified Eagle's Medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 100units/ml penicillin, 100µg/ml streptomycin and 0.584g/L L-glutamine in an humidified incubator at 37^{o}C and an atmosphere of 5% CO_2 in air. Cells were treated with dexamethasone (DEX) through addition of 1000-fold molar ethanol solvated stocks, with controls treated with 0.1% (v/v) ethanol vehicle as control. HPACs were exposed to a multiple of 4 chemicals in an attempt to induce expression of several hepatic 1-4 sub-families of cytochromes P450 (CYP): 20µM β-naphthoflavone, 1mM sodium phenobarbitone, 20µM rifampicin and 100µM bezafibrate, primarily inducing hepatocyte CYP1A (Harvey et al., 2000), CYP2B (Pascussi et al., 2000), CYP3A (Wright, 1999) and CYP4A (Alvergnas et al. 2009) sub-families respectively. Cells were treated daily for 4 days and harvested 24 hours after the last treatment on day 5. Liver myofibroblasts were prepared through isolation of quiescent hepatic stellate cells from adult male C57Bl/6 mice and activation to myofibroblasts through culture on plastic in high glucose (5g/L) DMEM supplemented with 20% FCS, 100units/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamycin and 0.584g/L L-glutamine as previously described (Ebrahimkhani et al., 2011) prior to culture in medium used for HPAC cells and co-culture experiments. Photomicrographs of 5 randomly

selected fields of view were taken per time point and treatment and cell number determined be an operator blinded to the treatments. The mean cell number from at least 3 separate culture per treatment were used to determine the mean and SD cell number for each time point. For soft agar cultures, cells were sub-cultured and suspended at 2.5×10^3 cells/mL in media containing 0.35% (w/v) agarose as previously outlined (Fairhall et al., 2013b). Cells were cultured for 2 weeks, before staining each well with 500 µl of 0.005% (w/v) crystal violet for 1 hour to visualize cells.

4.3 Gene expression studies

DNA, total RNA and protein extracts were prepared and RT-PCR and PCR were performed as outlined (Ebrahimkhani et al., 2011; Wallace et al., 2010c) using primers provided in supplementary data 3. qRT-PCR was performed using validated Taqman primers supplied by Applied Biosystems. Western blotting, immunocytochemistry and immunohistochemistry were performed as described (Ebrahimkhani et al., 2011; Wallace et al., 2010c). Cytochrome P450 CYP2E activities were determined using para-nitrophenol as previously described (Marek et al., 2005). CYP3A4 activities were determined as described (Probert et al., 2014) using a kit supplied by Promega.

4.4 Genomic DNA methylation

Genomic DNA was isolated as outlined (Fairhall et al., 2016) and total global methylation determined using a Sigma Imprint® Methylated DNA quantification kit, essentially according to the manufacturer's instructions. Typically, 100ng of DNA from each treatment was analysed and compared to standard methylated DNA provide in the kit. Relative global methylation levels were calculated as percentage compared to the fully methylated control.

4.5 Transfection and Dual-Luc reporter gene assays

HPAC cells were transfected with PEI reagent (MerckMillipore, Nottingham, UK); HepG2 cells and B-13 cells were transfected with Genejuice (Merck, Nottingham, England) according to the manufacturer's instructions. For luciferase-based reporter gene studies, luciferase and renilla activities in cell extracts were assayed using the Dual-Luciferase assay system from Promega (Southampton, UK) using a luminometer. Renilla activities were used at all times to normalise for transfection efficiencies.

