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d-LIVER

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

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OBJECTIVE: ICT-2011.5.1

D8.1: Optimum seeding and differentiation protocols for a bio-artificial liver based on experimental rat pancreatic progenitor cell-derived hepatocytes

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1. Executive Summary

The objective of the work reported in this deliverable is to determine whether a rodent hepatocyte progenitor (B-13) cell line can be seeded and differentiated into hepatocytes (B-13/H cells) in the SCS experimental extracorporeal liver system in collaboration with colleagues at Charité. The seeding conditions required for the experimental extracorporeal liver system required changes to the medium to avoid excessive frothing. The altered conditions slowed cell growth but did not inhibit differentiation to B-13/H cells. Two sets of experiments with the experimental extracorporeal liver system were performed and a range of conditions were examined in terms of the number of cells seeded into the device, the concentrations of hormone added to direct B-13/H differentiation and whether it is better to seed pre-differentiated B-13/H cells into the device instead of the progenitor cell. Insights have been gained from the studies, which indicate that seeding with a high number of B-13 cells and a high concentration of hormone does not offer advantages in terms of functionality and that seeding with B-13 progenitors is superior to seeding with B-13/H cells.

2. Introduction

There is currently considerable effort directed towards generating human hepatocytes from stem cells since these would have both basic science (e.g. drug metabolism and toxicity screening) and clinical (e.g. incorporation into bio-artificial liver devices) applications [1]. However, embryonic stem cells (ESC) and induced pluripotent stem cells have so far failed in their ability to generate cells with comparable function to human hepatocytes in vitro [2-4] or at the very least, require efficient (i.e. viral-mediated) forced over-expression of liver transcription factors [5].

One alternative to using stem cells as a source for hepatocytes, is to use progenitor cells. In the liver, a bi-potential population of progenitor cells located within the ductal regions of the lobule is known to exist although harnessing this resource to generate hepatocytes in vitro has yet to be realised because there is little understanding of how to control their differentiation in vitro [6]. The pancreas - which is developmentally closely related to the liver - also contains a ductal progenitor cell [7]. The rat pancreatic progenitor "B-13" cell line appears to be related to this pancreatic ductal progenitor cell. Critically however, B-13 cells appear to be the only cells capable of overcoming the three major hurdles required to generate functional hepatocytes (referred to as B-13/H cells) in vitro [8-11]. The B-13 differentiation into hepatocytes is achieved in a highly cost-effective manner, requiring the addition of a simple glucocorticoid hormone treatment [1,8-11]. In contrast to normal primary hepatocytes, B-13/H cells are formed on a simple plastic sub-stratum and they remain differentiated for at least several weeks [1]. This contrasts markedly with normal primary rat hepatocytes cultured under the same conditions, which lose most hepatic functions within 2 – 3 days of isolation [1].

Over the last few years, we have attempted to place the B-13 response into a physiological context, since a human equivalent would have significant scientific and clinical value. We have shown that treating rats with a synthetic glucocorticoid results in the appearance of acinar cells which stain positive for the expression of hepatocyte markers [12]. In mice with high circulating levels of endogenous glucocorticoid (that gives rise to Cushing's disease-like symptoms by adulthood), a large proportion of the acinar pancreas expressed genes associated with hepatocytes [13]. In a third of these mice, malabsorption developed because of a loss in pancreatic exocrine function [1,13].

The uniquely effective differentiation of B-13 cells to B-13/H cells is associated with genetic alterations in the cells that have resulted in a stable progenitor phenotype for at least 17 years (the cells were first derived in the mid 1990s [14]) and an ability to overcome the three hurdles required to generate hepatocytes in vitro. In this component of the Workpackage WP8, the ability of the B-13 cell to seed into SCS experimental extracorporeal liver system has been examined as a pilot for a human B-13 equivalent to be generated.

3. Pilot studies to assess B-13 seeding into experimental extracorporeal liver system

3.1. Examination of proposed seeding medium conditions on B-13 cell differentiation into B-13/H cells

B-13 cells were grown in medium determined from previous studies at Charité for optimum seeding into experimental extracorporeal liver system (note, lower FCS is required to seed into extracorporeal liver system to avoid frothing of media). The B-13 cells grew at a slower rate but still efficiently trans-differentiated in 2D culture indicating that modified culture conditions will not abrogate differentiation into hepatocytes within the 3D culture system (see Figure 1).

