

Development of Conditionally-Immortalised Human Hepatocyte Cell Lines

Deborah Johnson^{1,2}, Anders Svanberg^{1,2}, John Sinden¹, Nick Plant², & Peter Goldfarb^{1,2}

¹ReNeuron Ltd, 10 Nugent Road, Surrey Research Park, Guildford, Surrey, GU2 7AF, UK

²Molecular Toxicology Group, School of Biomedical & Molecular Sciences, University of Surrey, Guildford, Surrey, GU2 7XH, UK

Abstract

Human hepatocytes are a valuable tool for the rapid assessment of new drugs in terms of hepatic metabolism, enzyme induction and cellular toxicity. However, the establishment of cell lines expressing the full range of adult human liver functions will require novel approaches. Human liver tissue from a 15-week old foetus was obtained according to UK ethical compliance and dissociated with collagenase. Following immunomagnetic purification, parenchymal cells were established on collagen-coated tissue culture plasticware. A restrictive medium was used to discourage growth of non-parenchymal cell types. The primary cells were then conditionally immortalised by retroviral infection with a recombinant human cMycER construct which promotes cell proliferation in the presence of 4-hydroxy-tamoxifen (4-OHT). Following infection, cells were subjected to 14 days culture in a selective medium after which surviving colonies were harvested and expanded. Cell lines were then screened by immunofluorescent staining for the adult hepatocyte markers serum albumin and cytochrome P450. Of the positively-staining cell lines, one (LIV0A07) showed particularly promising growth properties, retained the immortalising transgene and required the presence of 4-OHT for proliferation. LIV0A07 cells did not stain with an anti-fibroblast antibody or for the immature hepatocyte marker α -fetoprotein. Further characterisation of LIV0A07 is now in progress to quantify expression of cytochrome P450 drug-metabolising enzymes and to confirm that the cells differentiate fully when 4-OHT is removed from the medium.

Introduction

The liver is the primary site for the metabolism of drugs in the body, hence liver tissue is used in the assessment of new drugs in ADME/Tox testing.

- Adult primary hepatocytes have low proliferation capacity and variation is high between batches. The use of hepatoma cell lines overcomes problems with proliferation, however the expression of key enzymes may be low, or inappropriate for a model of normal human adult hepatocytes¹.
- In this project human foetal hepatocytes have been conditionally immortalised using a c-mycER construct. It was hypothesised that these conditionally immortalised cells would proliferate in the presence of 4-OHT², while in the absence of 4-OHT the cells would not grow, but differentiate to display characteristics of a mature hepatocyte³.

Materials & Methods

- Human liver tissue from a 15-wk old foetus was obtained according to UK ethical compliance and dissociated with collagenase.
- Following immuno-magnetic purification, parenchymal cells were established on collagen-coated tissue culture plasticware.
- Arginine-free Williams E medium supplemented with ornithine, hydrocortisone, insulin, glutamine, epidermal growth factor and penicillin-streptomycin was used to discourage growth of non-parenchymal liver cell types.
- Actively dividing primary cells were conditionally-immortalised with a recombinant human cMycER construct. Following infection, cells were subjected to 14-days culture in a selective medium, after which 50 surviving colonies were harvested and expanded.
- Characterisation of the cells was performed using telomerase assay, PCR, and immunocytochemistry (ICC).

Results

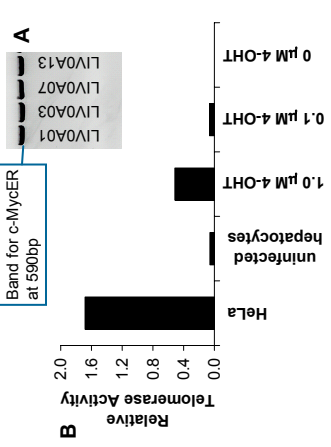


Figure 1: A) RT-PCR was used to confirm c-mycER expression in selected clones. B) Telomerase activity (a measure of c-MycER function) was dependent on 4-OHT.

- Expression of c-mycER was confirmed by RT-PCR, and telomerase activity was driven by 4-OHT in clones expressing the c-mycER construct (figure 1).
- Of the 50 clones harvested one, LIV0A07, showed particularly promising growth characteristics.
- LIV0A07 forms a monolayer on collagen coated plasticware, and grows with a population doubling time of approximately 75 h when exposed to 1.0 μ M 4-OHT.

4-OHT Controls Proliferation in Conditionally-Immortalised Clones

- In culture, primary hepatocytes have a limited capacity for proliferation, however in cells expressing the c-mycER construct the presence of 4-OHT is predicted to drive proliferation². A proliferation assay was performed to confirm this hypothesis and figure 2 shows that LIV0A07 growth is arrested in the absence of 4-OHT.

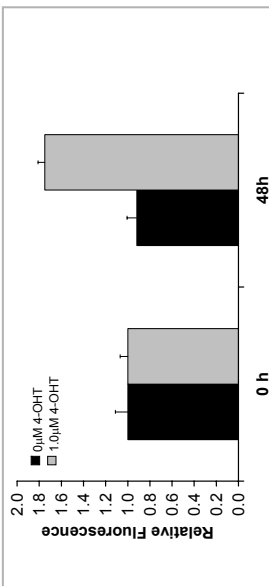


Figure 2: Cell proliferation in 96 well plates was assessed using a cyquant assay⁴. Cells exposed to medium lacking 4-OHT did not proliferate over a 48 h period while the population exposed to 1.0 μ M 4-OHT expanded to 1.7 fold.

