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Assessment of Hepatocellular Proliferation in Mouse and Human Hepatocytes in a Novel Humanized FRG KO Mouse Model

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Summary

We used dual immunofluorescence staining, image analysis and RT-PCR to investigate the phenobarbital (PB) proliferation and cytochrome P450 induction responses, respectively, of mouse hepatocytes in untransplanted FRGKO mouse liver and mouse and human hepatocytes in transplanted FRGKO mouse liver. FRG KO mouse liver can be populated with human liver hepatocytes due to a lack of immune surveillance and a deficiency in fumarylacetoacetate hydrolase (FAH) which confers a growth advantage to the human hepatocytes in the FRG KO mouse liver. The aim of the study was to determine whether or not mouse and human hepatocytes proliferate in response to PB treatment in the context of this in vivo model. There was no increase in hepatocyte proliferation (LI%) in the untransplanted FRGKO mouse liver following 3 days dosing with 75mg/kg PB and this treatment also did not cause an increase in mouse or human hepatocyte proliferation in 40-90% human hepatocyte transplanted FRGKO mouse liver, Table 1. However there were higher levels of hepatocyte proliferation (not stat sig) in mouse and human hepatocytes (and non-hepatocytes) in saline treated transplanted FRGKO mouse liver compared to saline treated untransplanted mouse liver. This effect may reflect sinusoidal cell proliferation and inflammatory cell infiltration, although the equivalent groups dosed with PB showed lower LI's. PB treatment caused a significant induction of Cyp2b10 (6-60 fold) and CYP2B6 (4-5 fold) mRNA in transplanted FRGKO mouse liver, and this treatment also caused an induction of Cyp2b10 in untransplanted FRGKO mouse liver, Figure 4.

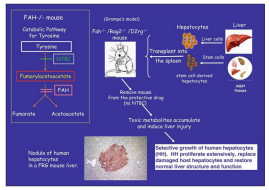

Fig 1: Schematic showing details of the FRGKO mouse model (Azuma et al 2007)

Methods

Animals/treatments

6 control and 6 PB treated FRG KO/, hu- 40%-70% - FRG KO/ and hu- ≥90% - FRG KO/ mice (total 36 liver samples).

Dual Immunofluorescence (IF) staining

Formalin fixed paraffin embedded (FFPE) liver (left lobe) sections were dual IF stained with Anti-FAH (Yecuris # FAH: 20-0034) and Anti-BrdU (Fitzgerald Laboratories #250-BS17) on a Leica Bond Max staining robot using peroxidase conjugated secondary antibodies [goat anti-rabbit HRP (Leica#DS9800) and goat Impress HRP (Vector labs #MP-7405)] and fluorescent tyramide (Cy5 and Cy3), respectively. Slides were counterstained with Sytox green Life technologies #S-34860 (1:1000 dilution).

Whole section image analysis.

Slides were scanned on a Zeiss Axioscan Z1 confocal scanner (x40) and whole liver lobe images subjected to automated image analysis (Fig 3) using Image J software using a script/algorithm in 4 stages:(1) A difference of gaussian filter was used to find non-hepatocyte nuclei based on size; (2) a difference of gaussian filter was used to find hepatocyte nuclei (hepatocyte coordinates were excluded if they were in close proximity to non-hepatocyte nuclei to prevent closely neighbouring non-hepatocytes interfering with the analysis); (3) next human hepatocytes were identified based on the intensity of FAH (red channel) staining in a fixed radius from the centre of the hepatocyte; (4) finally a labeling indices (LI%) for mouse hepatocytes and human hepatocytes were calculated as follows: mouse hep LI% = number of BrdU+ nuclei/total FAH- (mouse hep) nuclei x 100%; human hep LI%= number of BrdU+ nuclei/total FAH+ (human hep) nuclei x 100%.

RT-PCR analysis

Cyp2B6 and Cyp2b10 RT-PCR was performed on RNA was extracted from frozen liver (5x5mm) using Taqman probes specific for these genes (Life technologies #Hs00167937 and # Mm00456588). Ct data was normalised using either human or mouse ubiquitin ligase as endogenous control probes and delta Ct (ΔC_T) values were calculated as follows: e.g ΔC_T = Cyp2b10 C_T-mouse specific house keeping gene C_T. Changes in Cyp2B6 and Cyp2b10 mRNA expression were assessed using delta delta Ct($\Delta\Delta C_T$) calculated as follows: ($\Delta\Delta C_T$ = ΔC_T treated-ΔC_T control).