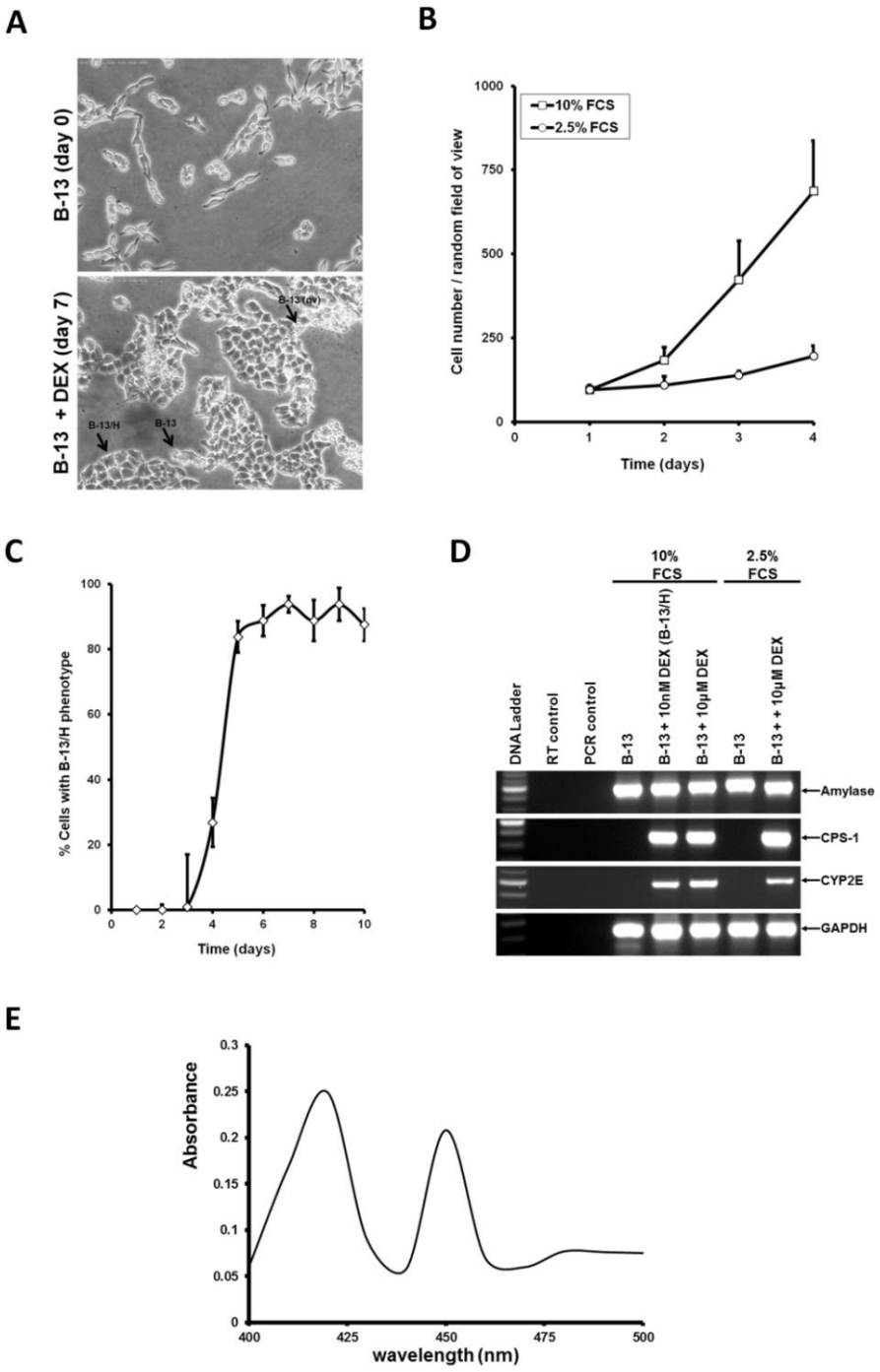

Figure 1: Examining the effects of the modified culture conditions required for culture in the experimental extracorporeal liver system: (A) Morphology of cells treated as indicated; (B) growth rate of B-13 cells at the indicated concentration of FCS; (C) rate of change in morphology of B-13 cells into B-13/H cells with time after exposure to 10µM DEX; (D) RT-PCR analyses for the indicated transcript in cells treated for 7 days as indicated; (E) CO-reduced versus reduced spectrum detection of haemoproteins with cytochrome P450 detectable at 450nm. B-13 cells produced no detectable spectrum (not shown). All data are typical of at least 3 separate experiments. DEX = dexamethasone, FCS = foetal calf serum.

Since cytochrome P450 levels were to be used to determine in part, the functionality of B-13/H cell in an experimental extracorporeal liver system, an examination of the effect of a variety of medium supplements designed to ensure co-factors were not limiting activity, were examined.

Table 1 demonstrates that a normal level of cytochrome P450 can be achieved with 10nM dexamethasone and that an induced level is detected with 10µM dexamethasone. Addition of supplements proposed to enhance haem synthesis (required for function and detection via this method), reduced the levels of induction, indicating that these supplements are not required.

*Table 1: Effect of various culture conditions on the levels of total CO/reduced spectrophotometrically-detectable cytochrome P450 in rat hepatocytes and B-13 cells. Cell samples were washed in 1xPBS and stored as pellets at -80°C until analysis. All data are the mean and standard deviation of at least 3 separate preparations. n/a, not applicable. n/d, not detectable. $^{\$}$5µg/ml bovine insulin, 5µg/ml transferrin, 30nM sodium selenite and 80µM aminolevulinic acid. *2.5mM nicotinamide. $^{\&}$Assumes haemoprotein is all CYP420.*

Cells	[medium glucose]	[Serum] (v/v)	[DEX]	ITS + ALA$^{\$}$	NCT*	Total CYP450 pmol/mg protein	Total CYP420$^{\&}$ pmol/mg protein
Hepatocytes **(freshly isolated)**	n/a	n/a	n/a	n/a	n/a	244 ± 26	493 ± 23
B-13	5mM	10%	-	-	-	n/d	n/d
B-13/H	5mM	10%	10nM	-	-	170 ± 29	400 ± 29
B-13	25mM	2.5%	-	-	-	12 ± 6	n/d
B-13/H	25mM	2.5%	10µM	-	-	840 ± 108	1100 ± 87
B-13/H	25mM	2.5%	10µM	+	-	330 ± 19	610 ± 69
B-13/H	25mM	2.5%	10µM	+	+	75 ± 15	380 ± 33

3.2. Seeding B-13 cells into the experimental extracorporeal liver system (experiment 1)

B-13 cells were transported to the Charité laboratory in Berlin, expanded in number and archived (frozen in aliquots and stored in liquid nitrogen).

B-13 cells were then seeded into experimental extracorporeal liver system and differentiated into B-13/H cells whilst at the same time a batch of cells were cultured in normal 2D culture for comparison. Despite robust trans-differentiation of B-13 cells to B-13/H cells in 2D culture and functional drug metabolism activity, in 3D there was evidence for trans-differentiation but there was low drug metabolic activity in the cells. This may be caused by the material used to construct the 3D device non-specifically binding the dexamethasone hormone used to differentiate cells.

