Grant Agreement no. 287596

d-LIVER

ICT-enabled, cellular artificial liver system incorporating personalized patient management and support

INSTRUMENT: Collaborative Project (Integrating Project)

OBJECTIVE: ICT-2011.5.1

D8.5: Supply of human pancreatic hepatocyte progenitor cell lines

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PP	Restricted to other programme participants (including the Commission Services)	
RE	Restricted to a group specified by the consortium (including Commission Services)	
CO	Confidential, only for members of the consortium (including Commission Services)	

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1. Executive Summary

A major objective of WP8 is to generate a cost-effective cell line capable of being readily expanded in number in a simple culture system and converted into functional hepatocytes using a simple regulatory switch. The function of hepatocytes generated also needs to be stably maintained in a bioreactor for long periods. Such a cell source is likely essential for any subsequent cell-based bioreactor to become a realistic outcome in the treatment of patients with decompensated liver disease. A progenitor cell from human pancreas that is capable of generating hepatocytes was identified, but sufficient tissue was not available for this approach to be an effective source. Human stem cells were too expensive and failed to generate sufficiently functional hepatocytes. A human pancreatic cell line was therefore examined. Factors which promoted hepatic phenotype were identified through infection with recombinant adenoviruses. A gene was identified as significantly up-regulating hepatic phenotype in glucocorticoid-treated cells. A piggybac vector encoding this gene under control of the glucocorticoid receptor was constructed and a stable cell line generated (H-13 cells). The H-13 cell line is expandable on simple plastic culture, enters a replicative senescence on addition of glucocorticoid, expresses high levels of the piggybac-encoded gene and expresses several liver-enriched genes at levels similar to normal human liver.

2. Introduction

Hepatocytes are the primary defining cell of the liver, performing the vast majority of its functions. Isolation and/or culture of hepatocytes is therefore a potential source of functional cells that could be used in a bioreactor. However, several factors limit hepatocyte utility. Hepatocytes do not proliferate *in vitro* and therefore cannot be expanded *in vitro*. Furthermore, culture results in de-differentiation and loss of function. Complex culture modifications (e.g. matrigel) can ameliorate this loss, but would introduce an array of often uncharacterised factors which likely will complicate use in any bioreactor (e.g. immune response to xenogenic proteins), in addition to markedly increasing costs.

Human liver is in short supply and is often of poor quality. In the absence of sufficient human hepatocytes from donor livers, the main alternative for generating human hepatocytes is through differentiation of human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Despite significant progress over the last 13 years, it has not been possible to generate hepatocytes with function quantitatively similar to adult human hepatocytes. Stem cell-derived hepatocytes remain in a foetal state and cannot progress further unless transplanted in-vivo. Since normal hepatocytes de-differentiate into a foetal state *in vitro* (even when present within culture tissue slices), a barrier to mature hepatocyte formation likely exists in stem cell-derived hepatocyte cultures. In addition, a major hurdle to the use of ESC/iPSC-derived hepatocytes is their high cost of generation (see – d-LIVER consortium. (2012) Developing an "ICT-enabled, cellular artificial liver system incorporating personalized patient management and support" http://www.d-liver.eu/ See: http://d-liver.eu/wp-content/uploads/relative-cost-of-hepatocytes-d-LIVER_TR_2012_WP8-for-web-v2a.pdf).

Early work in WP8 investigated whether a human B-13 equivalent might be suitable for seeding and functioning in a SCS bioreactor, using B-13 cells in a pilot study by UNEW with Charité and SCS (deliverable D8.1).

An in-house antibody-magnetic bead-based methodology was then used to successfully isolate putative progenitor cells from human pancreatic tissue. The isolated cells were examined for the expression of stem cell, neuroendocrine and pancreatic markers by RT-PCR. The progenitor cells were positive for the 4 pluripotency markers and positive for a limited sub-set of pancreatic markers. Cells were cultured and expanded in number. Cells were cryopreserved and successfully revived and treatment with glucocorticoid resulted in the expression of a range of hepatic markers as determined by RT-PCR. The cells isolated using this strategy are therefore a suitable progenitor for human hepatocyte generation *in vitro* (D8.2). However, access to sufficient human pancreas was limited and this approach was halted.

