HNF4alpha expression amplifies the glucocorticoid-induced conversion of a human pancreatic cell line to an hepatocyte-like cell.

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ABSTRACT

The pancreas and liver are closely related developmentally and trans-differentiation of cells from one tissue into the cells of the other has been documented to occur after injury or exposure to selected growth factors or glucocorticoid hormones. To generate a readily-expandable source of human hepatocyte-like (H-13) cells, the human pancreatic adenocarcinoma cell (HPAC) line was stably transfected with a construct encoding the variant 2 hepatocyte nuclear factor 4 α (HNF4α) using a piggyBac vector and transient expression of a transposase. Through induction of transgene HNF4α regulated via an upstream glucocorticoid response element in combination with existing modulating effects of glucocorticoid, H-13 cells were converted into quantitatively similar hepatocyte-like (H-13/H) cells based on expression of a variety of hepatocyte proteins. H-13/H cells also demonstrated the ability to store glycogen and lipids. These data provide proof of concept that regulated expression of genes associated with hepatocyte phenotype could be used to generate quantitatively functional human hepatocyte-like cells using a readily expandable cell source and simple culture protocol. This approach would have utility in Toxicology and Hepatology research.

Keywords: HPAC, AR42J-B13, H-13, glycogen, steatosis, cytochrome p450.

1.Introduction

Hepatocytes are specialised cells that perform a variety of specific functions not performed by other cell types [1]. The mature hepatocyte phenotype is thought to be dependent on the expression levels and activities of transcription factors that co-ordinate the appropriate hepatic function-defining gene expression pattern. However, this program has been challenging to fully re-capitulate in stem cell-derived hepatocytes in vitro, with cells bearing more comparability to foetal hepatocytes [2-4].

Hepatocyte de-differentiation is a paradigm for specialised cell responses to the in vitro culture environment. De-differentiation may be triggered as early as the cell isolation procedure and likely driven by multiple factors such as cell density, cell-cell interactions, cell-matrix interactions, loss of hormonal regulators and other factors in vitro [5-7]. Under very simple culture conditions (e.g. culture on plastic), hepatocytes may barely resemble their original phenotype within a few days of culture. However, evidence that rat hepatocyte de-differentiation may be markedly halted under these conditions exists since the proliferative AR42J-B13 cell line (B-13 cells) can be differentiated into non-proliferative – quantitatively equivalent – mature adult hepatocyte-like (B-13/H) cells in vitro through the addition of glucocorticoids [8-10]. Critically, once these cells differentiate into hepatocytes, they stably remain in this phenotype for several weeks and do not de-differentiate [10]. Understanding the apparent obliviousness of B-13/H cells to their environment and applying this to stem cells may be one way to overcome the reluctance of stem cells to fully differentiate into quantitatively equivalent, stable adult hepatocytes in vitro, and prevent their de-differentiation.

B-13 cells were originally derived from the rat pancreas and appear to behave as an immortal pancreatohepatobiliary progenitor cell line restricted to differentiation to B-13/H cells [11,12]. Differentiation is triggered by exposure to glucocorticoid which – via the glucocorticoid receptor (GR) – induces serine threonine kinase 1, β-catenin phosphorylation, transient wnt signalling repression, epigenetic changes and induction of hepatic transcription factors Cebpα, Cebpβ, Hnf1α

and Hnf4α [8,10,13,14]. Recently, the human pancreatic adenocarcinoma cell line (HPAC) phenotype was shown to be suppressed by glucocorticoid exposure and to undergo a qualitatively similar expression of genes associated with hepatocytes (although the levels were relatively low compared to intact human liver [15]). In this communication, we report the amplification of hepatocyte gene expression in HPAC cells to protein levels quantitatively similar to human liver.

2. Materials and methods

2.1. Cells, tissues and constructs.

HPAC cells were purchased from LGC Standards (Middlesex, UK). Human induced pluripotent stem cell (hiPSC)-derived hepatocytes were purchased from Definigen (Cambridge, UK) and maintained as described [16]. The empty PiggyBac vector containing a tetracycline regulatable promoter sequence (PB-TET) [17] was kindly provided by Dr Andras Nagy (University of Toronto, Canada). The transposase-encoding construct pCyL4320 [18] was obtained from the Wellcome Trust Sanger Institute (Cambridge, UK). Human liver and pancreas tissue was obtained with patient consent through the Newcastle Hepatopancreatobiliary Research Tissue Bank, with ethical approval by the Newcastle & North Tyneside 2 Research Ethics Committee. Human pancreas and liver tissue was taken from the margins of fresh tissue removed from patients due to the presence of tumours, but was histologically normal. Human acinar cells were prepared as previously outlined [19].

2.2. 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.

HiPSC-derived hepatocytes were maintained as outlined [16] HPAC, B-13 and H-13 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.