Subsequent analysis of media used confirmed that dexamethasone did not significantly bind non-specifically to the material used to construct the 3D device (Figure 2).

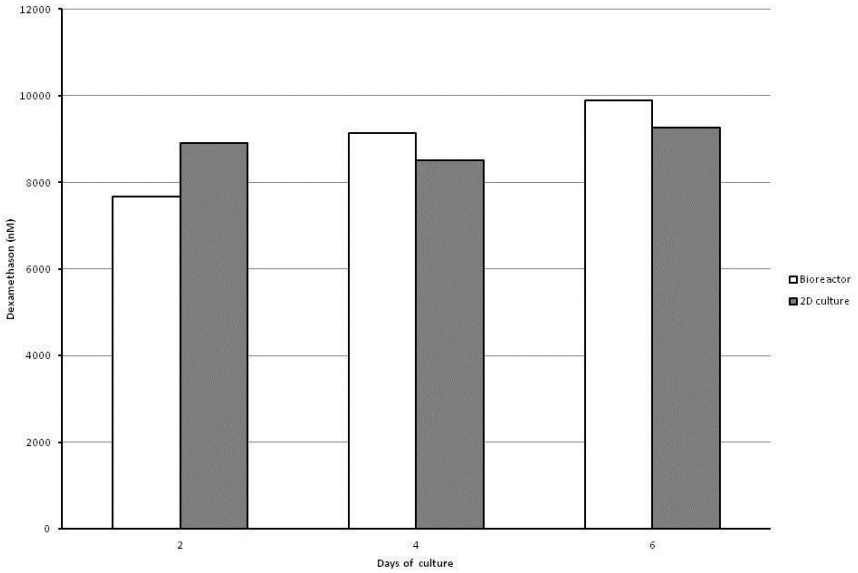

Figure 2: Comparison of dexamethasone levels in media samples from the 3D experimental extracorporeal liver system and 2D cultures of B-13 cells. Samples of media containing 10µM dexamethasone were analysed by HPLC at the indicated number days after B-13 cell seeding.

As a fall back option (in case any future novel 3D matrix required for a human equivalent system binds dexamethasone) a range of glucocorticoid variants were examined (dexamethasone 21-acetate, dexamethasone 21-phosphate disodium and dexamethasone H_2O soluble) were also examined for their ability to direct B-13 cells into B-13/H cells. Figure 3 shows that all variants directed B-13 cells into B-13/H cells and therefore remain a potential alternative glucocorticoid for use if solubility or binding to 3D matrices becomes a problem in the future.

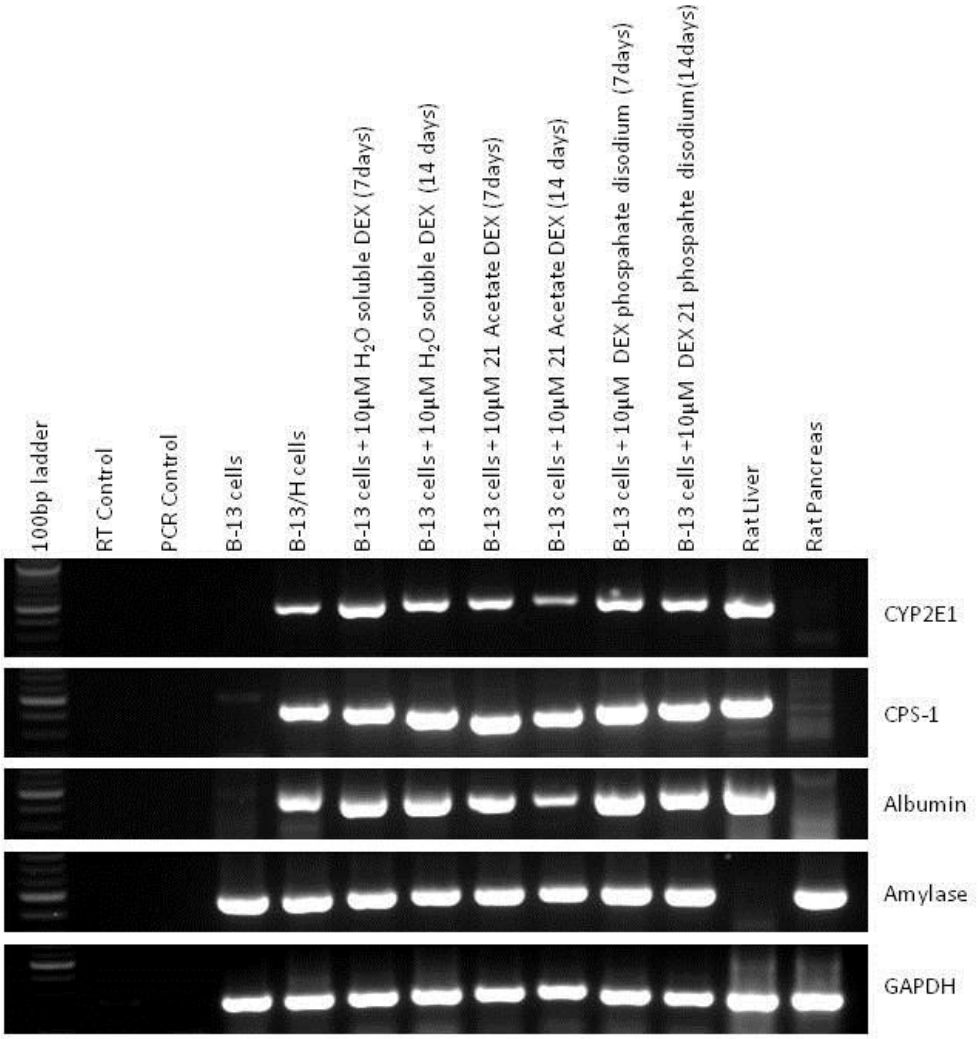

Figure 3: Comparison of different water-soluble dexamethasone analogues for their ability to promote B-13 differentiation into B-13/H cells. B-13 cells were cultured in normal 2D culture and treated for up to 14 days with the indicated steroid prior to RNA isolation and RT-PCR for the indicated transcript. B-13/H cells, B-13 cells treated for 14 days with dexamethasone.