Staining for Hepatocyte Markers (1)

- Withdrawal of 4-OHT is predicted to induce differentiation of clones expressing c-mycER. ICC was performed to determine expression of liver markers albumin and CK-18 in proliferating and differentiating (48 h) cells (figure 3).

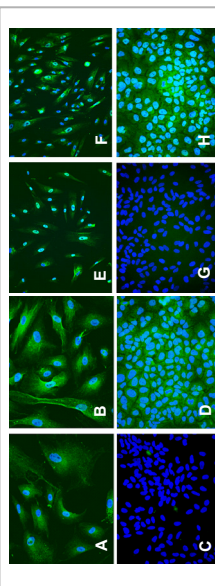


Figure 3: Fluorescent staining of immortalised cells revealed expression of hepatocyte specific markers (green). Albumin was detected (A) in proliferating LIV0A07 (+4OHT), and was enhanced (B) in differentiating LIV0A07 (-4OHT). Cytochrome P450 (C) was also enhanced (F) in differentiating LIV0A07 (-4OHT), and was also staining was present (E) in proliferating LIV0A07, and was also enhanced (F) in differentiating LIV0A07 (-4OHT). CK-18 (D) and CK-19 (G) were used as negative controls for albumin and CK-18, while (D) and (H) are Huh7 human hepatocarcinoma cells stained for albumin and CK-18. Cell nuclei were stained blue with Hoechst.

Staining for Hepatocyte Markers (2)

- No positive staining was observed when cells were exposed to antibodies against α -fetoprotein or fibroblast markers (data not shown).

Cytochrome P450 Expression (1)

- Normal primary hepatocytes in culture rapidly lose expression of the cytochrome P450 enzymes¹. CYP3A4 is the major cytochrome P450 in adult human liver known to metabolise a wide variety of compounds, while CYP3A7 is the foetal form of this enzyme. LIV0A07 was assessed for expression of these enzymes using QRT-PCR on RNA samples from proliferating and differentiating (72 h) cells (figure 4).

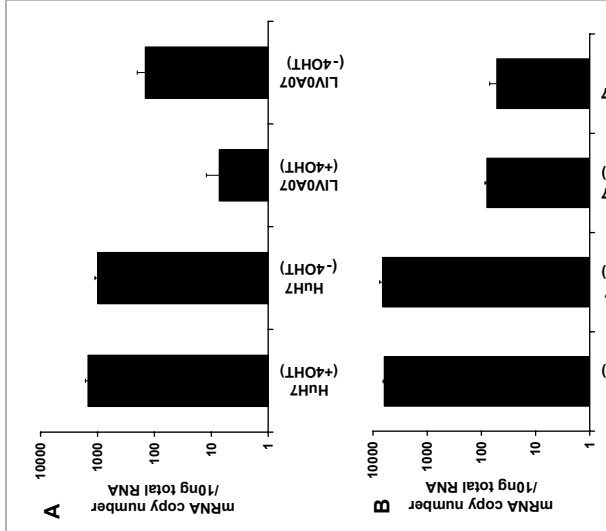


Figure 4: QRT-PCR revealed expression of cytochrome P450 3A4 (A) and the foetal form 3A7 (B) in proliferating (+4OHT) and differentiating (-4OHT) cells. A specific increase in expression of CYP3A4 was seen in differentiated LIV0A07.

Cytochrome P450 Expression (2)

- Whereas in Huh7 cells expression of the foetal CYP3A7 isoenzyme is nearly 10-fold higher than the adult form, in differentiating LIV0A07 cells this is not the case.

Conclusions

- Human hepatocyte cell lines have been established that proliferate in culture and can be induced to display properties of adult human hepatocytes.
- These cells are controlled by a c-mycER construct that is driven by the presence of 4-OHT in the culture medium. Under this system the cells proliferate in the presence of 4-OHT but begin to differentiate to a more mature phenotype when 4-OHT is withdrawn.
- ICC shows that the cells express the liver markers albumin and cytochrome P450, but do not express the immature hepatocyte marker α -fetoprotein. Cells are not stained when exposed to an anti-fibroblast antibody.
- QRT-PCR has been used to examine expression of CYP3A4 and CYP3A7. Initial studies have shown that CYP3A4 (the adult form of this enzyme) is upregulated in differentiating cells while CYP3A7 levels are unaltered.
- Further studies are now ongoing to optimise culture conditions for higher CYP3A4 expression in LIV0A07 and to determine the phenotype in more recently isolated cell lines.

References

1. Rodriguez-Antona C., Donato MT, Boobis A, Edwards RJ, et al. Xenobiotica, 2002, 32, 505-520.
2. Littlewood, TD, Hancock, DC, Danielian, PS., Parker, MG, Evan GI. Nucleic Acids Research, 1995, 23, 1686-1690.
3. Shachaf, C., Kopelman, AM., Arvanitis, CA., Karlsson, A, et al. Nature, 2004, 431, 1112-1117.
4. Molecular Probes, Paisley, UK

Acknowledgements

This work was partly funded by the UK Department of Trade and Industry Teaching Company Scheme (Program number 4118).