The fallback option of using a derivative of the a human cell line was therefore developed. Based on the data from investigations into Wnt signalling, polymer substratum, oncostatin M and adenoviral-mediated transcription factor expression, it was determined that over-expression of one particular gene was all that was required to generate a relatively functional hepatyocyte.

A piggybac vector encoding this gene under control of glucocorticoid exposure has been generated and several stably transfected cell lines generated. These are now available to other partners.

3. Generation of H-13 cell lines

3.1. Methods

Cloning

The TET-CMV promoter sequence in the PB-TET construct (*piggybac* construct without the pluripotency-inducing genes inserted [1]) was restricted to remove this regulatory sequence and replaced with the GRE4 and minimal promoter regulatory sequence above to generate the PB-GRE4prom construct. Using a pool of human liver RNA, the required gene was cloned by RT-PCR using the upstream and downstream primers (containing the CACC + ATG start codon and stop codon respectively). A single PCR product of the predicted size was amplified, ligated into pENTR (Life technologies) and clones sequence checked prior to a sub-cloning the gene sequence into PB-GRE4prom with LR clonase to generate PB-GRE4prom-X. This construct was transfected with pCyL043 (encoding the expression of a transposase enzyme) into a human pancreatic cell line. Cells were treated with G418 for approximately 2-3 weeks to select for cells that had stably incorporated PB-GRE4-prom-X.

Cell culture

Cells were cultured in low glucose Dulbecco's minimum essential medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 80 U/ml penicillin and 80 µg/ml streptomycin. Cells were incubated at 37 °C in a humidified incubator gassed with 5% CO_2 in air. The PB-GRE4prom-X construct was transfected with pCyL043 (encoding the expression of a transposase enzyme) into the cells. Cells were treated with G418 for approximately 2-3 weeks to select for cells that had stably incorporated PB-GRE4-prom-X. Dexamethasone (DEX) was purchased from the Sigma Chemical Co. (Poole, UK) and was added to medium from 1000-fold concentrated ethanol vehicle solvated stocks, control cells were treated with 0.1% (v/v) ethanol alone as control.

Gene expression

RT-PCR was performed on total RNA isolated using Trizol (Invitrogen, Paisley, UK) essentially as previously outlined [2,3]. Western blotting was performed as previously outlined [2,3].

3.2. Results

A stable transformant line (H-13) was identified that expressed high levels of X mRNA (Fig. 1B) and detectable levels of X protein (Fig. 1C) after treatment with DEX. DEX treatment also promoted a robust epithelial morphology (Fig. 1D). A comparison of the levels of expression of proteins in H-13 cells with human liver and hepatocytes derived from human induced pluripotent stem cells was therefore made. Fig. 1E demonstrates that H-13 cells contain high levels of several proteins, with comparable levels of the major human liver CYP3A4 when compared to human liver. In contrast, hiPSC-derived hepatocytes, even after infection with a variety of AdV, failed to express detectable levels of these proteins.