B-13/H viability was assessed in the experimental extracorporeal liver system and in 2D culture using leakage of ALT, AST, GLDH and LDH enzymes (Figure 4).

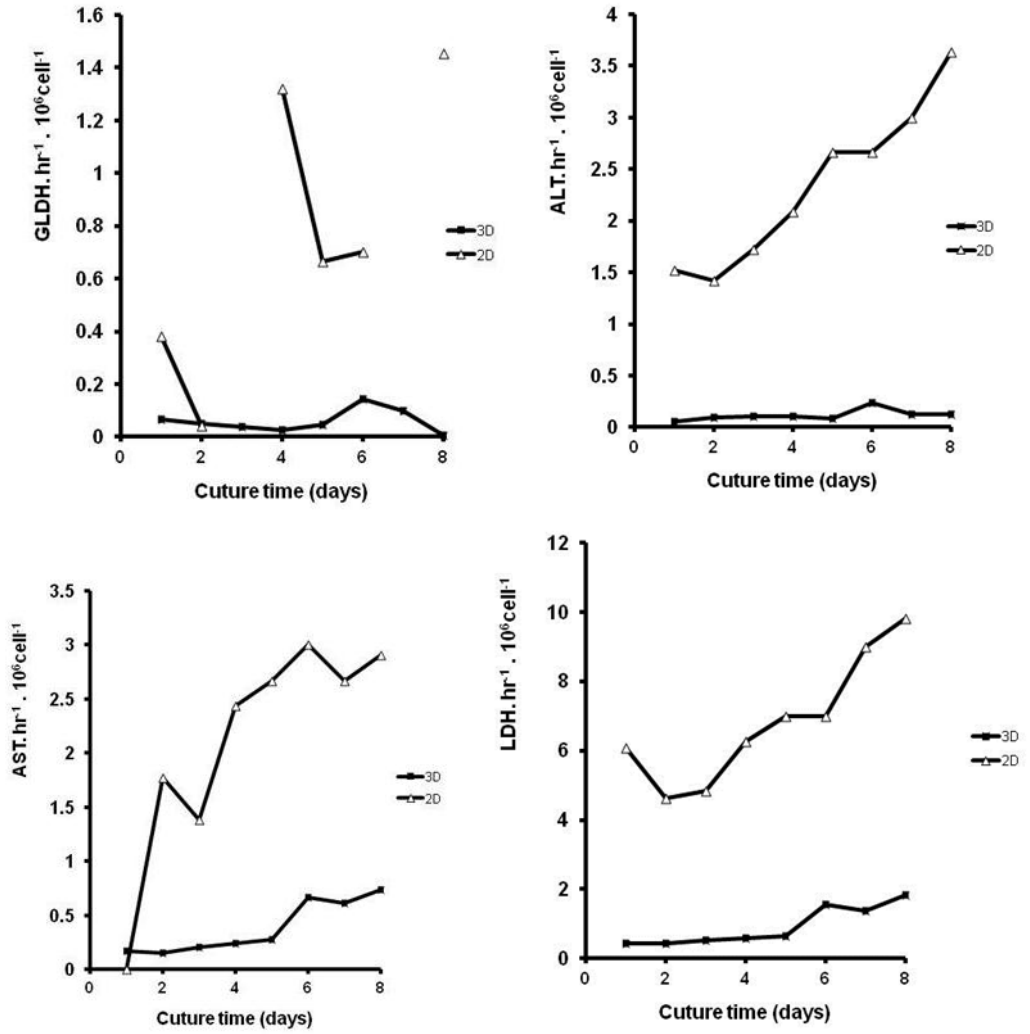

Figure 4: Comparing cell viability 2D and 3D experimental extracorporeal liver system B-13/H cultures. B-13 cells were converted to B-13/H cells in standard 2D culture or within the 3D experimental extracorporeal liver system and the levels of the indicated enzyme activity determined in media with time.

B-13/H metabolism was assessed in the experimental extracorporeal liver system and 2D culture and the levels of glucose, lactate and urea compared (Figure 5). The 3D system showed enhanced hepatic functionality in that there was lower levels of lactate production and higher levels of urea synthesis. Urea synthesis is a liver-specific function and reduction during liver failure is a major determinant in patient survival.