Results

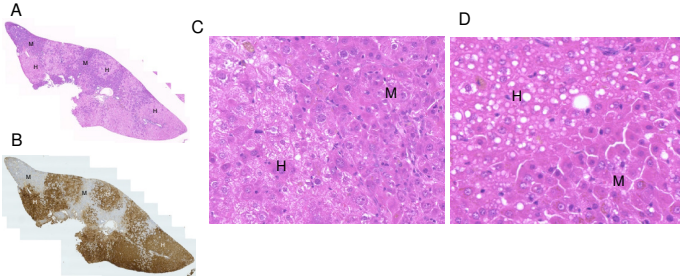

Fig 2 (A) H&E and (B) FAH IHC (DAB) staining of human hepatocyte-transplanted FRGKO mouse liver; (C) control; (D) PB treated. Note: No hypertrophy in PB treated liver. Mouse hepatocytes are more eosinophylic than human hepatocytes. M = Mouse hepatocytes; H = human hepatocytes.

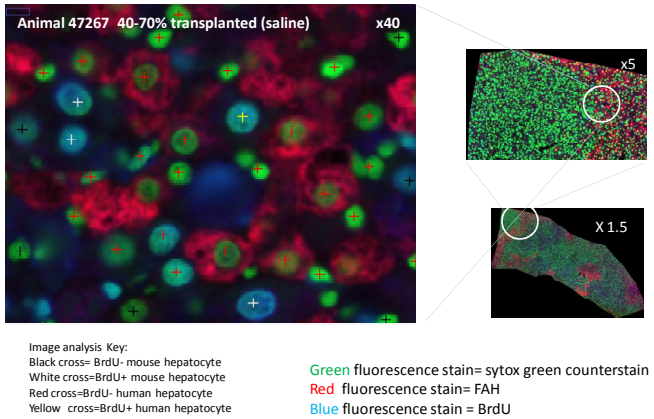

Image analysis Key:
Black cross= BrdU- mouse hepatocyte
White cross=BrdU+ mouse hepatocyte
Red cross=BrdU- human hepatocyte
Yellow cross=BrdU+ human hepatocyte

Green fluorescence stain= sytox green counterstain
Red fluorescence stain= FAH
Blue fluorescence stain = BrdU

Figure 3.Image analysis of human hepatocyte transplanted FRGKO mouse liver sections dual IF stained for FAH and BrdU. The algorithm identifies 4 categories of cells across the whole lobe of the liver (see Image Analysis Key - above) and computes a labelling index (e.g. LI%= BrdU+ mouse hepatocytes/total mouse hepatocytes %) for both mouse and human hepatocytes.

Animal Type/treatment	Group size	Mean Mouse Hepatocyte LI%	SD	Mean Human Hepatocyte LI%	SD
Control (untransplanted)	6	0.06	0.11	N/A	N/A
PB (untransplanted)	6	0.07	0.10	N/A	N/A
Control (40-70% transplanted)	6	3.63	5.91	1.05	1.73
PB (40-70% transplanted)	6	1.58	1.91	0.71	0.87
Control (90% transplanted)	6	1.10	1.65	0.73	1.35
PB (90% transplanted)	6	0.17	0.32	0.04	0.05

Table 1: Proliferation (LI%) of mouse and human hepatocytes in control and PB treated FRGKO mouse liver.
N/A = not applicable due to absence of human hepatocytes in untransplanted FRGKO mouse liver.

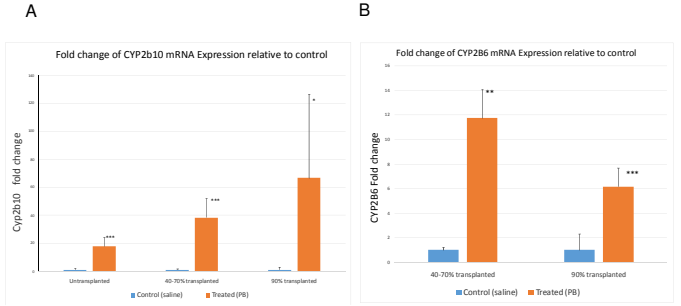

Figure 4. Effect of phenobarbital (PB) treatment on the expression of (A) Cyp2b10 mRNA and (B) CYP2B6 in untransplanted and human hepatocyte transplanted FRGKO mouse liver.

Conclusions

- Following PB administration in untransplanted FRGKO mice the LI% was similar to the control values.
- The mouse hepatocyte control LI% was higher in the transplanted (40-70% and 90%) compared to the untransplanted mice.
- Following PB administration in the transplanted mice, the mouse/human hepatocyte (40-70% and 90%) LI% was lower compared to the control (transplanted) mouse/human hepatocytes.
- Microscopic evaluation showed that whilst the untransplanted mouse hepatocytes appeared relatively normal in appearance, in the transplanted mice the hepatocytes appeared compromised (inflammation, apoptosis and sinusoidal cell proliferation). Nor were the colonising human hepatocytes normal in appearance (fat vacuolation).
- PB administration did not appear to result in the characteristic microscopic hypertrophy, in either the untransplanted or transplanted hepatocytes however there was an induction of Cyp2b10 and CYP2B6 mRNA in mouse and human hepatocytes, respectively.
- These findings suggest that in this experiment the model did not demonstrate the expected proliferative response to PB

This study was supported by Syngenta Ltd