4.6 Sequence determination of the HPAC GR cDNA sequence

RNA was isolated from HPAC cells (and human hepatocytes as positive control) and first strand cDNA synthesised using MMLV (using a kit supplied purchased from Sigma, Pool, UK) primed using random hexamers (Promega, Southampton, UK). Sections of the full amino acid sequence-encoding GR [encompassing 2395bp containing the coding sequence of the predominant human variant 1 transcript, also known as GR-alpha or GR-A; note, that 5 other variant transcripts encode the same protein isoform] was then amplified by PCR using a proof reading polymerase - Phusion® high fidelity kit with GC buffer (NEB, Hitchin, UK) using primer pairs and the conditions as outlined in supplementary data 2. PCR products were blunt end TOPO cloned (Invitrogen) and *EcoR*1 restricted to confirm PCR product insertion. Selected clones were then sequenced 6 times in total (3 times in either direction) and the relevant (non-primer derived) GR encoding sequences aligned using Omega Clustal (http://www.ebi.ac.uk/Tools/msa/clustalo/). In all cases, the sequences were 100% identical. Restriction of pUC19 (Invitrogen) or PCR products with *Acc*I (Thermo Scientific) was performed at 37^{o}C overnight following the manufacturer's instructions.

Author contributions

E.A.F, A.C.L., A.F.L, P.M.P., G.R. and C.D. performed the laboratory-based work and analyses presented in the manuscript. M.C.W conceived the studies, designed the experiments and wrote the manuscript. All authors read and commented on the final manuscript.

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Summary statement

- Glucocorticoid exposure suppresses HPAC cell proliferation and induces an hepatocyte-like phenotype.
- Change in differentiation is dependent on the glucocorticoid receptor, not other nuclear receptors (e.g. PXR).
- Differentiation change is associated with a pulse in genomic DNA methylation and suppression of the notch signalling pathway.

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452 **Figure legends**

453

454 **Figure 1.** ***Effect of DEX exposure on the expression of hepatocyte transcripts, proteins and activities***
455 ***in HPAC cells.*** **A**, RT-PCR for the indicated transcripts in HPAC cells, results typical of 7 separate
456 experiments. **B**, qRT-PCR analysis for the expression of the indicated transcripts in RNA isolated from
457 either HPAC cells treated with 10μM DEX for 14 days or human liver, expressed relative to their
458 levels in HPAC cells (set equal to 1). Data are the mean of 3 separate determinations from RNA
459 pooled from 3 human livers and 7 separate HPAC experiments. **C**, Western blot for the indicated
460 protein, data typical of 6 separate determinations. **D**, Cytochrome P450-dependent enzyme activities in