2.3 Recombinant DNA and cloning

The GRE4 and minimal promoter sequence from the glucocorticoid receptor inducible reporter gene construct GRE4-pGL4.28 [14] was amplified using a proof reading polymerase by PCR with primers incorporating 5' PacI (ATTTCTCTGGCTTAATTAACCGGTACCTGAGC) and a 3' SacII (ACCAACAGCCGCGGATTGCCAAGCTGGAAGTCGAGCTTC) restriction sites respectively. The blunt ended PCR product was cloned into pCR TOPO (Invitrogen) and sequence checked. The TET-CMV promoter sequence in the PB-TET construct (PiggyBac construct without the pluripotency-inducing genes [17] was restricted with PacI and SacII to remove the tetracycline regulatory sequence and ligated with the GRE4 and minimal promoter regulatory sequence to generate the PB-GRE4prom construct. HNF4α transcript variant 2 (NM_000457.4) was cloned by RT-PCR using the upstream 5'-CACCATGGGACTCTCCAAAACCCTCGTCGACATGGAC and downstream primers 5'-CGGCTTGCTAGATAACTTCCTGCTTGGTGATGGTCGGCTG (containing the CACC + ATG start codon and stop codon respectively). A single PCR product of 1436bp was amplified, ligated into pENTR (Life technologies) and clones sequence checked prior to a sub-cloning the HNF4α sequence into PB-GRE4prom with LR clonase to generate PB-GRE4prom-hHNF4α.

2.4 Gene expression studies

RNA and protein extracts were prepared and RT-PCR performed as previously outlined [13,14] using primers described in Table 1. Western blotting, immunocytochemistry and immunohistochemistry were performed as described [13,14].

2.5 Adenoviral-mediated gene expression

Replication-deficient recombinant adenovirus were generously provided by Harry Heimberg, Vrije Universiteit, Brussels (Ad-null); Emma Regardsoe, University of Oxford, UK (Adv-GFP); Hiroshi Sakaue, Department of Nutrition and Metabolism, Institute of Health Biosciences The University of Tokushima Graduate School 3-18-15 Kuramoto-cho, Tokushima, Japan (CEBPβ LIP; CEBPβ LAP); Marco Pontoglio and Benoit Viollet, Institute Cochin, INSERM U1016/ CNRS UMR 8104/Université Paris-Descartes (HNF1α) and Ramiro Jover Unidad Mixta Hepatologia Experimental & CIBERehd, Departamento de Bioquimica y Biologia Molecular, Universidad de Valencia, Spain (AdV-HNF4α).

2.6 Glycogen analysis

Glycogen was stained in formalin-fixed cells using periodic acid Schiff stain essentially as previously described [20]. For quantitative analyses, a colorimetric method was used, using purified rabbit glycogen as standard [20].

2.7 Lipid analysis

Triglyceride within cells was identified by oil red O staining essentially as already described [21]. After examination, the oil red O stain was extracted from the cells by incubation with 100% isopropanol and quantification through absorbance at 515 nm. The total cell protein was then determined using the Lowry method.

3. Results and discussion

Previous experience with rat B-13 cells has demonstrated that the hepatocyte-like B-13/H cells derived therefrom show good comparability with primary rat hepatocytes with respect to several cytochrome P450s and sensitivities to drug/chemical toxicity [8-10,12-14]. Exposing HPAC cells to glucocorticoid had a variety of effects also observed when B-13 cells were treated with glucocorticoid, including an inhibition in proliferation, phenotypic changes and low level expression

of a variety of hepatocyte-specific transcripts (e.g. albumin) [15]. However, when comparing the response of B-13 and HPAC cells to DEX, it can be seen – based on cytochrome P450 2E1 (CYP2E1) or carbamoyl phosphate synthetase I (CPSI) expression - that the HPAC cells lack the robust quantitatively comparable conversion to hepatocyte-like cells seen with B-13 cells (Fig. 1A). In order to determine whether a minority of HPAC cells were capable of differentiating into hepatocytes, immunocytochemistry for CYP2E1 protein was performed. HPAC cells were rarely observed to be positive after treatment with DEX and the few cells that were positive, showed low levels of both cytoplasmic and nuclear staining, suggestive of non-specific binding of the primary and/or secondary antibodies (Fig. 1B) since CYP2E1 is localised to the endoplasmic reticulum. In contrast, B-13 cells were negative and B-13/H cells robustly positive for cytoplasmic CYP2E1, with the near complete conversion of all B-13 cells to CYP2E1-positive B-13/H cells (Fig. 1B).