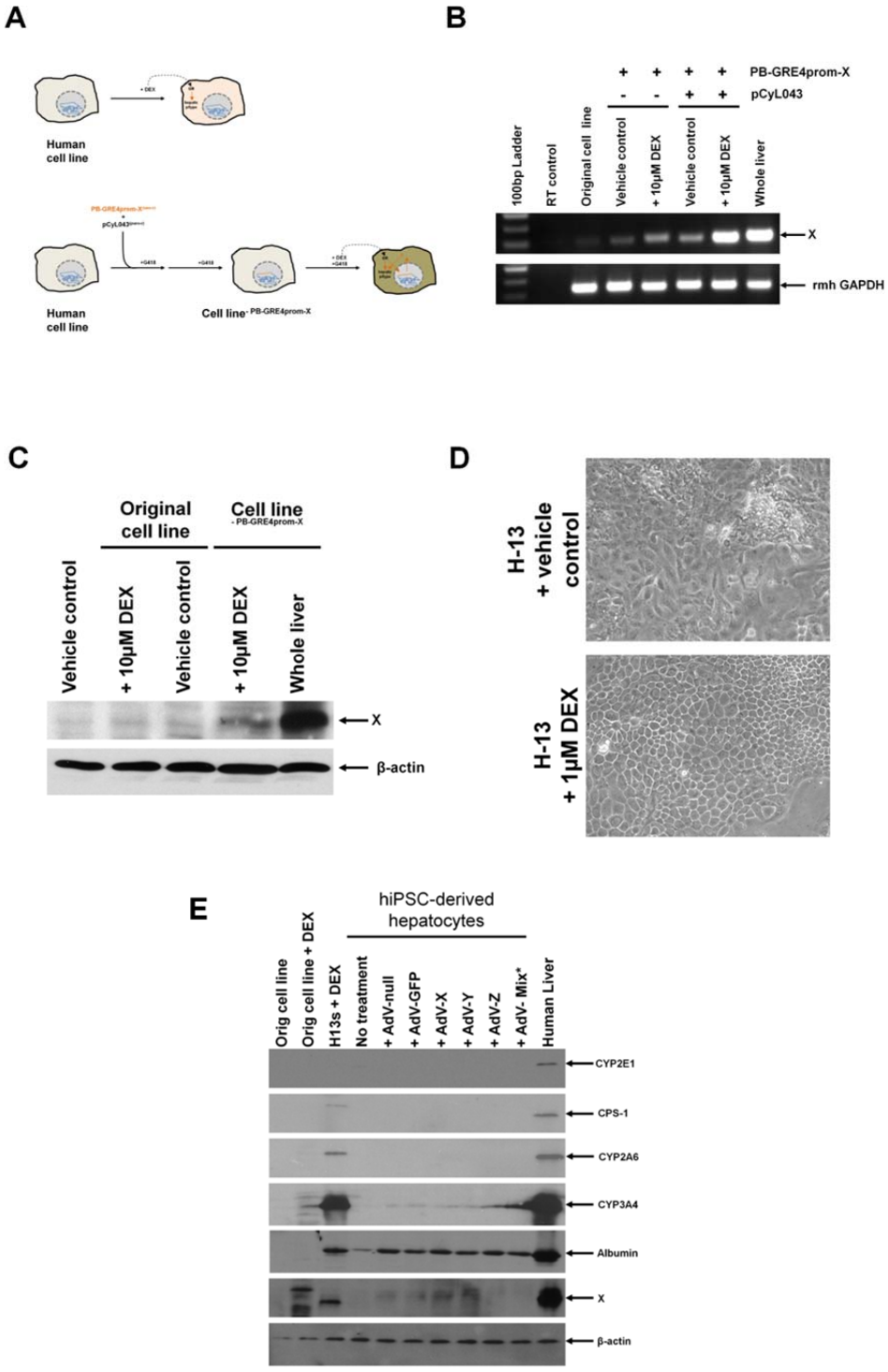

Figure 1. HPACs stably expressing a DEX-inducible X transgene (H-13 cells) express high levels of liver-specific genes: (A) schematic diagram outlining generation of H-13 cells. B, RT-PCR amplification of X mRNA transcripts in the indicated cell line (note treatment with DNase I was used to prevent direct PCR amplification of X sequence within PB-GRE4prom-X); (C) Western blot in cell extracts (20 µg protein/lane); (D) Photomicrographs of H-13 cells and H-13 cells after treatment with DEX or vehicle control for 7 days; (E) Western blot for the indicated protein in cell extracts (20 µg protein/lane). All data typical of at least 3 separate experiments.

4. Conclusions

These data indicate that a human pancreatic progenitor cell line has been engineered so that after expansion and treatment, cells with potentially near liver levels of function can be generated with ease and in a cost-effective manner. These cells are now available for other partners. These cells will be further characterised for hepatic function, including examining their ability to detoxify serum from cirrhotic patients.

5. References

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