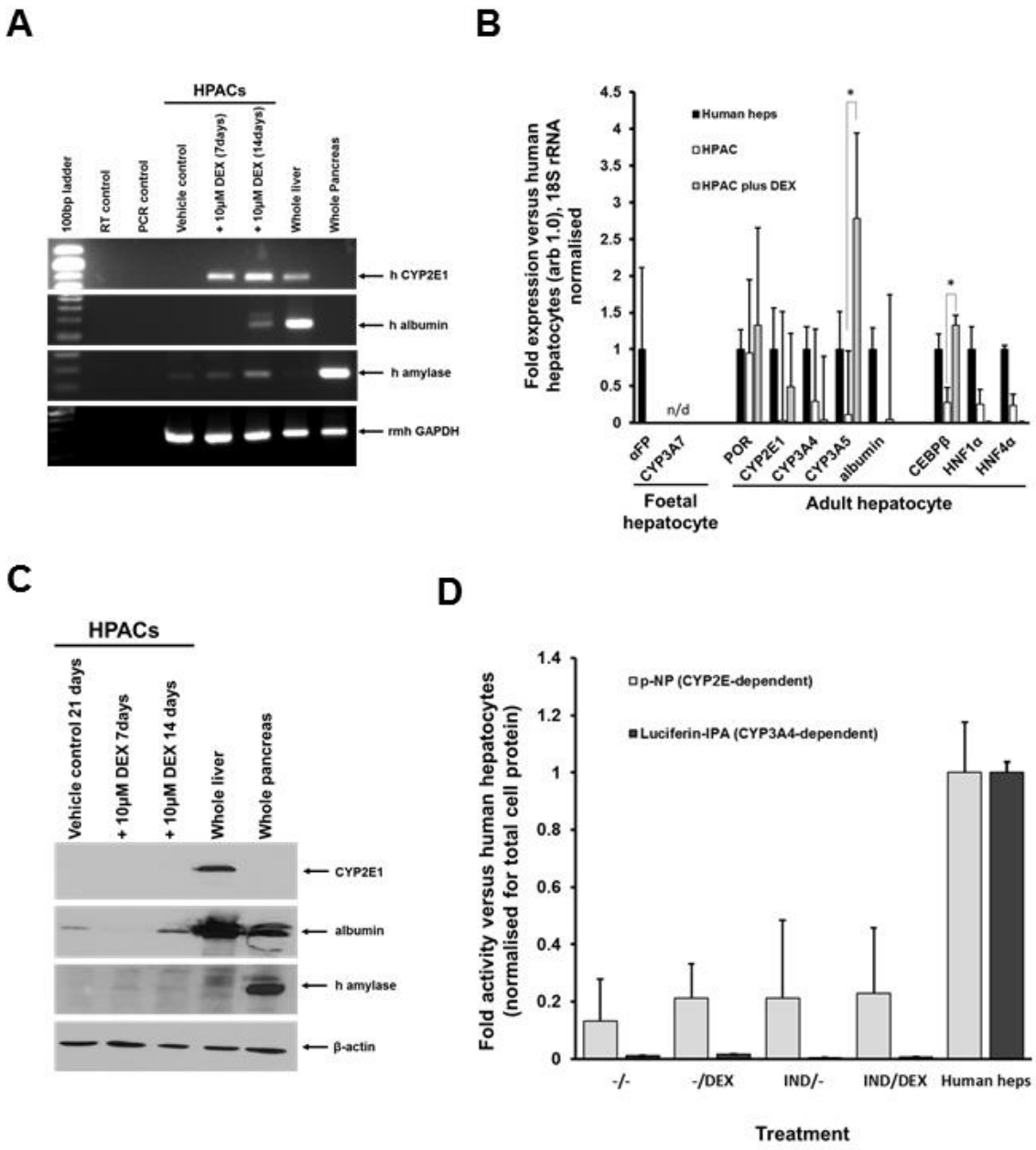
HPAC cells treated as indicated. Data are the mean and standard deviation of 3 separate HPAC experiments or 3 separate human hepatocyte preparations. **D**, CYP2E and CYP3A4 enzyme activities in HPAC cells. HPAC cells were treated 10μM DEX for 14 days (DEX), and for the last 5 days with a cocktail of cytochrome P450 inducers (IND) before isolation of cells and assays. Activities are normalized to protein, expressed relative to human hepatocytes (Human heps, mean and SD of 3 determination from the same donor cells) and are the mean and SD of 3 separate experiments.

Figure 2. ***HPAC conversion to an hepatocyte-like phenotype is associated with a pulse in DNA methylation and a suppression of Notch signalling.*** **A**, genomic DNA methylation in the indicated cell type in response to DEX treatment or vehicle control (0.1% ethanol), data are the mean and SD of 3 separate determinations from the same experiment, typical of at least 3 separate experiments. *significantly different from equivalent vehicle control treated cells treated for the same period of time using the Student's T test (two tailed), $P > 0.95$. **B**, Wnt signalling as determined in cells transfected with 2 μg Topflash (T) or Fopflash (F) luciferase reporter constructs and 0.2 μg RL-TK as outlined in methods section followed by a determination of luciferase and renilla activities 24 hours later. Results are the mean and SD of 3 separate determinations from the same experiment, typical of 3 separate experiments. **C**, Notch signalling in cells transfected with 2 μg pCBFRE or pCBFREmut luciferase reporter constructs and 0.2 μg RL-TK as outlined in methods section followed by a determination of luciferase and renilla activities 24 hours later or after a further 24 hours treatment with DEX. Results are the mean and SD of 3 separate determinations from the same experiment, typical of 3 separate experiments. Significantly different from *mutant or #DEX-treated cells using the Student's T test (two tailed), $P > 0.95$. **D**, RT-PCR for the expression of notch ligand and receptor mRNA transcripts in HPAC cells and comparison to other human tissues. **E**, HPAC cells were co-cultured where indicated with culture-activated mouse liver myofibroblasts and treated with DEX or vehicle control for 14 days

prior to analysis for the indicated transcript. α-Smooth muscle actin (α-sma) is a transcript marker expressed in liver myofibroblasts (Wallace et al., 2008).