[FIGURE 1]

HPAC cells were amenable to adenoviral infection and based on infection with adenovirus encoding green fluorescent protein (AdV-GFP), an approximate MOI of 10 resulted in near complete infection of all cells (Fig. 2A) with minimal evidence of cell toxicity (data not shown). In order to investigate whether DEX-induced hepatocyte gene expression could be amplified in HPAC cells to a level more quantitatively comparable with human hepatocytes, HPAC cells were infected with recombinant adenovirus encoding a variety of genes. Fig. 2B demonstrates that AdV infection itself likely inhibits the hepatocyte-promoting effects of DEX since infection with a null virus (AdV-null) or AdV-GFP reduced the levels of CYP2E1 and albumin mRNA transcripts compared to DEX treatment alone. Based on expression of both CYP2E1 and albumin, AdV-CEBPα or AdV-HNF4α (Fig. 4B) alone further increased their expression induced by DEX treatment, compared to HPAC cells treated with DEX and infected with AdV-null.

[FIGURE 2]

To provide HPAC cells with an amplified hepatocyte phenotype, the piggyBac vector system of genetic manipulation was selected. The approach relies on insertion of target gene(s) with a selectable gene into the host cell genome (through transient co-transfection of a transposase), allowing for stable engineering of modified cells [17,18]. HPAC cells were therefore transfected with a piggyBac G418 selectable vector PB-GRE4prom-hHNF4α, encoding the human HNF4α cDNA sequence regulated by a minimal promoter and the GRE4 enhancer sequence (which confers marked glucocorticoid-dependent inducibility to a 3' gene [14] along with an expression vector (pCyL042) encoding a transposase, thereby placing ectopic HNF4α expression under control of DEX via the GR (see Fig. 3A). Expression of the HNF4α mRNA transcript was increased in HPAC cells co-transfected with the pCyL042 vector and treated with DEX compared to HPAC cells transfected identically but without co-transfection with pCyL042 (Fig. 2C). Examination of co-transfected cells at the protein level confirmed that DEX treatment induced HNF4α protein in a population of HPAC cells, but the levels remained significantly lower than those present in human liver (Fig. 2D). These cells were therefore expanded under G418 selection, sub-cultured and subjected to clonal expansion under selection in 96 well plates. The presence of single colonies in wells were retained and expanded and their response to DEX treatment via induction of the major human hepatocyte cytochrome P450 CYP3A4 protein used to identify DEX-responsive clones (Fig. 3A and 3B). Clone E12, subsequently referred to as H-13 cells was expanded for further analyses. Fig. 3C demonstrated a marked phenotypic alteration when the cells were exposed to DEX. Fig. 3D demonstrates that H-13 cells expressed the highest levels of several hepatocyte proteins (CPSI, CYP2A6, CYP3A4 and albumin), with comparable levels of the major human liver CYP3A4 when compared to human liver. In contrast, hiPSC-derived hepatocytes, even after further infection with a variety of AdV, failed to express detectable levels of these proteins.

[FIGURE 3]

To examine metabolic functions, H-13 and H-13/H cells (i.e. H-13 cells treated with DEX for 14 days) were exposed to high levels of glucose or fatty acids since the liver stores these metabolic fuels primarily in the form of glycogen and triglycerides respectively. Fig. 4A and 4B demonstrates DEX treatment of H-13 or B-13 cells all resulted in cells that were capable of synthesis and storage of glycogen. Notably, H-13/H cells were capable of storing glycogen at levels similar to human liver; stored more glycogen when incubated with high glucose concentrations and were responsive to insulin in that treatment with insulin increased the levels of glycogen synthesised and stored in the cells. Triglyceride synthesis in response to incubation with fatty acids and its intracellular accumulation as lipid droplets (steatosis) also only occurred in H-13 cells converted into their H-13/H phenotype (Fig. 4c and 4D). Treatment with drugs that cause phospholipidosis – tamoxifen, amiodarone [21] - did not result in an increase in steatosis in any cells in any phenotype, suggesting that the lipid droplets are primarily due to triglyceride accumulation and not an inhibition in phospholipid degradation.

[FIGURE 4]

Overall, these data indicate for the first time that inducible expression of HNF4α during HPAC derived H-13 cell conversion to hepatocyte-like cells amplifies conversion to one sharing some similarities with human hepatocytes with respect to several hepatocyte-specific or -enriched proteins. An understanding of the mechanism(s) regulating the conversion of B-13 or H-13 cells into B-13/H or H-13/H cells by glucocorticoid may have implications beyond the confines of understanding cell biology and trans-differentiation. For example, understanding both the stable progenitor nature of the B-13 cell and the resistance of B-13/H cells to de-differentiate may be invaluable knowledge in the stem cell field with regard to maintain a stable plastic and differentiated states respectively in a simple culture system. However, B-13 cells are rat cells and a human cell-based system is likely to have more utility in Toxicology and several other fields. The data presented in this paper provide proof of concept that regulated expression of genes associated with

hepatocyte phenotype could be used to generate quantitatively functional human hepatocyte-like cells using HPAC cells as a readily expandable cell source.