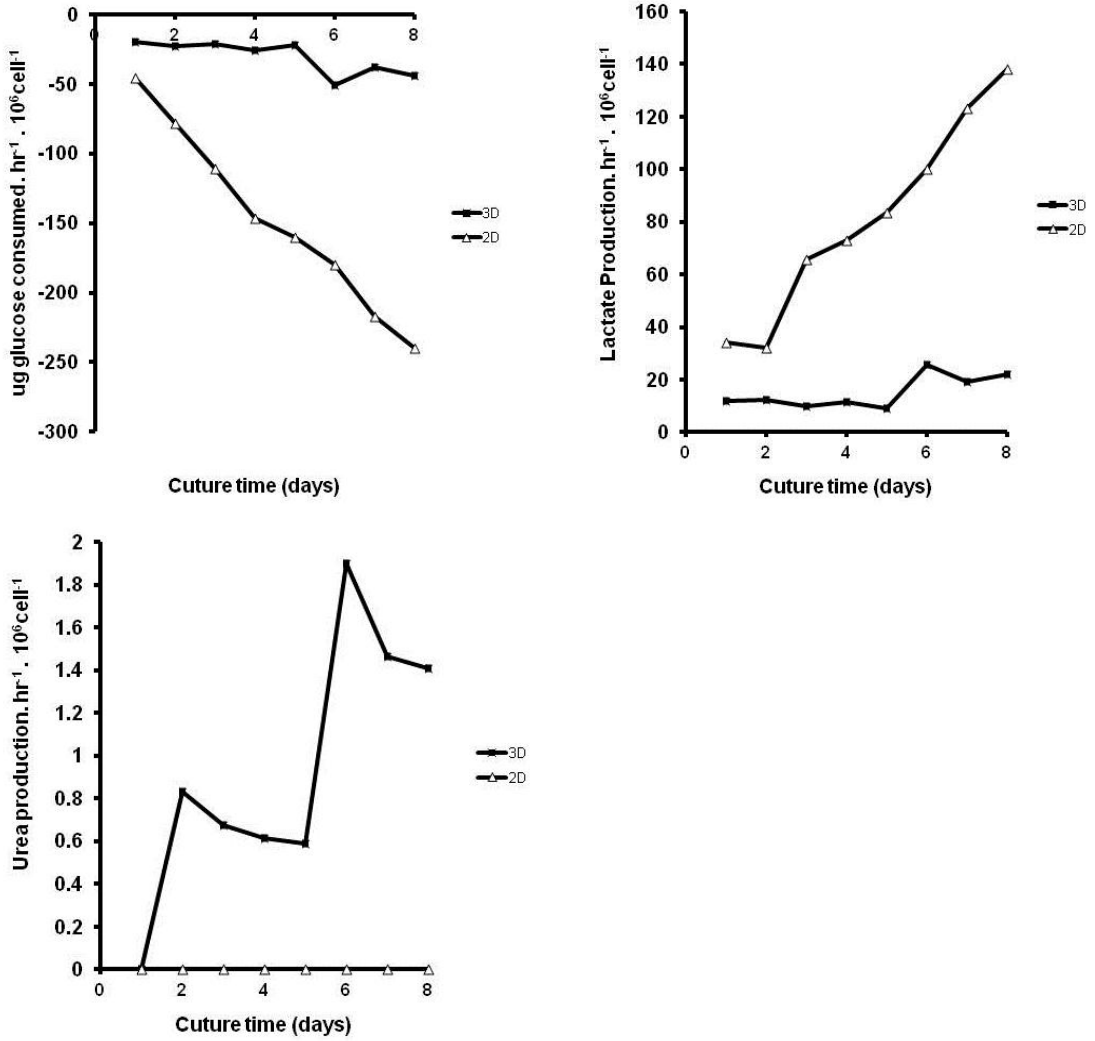

Figure 5: Comparing metabolite levels in 2D and 3D experimental extracorporeal liver system B-13/H cultures. B-13 cells were converted to B-13/H cells in standard 2D culture or within the 3D experimental extracorporeal liver system and the levels of the indicated metabolite determined with time.

Hepatic differentiation was assessed in B-13/H cells in the experimental extracorporeal liver system and compared to 2D culture by RT-PCR (Figure 6) and immunohistochemistry (Figure 7). Figure 7 shows that most liver-specific genes are up-regulated in B-13/H cells within the 3D bioreactor, and that CYP4A, which is refractive to induction in 2D culture, is expressed in 3D cultures.

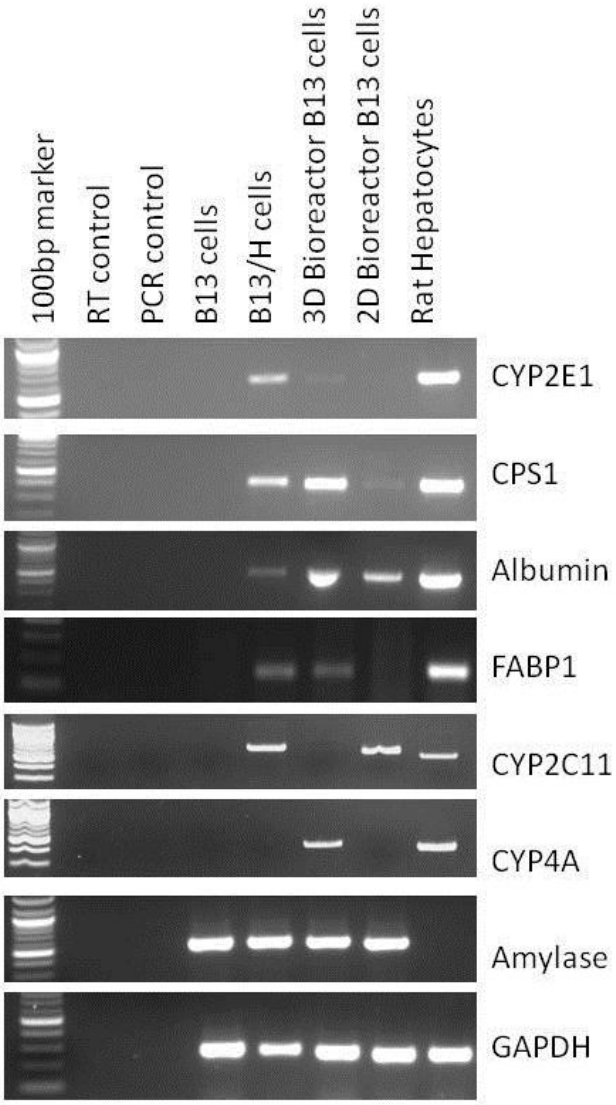

Figure 6: Comparing hepatic gene expression in 2D and 3D experimental extracorporeal liver system B-13/H cultures by RT-PCR. B-13 cells were converted to B-13/H cells in standard 2D culture or within the 3D experimental extracorporeal liver system and the levels of the transcript determined using standard methods.