Figure 3. ***Glucocorticoid-dependent hepatocyte-like phenotype in HPAC cells is mediated via the glucocorticoid receptor.*** **A**, RT-PCR for the expression of selected nuclear receptor mRNA transcripts in HPAC cells and comparison to other human tissues, data are typical of at least 3 separate determinations. **B**, RT-PCR for the expression of GR (also termed GRα) and the dominant-negative GRβ mRNA transcripts in HPAC cells and comparison to other human tissues. The GRβ protein is generated from an alternate mRNA transcript from the GR gene. **C**, HPAC cells were transfected with 2 µg GRE4-pGL4.28 and 0.2 µg RL-TK as outlined in methods section and after 24 hours, treated with the indicated concentration of DEX and/or 10µM RU486 prior to determination of luciferase and renilla activities 24 hours later. Results are the mean and SD of 3 separate determinations from the same experiment, typical of 3 separate experiments. Significantly different from *RU486-treated or $^{\$}$untreated cells using the Student's T test (two tailed), $P > 0.95$. **D**, Effect of RU486 on DEX-dependent hepatocyte mRNA transcript expression. Cells were treated for 14 days with the indicated concentration of DEX and/or RU486 prior to RT-PCR analysis for the indicated transcripts, results typical of 3 separate experiments; **E**, Effect of PXR activators (i.e. DEX at the concentration employed, phenobarbitone (PB), rifampicin (RIF) and metyrapone (MET) – see Harvey et al., 2000)) treatment on hepatocyte marker gene expression in HPACs. HPACs were treated as indicated prior to RT-PCR analysis for the indicated transcripts, results typical of 3 separate experiments. **F**, HPAC cells were transfected with 2 µg ER6-pGL3promoter and 0.2 µg RL-TK as outlined in methods section and after 24 hours, treated with DMSO vehicle control (0.1% v/v) or the indicated concentration of DEX or RIF prior to determination of luciferase and renilla activities 24 hours later. Results are the mean and SD of 3 separate determinations from the same experiment, typical of 3 separate experiments. *Significantly different from DMSO vehicle treated cells using the Student's T test (two tailed), $P > 0.95$.

Figure 4. ***Overlapping RT-PCR amplification and sequencing of the HPAC GR.*** **A,** schematic diagram of the human GR gene and strategy employed to amplify regions using HPAC RNA or human liver RNA as template, lower panels PCR products subsequently cloned and sequenced. **B**, sequence data demonstrating synonymous mutation in the 3' terminus of the HPAC cDNA sequence compared to the wild type (w/t) sequence. **C**, sequence data demonstrating 3 nucleotide (GTA) insertion predicting an insertion of an arginine amino acid at position 452 in the DNA binding domain of the HPAC GR protein. **D**, Identification of an *Acc*I restriction endonuclease site in the US2/DS2 3 nucleotide (GTA) insertion PCR product, which is absent from the w/t PCR product. **E**, *Acc*I restriction, left panel of pUC19 plasmid DNA which contains a single AccI restriction site leading to linearization of the plasmid (rp); right panel, restriction of US2/DS2 PCR product derived from HPAC RNA, with restriction giving w/t and a fragment of DNA only generated from PCR product amplified from the variant transcript encoding an insertion of an arginine amino acid at position 452 (variant). Note, restriction of individual pCR plasmid clones from the US2/DS2 PCR product generated similar results (i.e. minority contained *Acc*I restriction sites), data not included.