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Figure legends

Fig. 1. DEX treatment fails to induce significant hepatocyte protein expression in HPAC cells, in contrast to rat B-13 cells. A. Western blot for the indicated proteins in HPAC cells (10 μg total protein / lane), results typical of 3 separate experiments; B, Immunocytochemical staining for cytochrome P450 2E1 (CYP2E1) in the indicated cell type treated for 14 days with either DEX (to generate B-13/H cells) or B-13 or HPAC cells (treated with vehicle control), typical of at least 3 separate experiments.

Fig. 2. Expression of HNF4α in HPAC cells enhances DEX-dependent hepatocyte gene expression. A, Infection of HPAC cells with AdV-GFP, HPAC cells were infected with AdV-GFP for 2 days prior to fluorescence imaging (left panels) and quantification of mean and SD percentage of cells expressing GFP (right panel), data 3 separate infection experiments from the same experiment. B, RT-PCR for the indicated transcripts in HPAC cells treated for 7 days with DEX or vehicle control and 2 days after infection with the indicated AdV at an MOI of 10. Results typical of 3 separate experiments. C, RT-PCR amplification of HNF4α mRNA transcripts in HPAC cells transfected with the indicated construct and subsequent selection for cells retaining PB-GRE4prom-hHNF4α with G418 for 7 days. Note treatment of RNA with DNase I was used to prevent direct PCR amplification of the HNF4α sequence within PB-GRE4prom-hHNF4α. D, Western blot of cell extracts (10 μg protein/lane) from HPAC cells or HPAC cells selected for PB-GRE4prom-hHNF4α with G418 for 7 days (HPAC-PB-GRE4prom-hHNF4α) and treated as indicated for a further 14 days.

Fig. 3. Clonally selected HPAC cells with an amplified hepatocyte differentiating response to DEX. A, schematic diagram of H-13 clone isolation and conversion to H-13/H cells. B, Western blot identification of clones for the hepatocyte-specific CYP3A4 protein. Note, that CYP2E1 was not detectable in any clone. C, Photomicrographs of H-13 cells and H-13 cells after treatment with DEX for 14 days. D, Western blot for the indicated protein in the indicated cell extracts (10 μg protein/lane). All data typical of at least 3 separate experiments.

Fig. 4. H-13 cells store glycogen and lipids. A, PAS stain for glycogen in H-13 and H-13/H cells. Cells were cultured for 3 days in culture medium containing normal blood glucose 5mM concentration (N) or high 25mM glucose concentration (H) with or without 10 nM bovine insulin (Ins). Cells were then fixed and stained with or without prior treatment with diastase (which hydrolyses glycogen). B, Glycogen levels in cells treated as outlined in C, mean and SD of 3 separate determinations from the same experiment, typical of 3 separate determinations. *significantly different from equivalent cell type incubated in N medium using the Student's T test (two tailed), P> 0.95. C, Oil red stain for lipids in H-13 and H-13/H cells. Cells were cultured for 3 days in culture medium containing 0.8mM BSA carrier or 0.8mM BSA bound with linoleic and oleic acid at a ratio of 2.5: 1 such that cells were exposed to 2mM of each fatty acid (2mM FA). Alternatively, cells were exposed to the indicated concentration of phospholipogenic drugs tamoxifen or amiodarone. D, Lipid levels in cells treated as outlined in E, mean and SD of 3 separate determinations from the same experiment, typical of 3 separate determinations. *significantly different from equivalent cell type incubated in BSA control medium using the Student's T test (two tailed), P> 0.95.