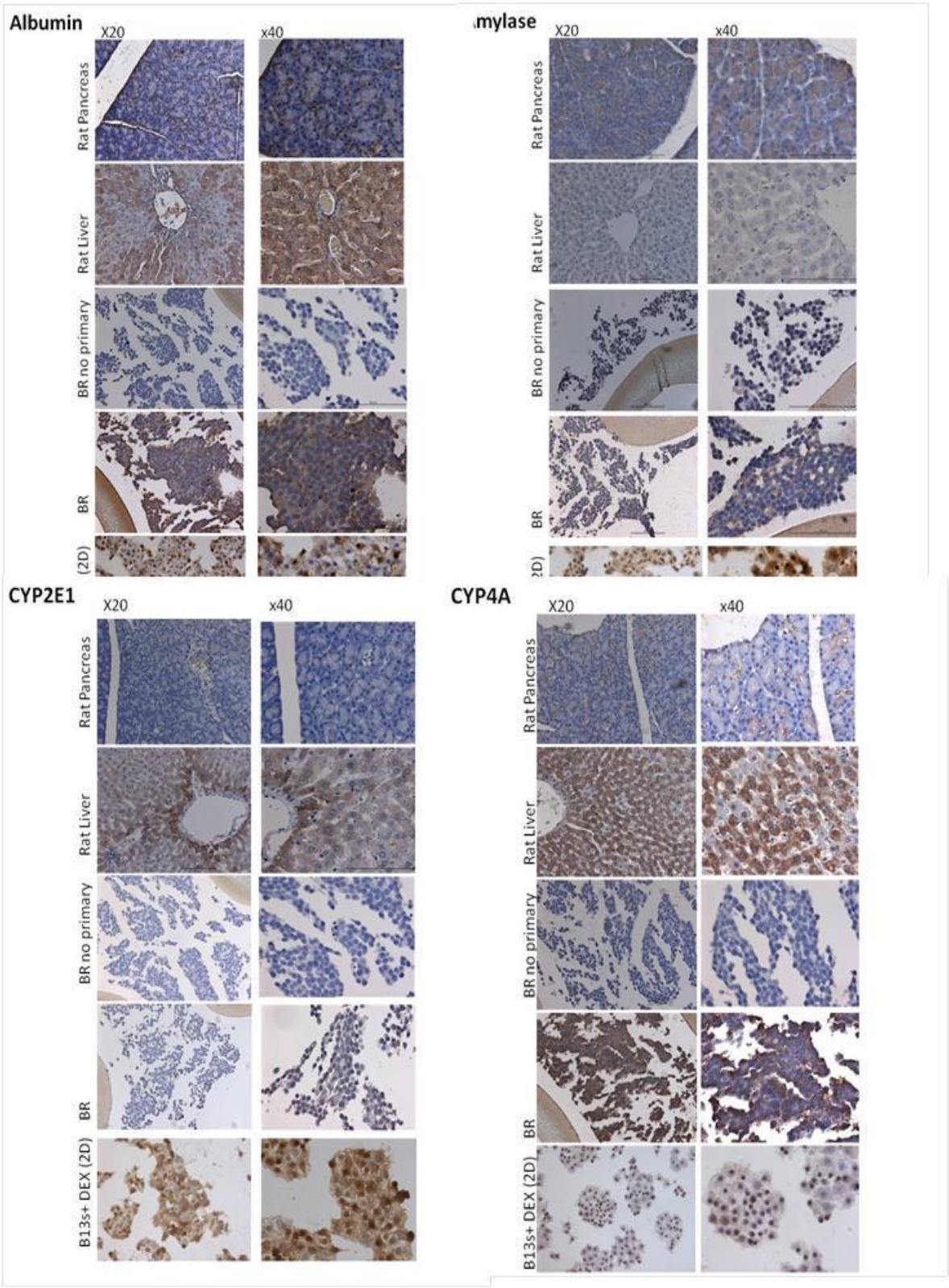

Figure 7: Comparing hepatic gene expression in 2D and 3D experimental extracorporeal liver system B-13/H cultures by immunohistochemistry. Immunohistochemistry for the indicated marker in rat pancreas, rat liver, B-13/H cells in the experimental extracorporeal liver system (BR) or 2D culture (B-13s + DEX(2D)).

B-13/H drug metabolism assessed in experimental extracorporeal liver system and 2D / compared to normal 2D culture (Figure 8). Despite increased gene expression in 3D culture,

functional drug metabolising activity was higher in 2D cultured cells. The potential reason for this may have been that the cells were not cultured for long enough in the 3D device. These issues were examined in the second set of experiments.