Fairhall et al: Fig. 1

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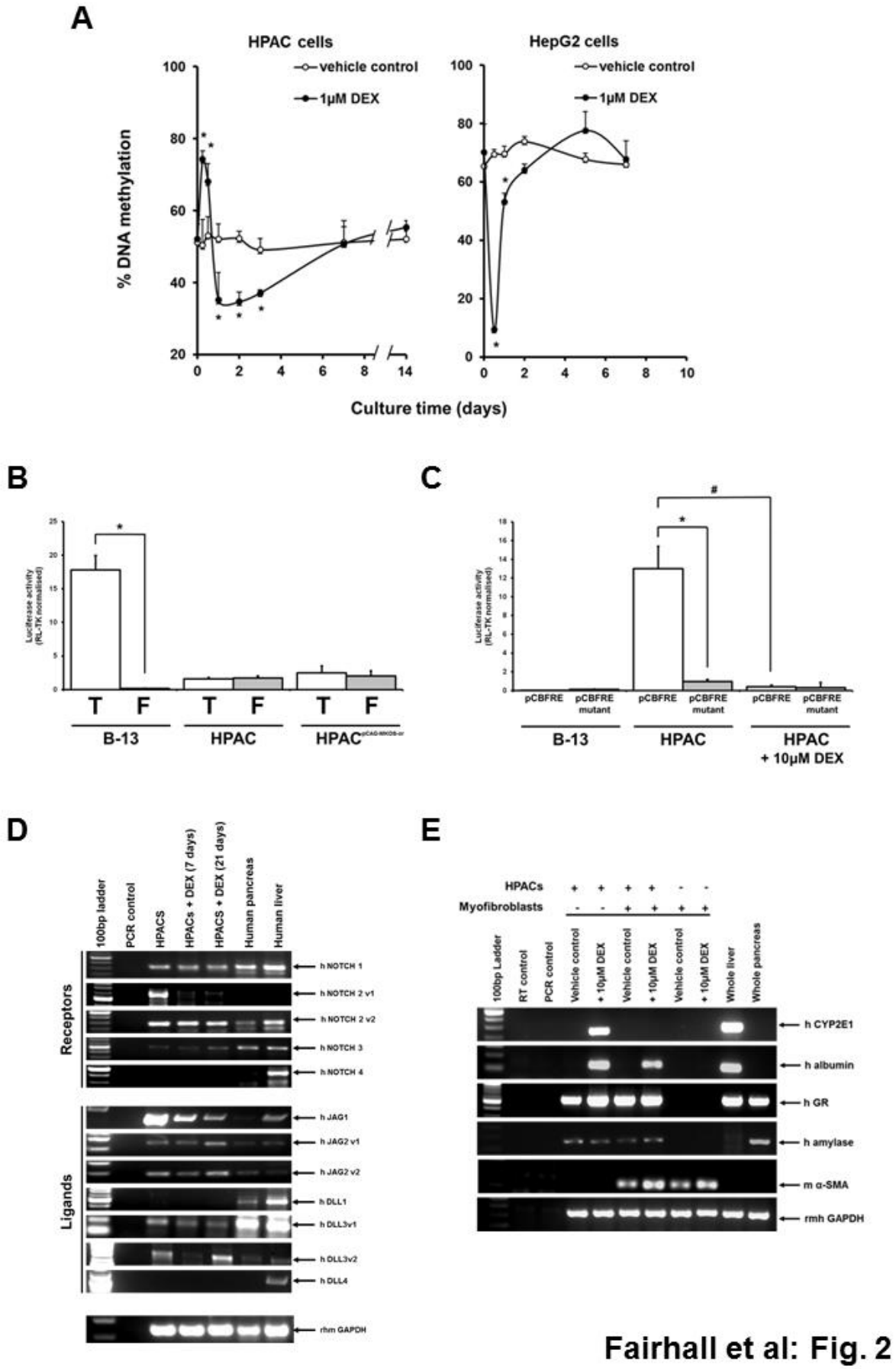