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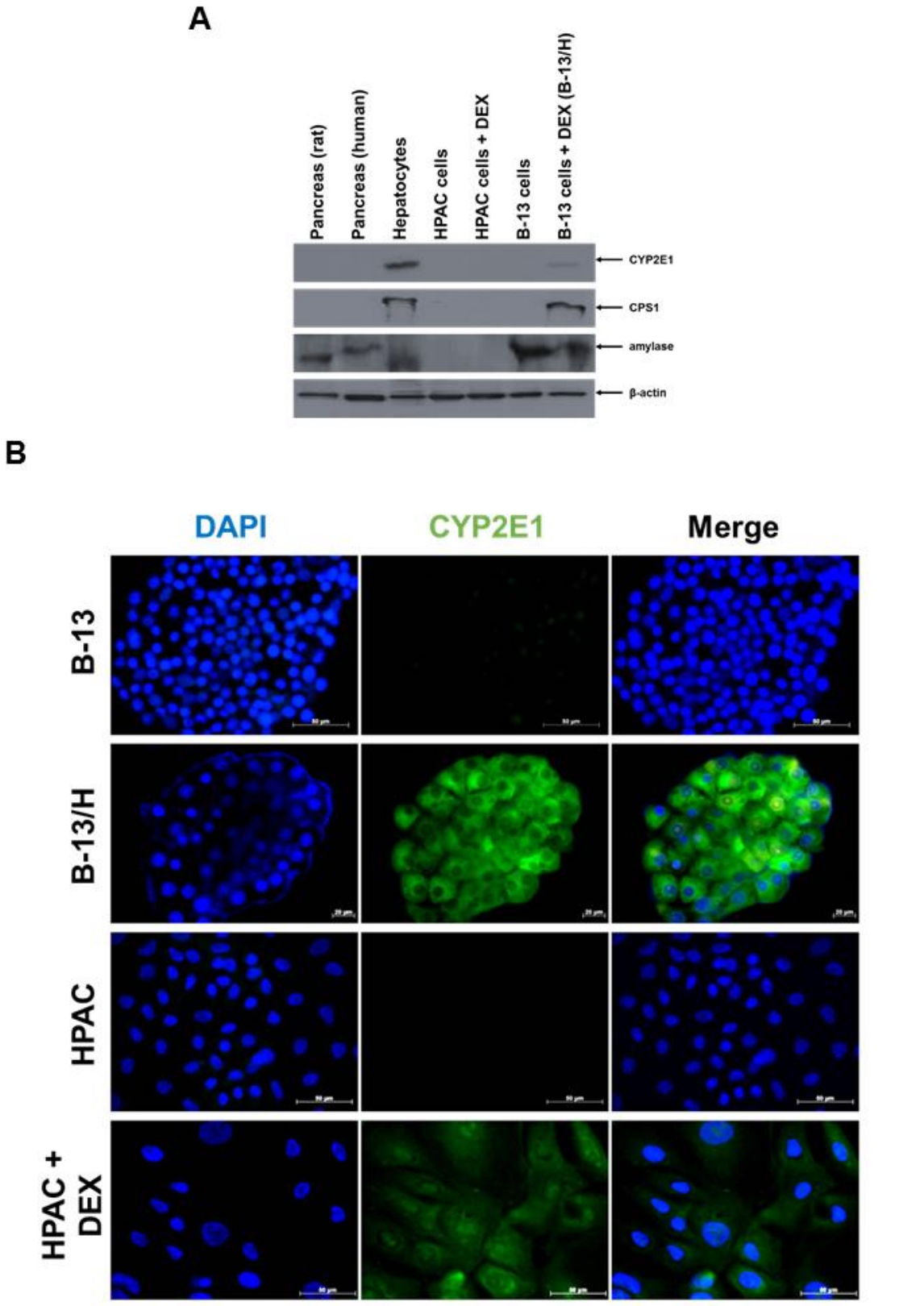
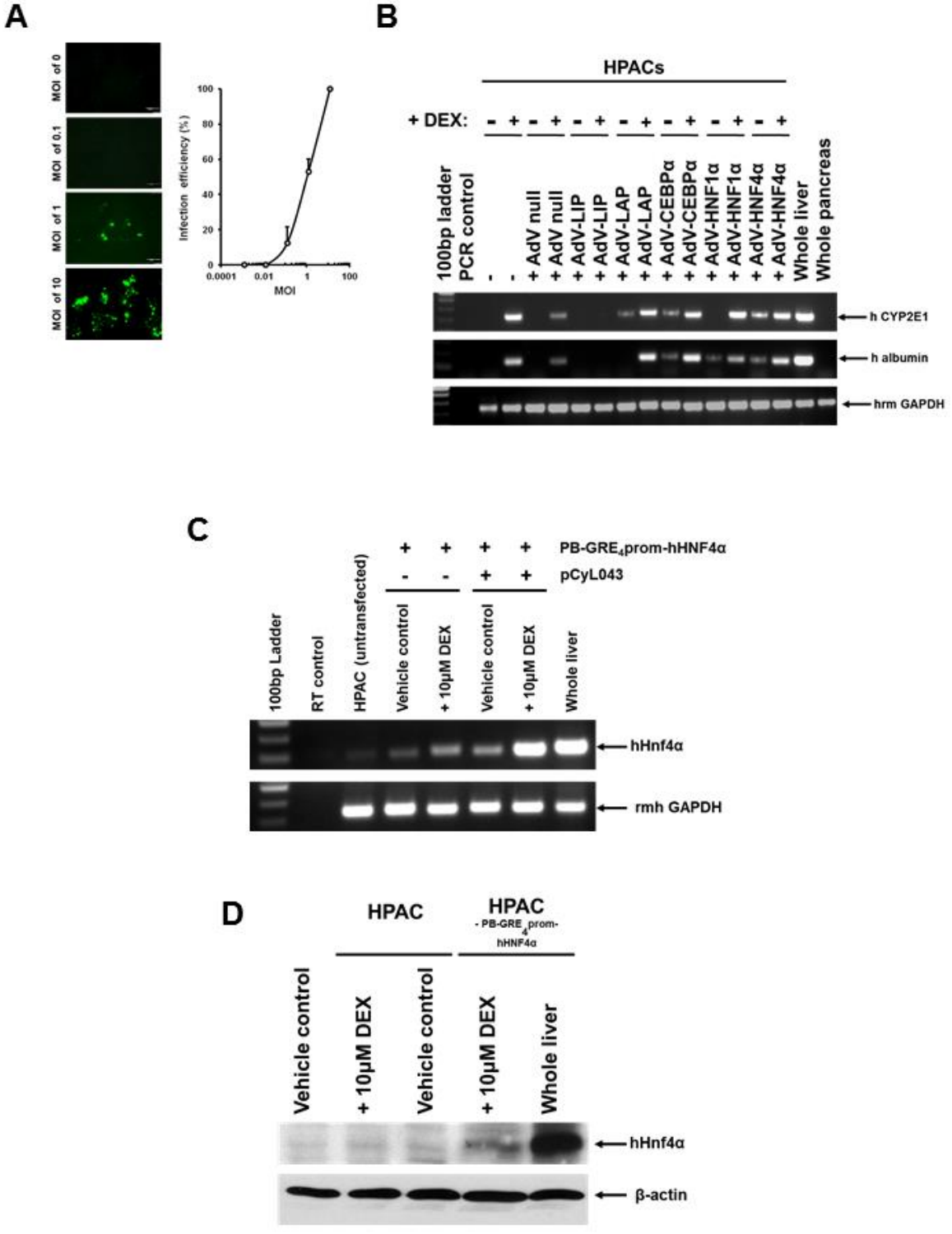
Table 1. Oligodeoxynucleotide primer sequences