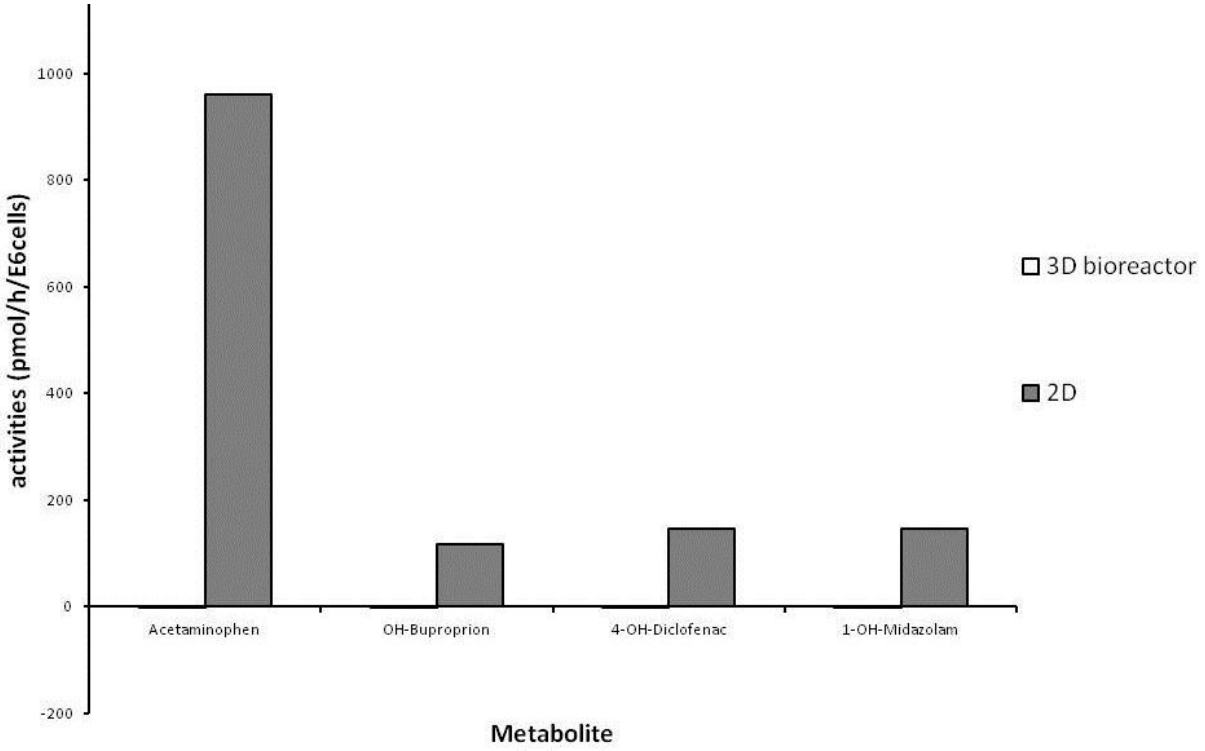

Figure 8: B-13 cells were converted to B-13/H cells in standard 2D culture or within the 3D experimental extracorporeal liver system and drug substrates added. At various time points, medium was withdrawn and metabolism of drug examined.

3.3. Seeding B-13 cells into the experimental extracorporeal liver system (experiment 2)

An experimental extracorporeal liver system experiment with primary rat hepatocytes was conducted to analyse liver-specific functions and xenobiotics metabolism as a control for B-13/H cells. Two experimental extracorporeal liver system runs with different time courses (8 days and 15 days of culture time) were used to investigate the trans-differentiation process of B-13 cells to B-13/H cells in the experimental extracorporeal liver system. One experimental extracorporeal liver system was used for examination for maintenance of the functionality of the differentiated B-13/H cells in the 3D environment (illustrated below in Figure 9).

The cell number used for inoculation of B-13 cells in the experimental extracorporeal liver system was reduced by 50% to improve the trans-differentiation process of the B-13 cells. The establishment of CYP activity testing (7-ethoxyresorufin-O-deethylase (EROD) and 7-ethoxycoumarin-O-deethylase (ECOD) assay) and of further parameters of liver cell function (e.g. galactose and sorbitol) are currently being analysed.

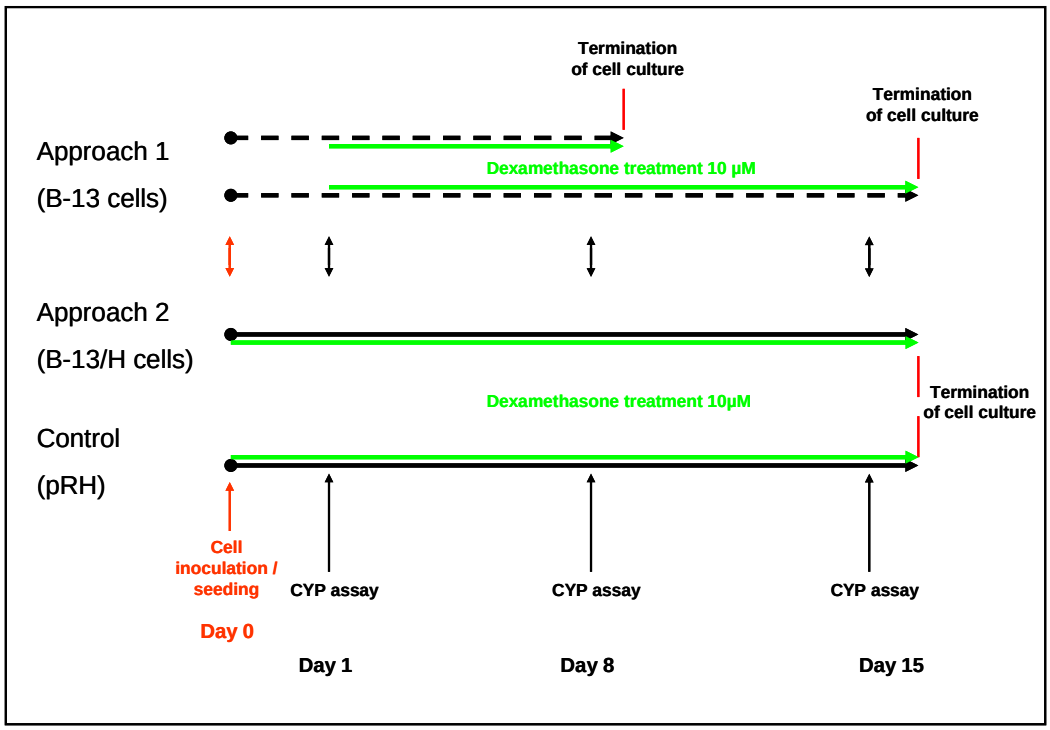

Figure 9: Plan for experiment 2.