Fairhall et al: Fig. 2

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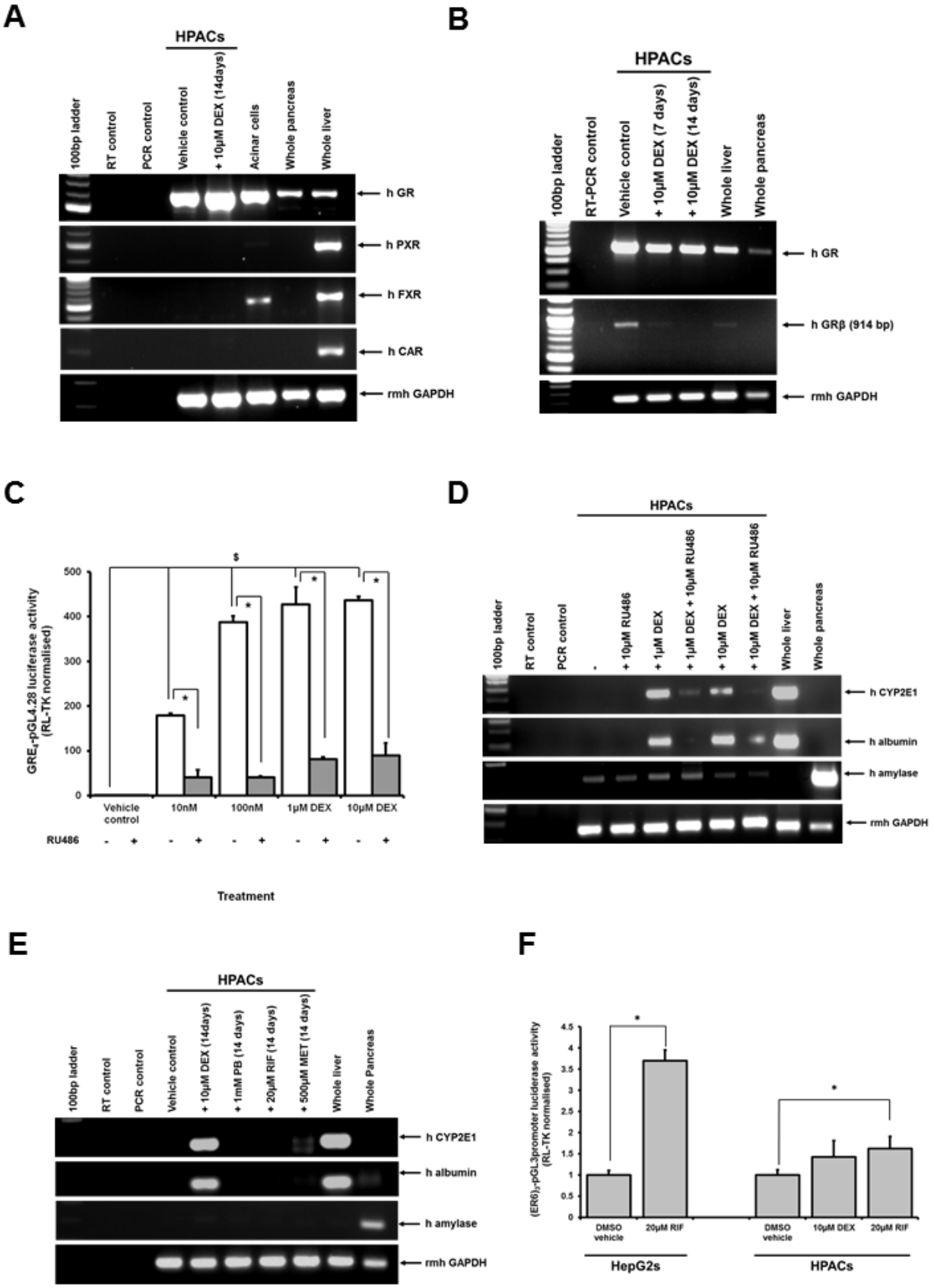