ID	Direction	Sequence (5'-3')	Comments
hCYP2E1	**US**	CCCGAGACACCATTTTCAGAG	Will amplify 228bp of human CYP2E1 mRNA (NM_000773.3)
	DS	AGAAACAACTCCATGCGAGCC	
hAlbumin	**US**	AGCTGCCTGCCTGTTGCCAAA	Will amplify 134bp of human amplify albumin mRNA (NM_000477.5)
	DS	AGGCGAGCTACTGCCCATGC	
hHNF4A	**US**	TCCCCATCAGAAGGCACCAACCT	Will amplify 110bp of human HNF4α mRNA, all transcript variants - NM_178849.2 (variant 1), NM_000457.4 (variant 2), NM_178850.2 (variant 3), NM_001030003.2, (variant 4), NM_175914.4 (variant 5), NM_001030004.2 (variant 6), NM_001258355.1 (variant 7), NM_001287182.1 (variant 8), NM_001287183.1 (variant 9), NM_001287184.1 (variant 10)
	DS	CAGCTCGAGGCACCGTAGTG	
rmhGAPDH	**US**	TGACATCAAGAAGGTGGTGAAG	Will amplify 243 bp of rat (NM_017008), mouse (NM_008084) or human (NM_002046) or glyceraldehyde 3 phosphate dehydrogenase mRNA.
	DS	TCTTACTCCTTGGAGGCCATGT	