Figure 10 confirms that B-13 cells trans-differentiated into B-13/H cells in the experimental extracorporeal liver system as determined through screening for a range of liver-specific transcripts.

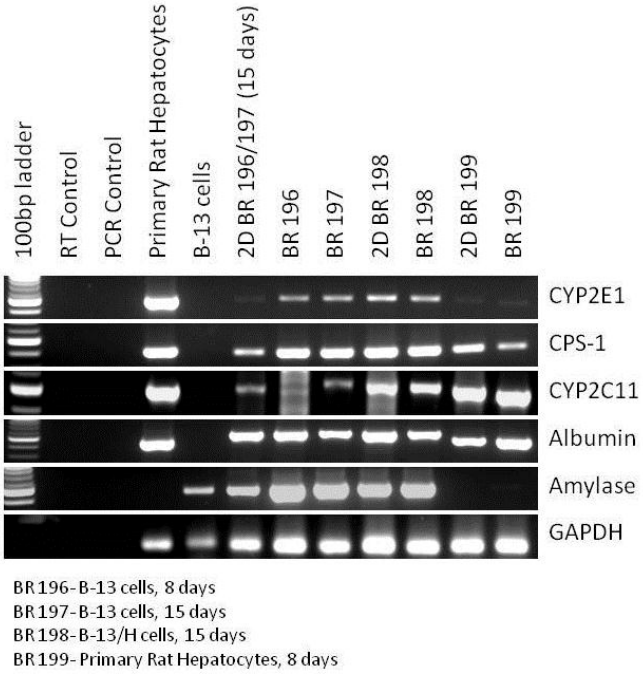

Figure 10: Comparing hepatic gene expression in 2D and 3D experimental extracorporeal liver system B-13/H cultures by RT-PCR. B-13 cells were cultured in normal 2D culture or seeded in the experimental extracorporeal liver system and treated for up to 15 days with the indicated steroid prior to RNA isolation and RT-PCR for the indicated transcript. B-13/H cells, B-13 cells treated for 14 days with dexamethasone.

Gene expression was also examined using immunohistochemistry. Figure 11 shows that a longer period of time for differentiation reduced amylase expression and increased hepatic expression (for e.g. albumin).

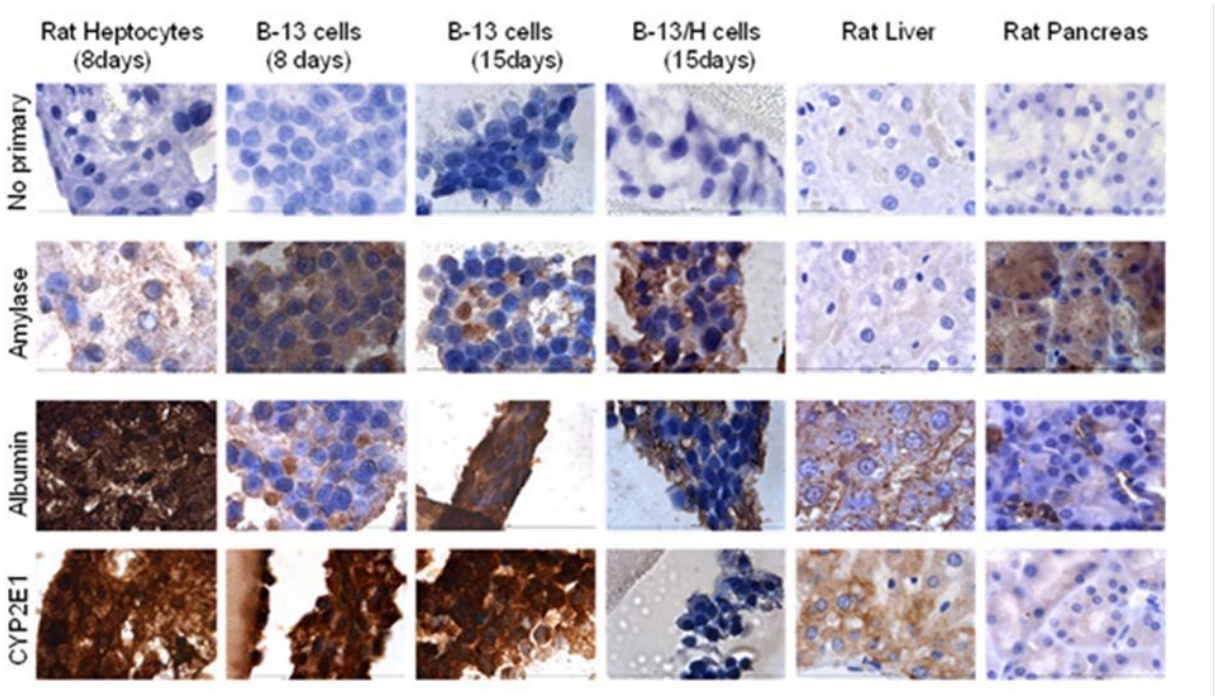

Figure 11: Comparing hepatic gene expression in 2D and 3D experimental extracorporeal liver system B-13/H cultures by immunohistochemistry. B-13 cells were cultured in normal 2D culture or seeded in the experimental extracorporeal liver system and treated for up to 15 days with the indicated steroid prior to fixation and immunostaining for the indicated antigen. Cell cultures are compared to rat liver and pancreas sections as controls.

4. Conclusions

These experiments demonstrate that the B-13 cell may be seeded into the experimental extracorporeal liver system and that the cells are capable of expanding within the device and responding to differentiation signals and forming functional hepatocytes. The data suggest that lower seeding densities are preferred and that differentiating the cells outside the 3D device and then seeding the cells into the device is not optimal.

5. References

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