Fairhall et al: Fig. 3

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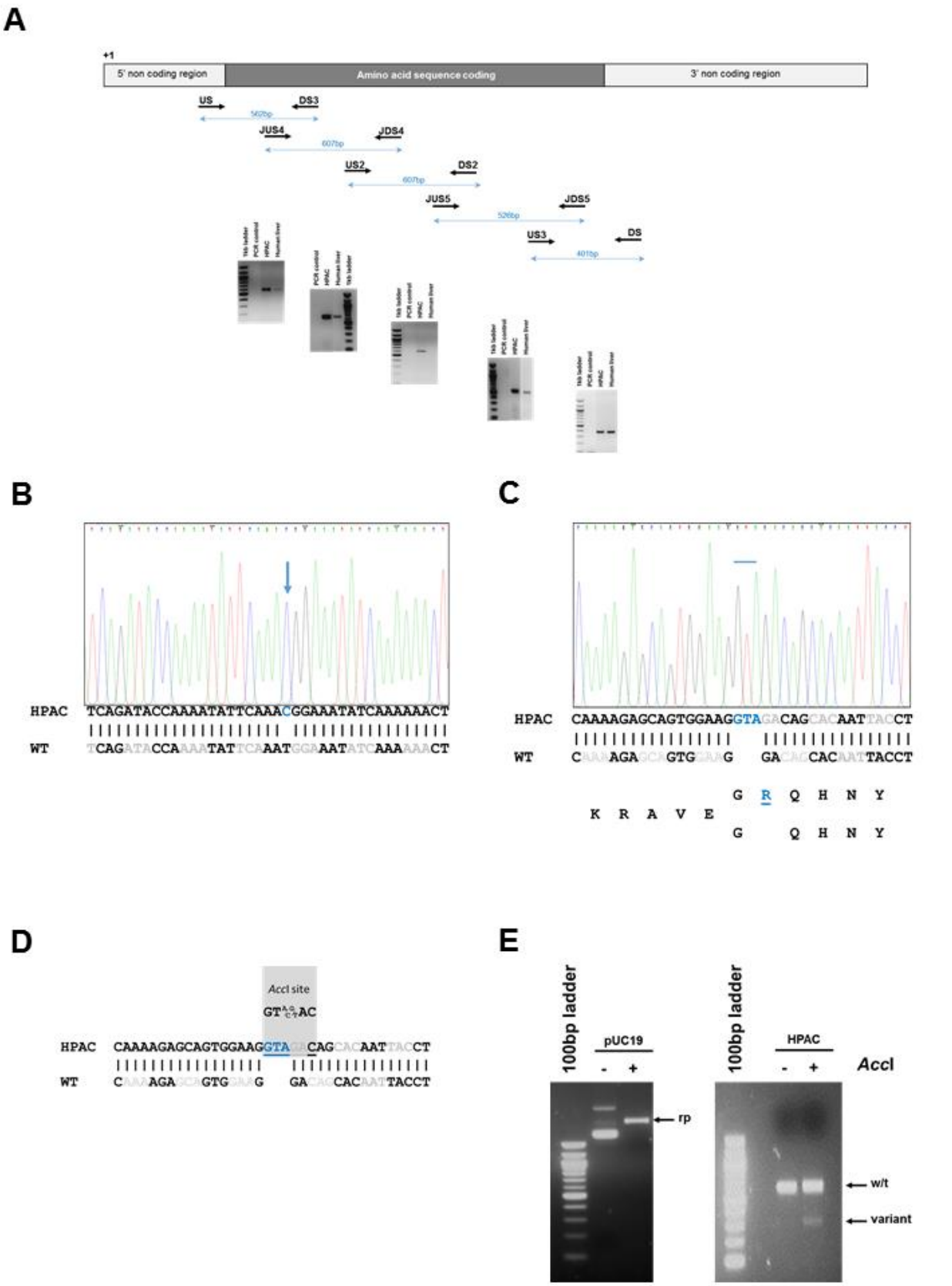

Fairhall et al: Fig. 4

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