Fairhall et al: Fig. 1



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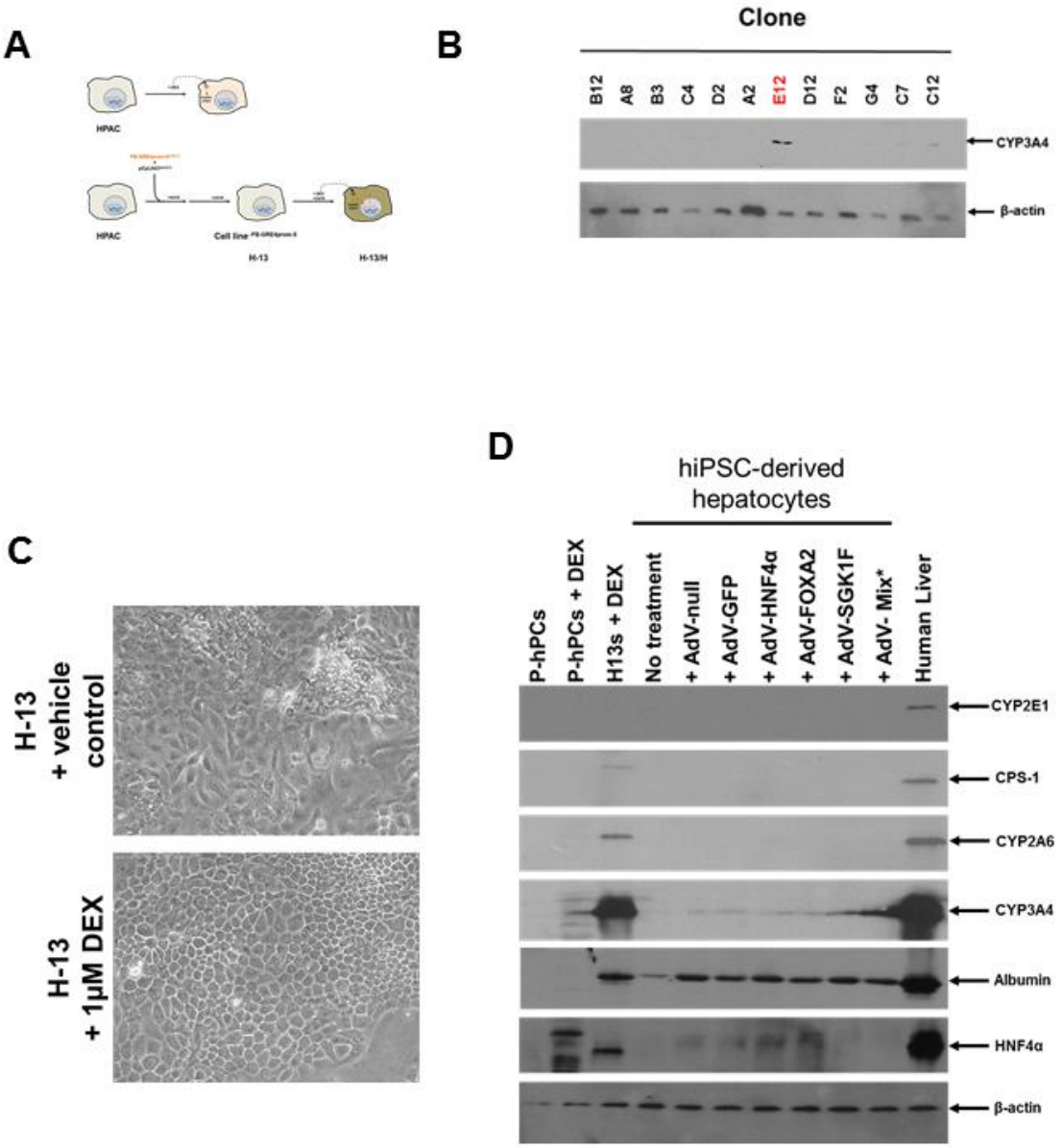

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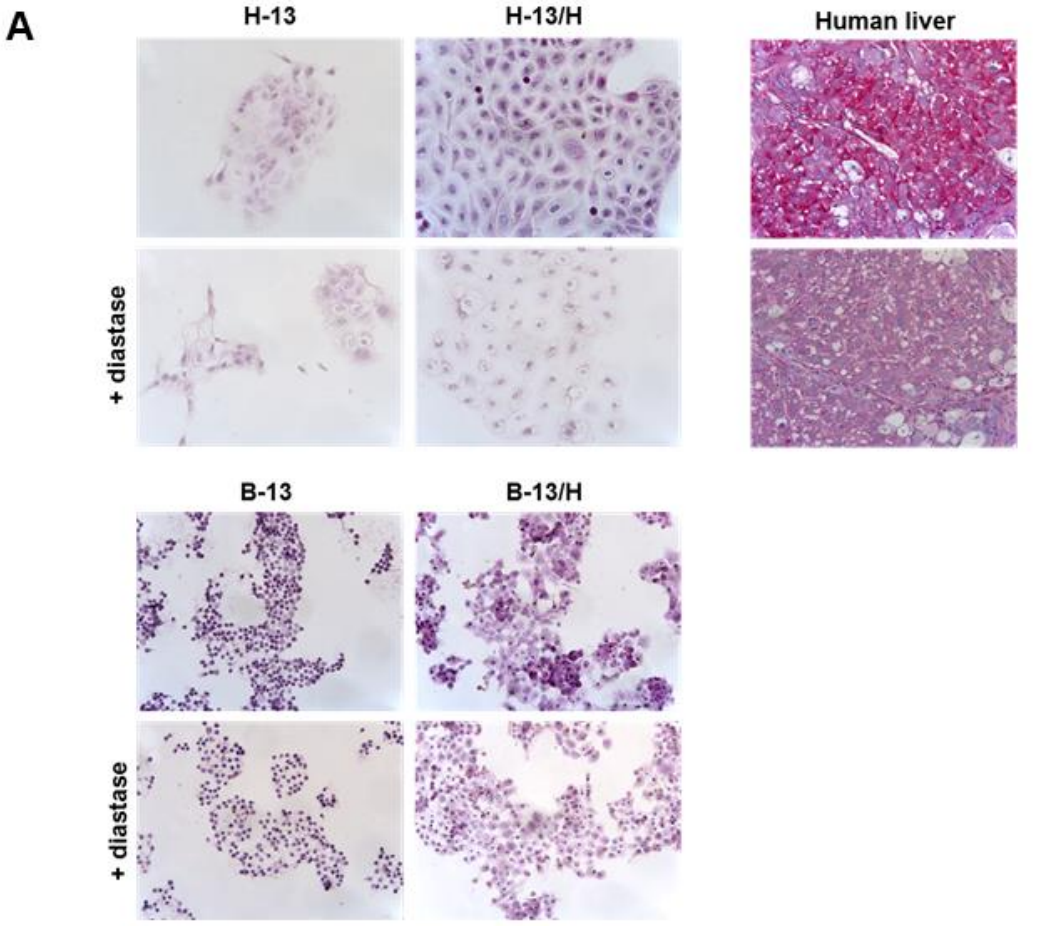

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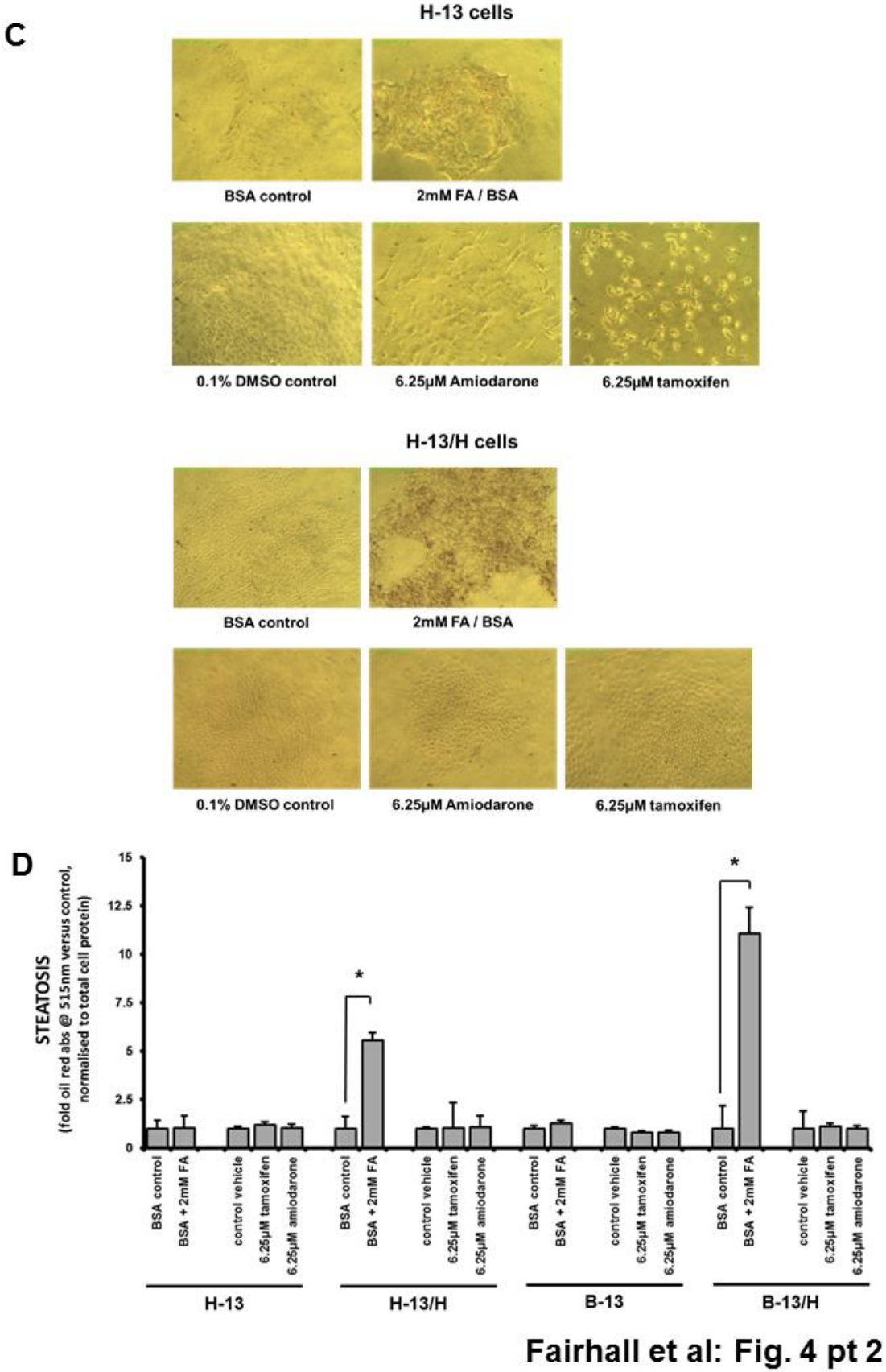

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