Evaluation of Hepatocarcinogenicity Biomarkers in 3D HepG2 Liver Spheroids Following Nanomaterial Exposure

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Introduction:

- Due to the rapid development and implementation of a diverse array of engineered nanomaterials (ENM), exposure to ENM is inevitable and the development of robust, predictive *in vitro* test systems for hazard characterisation is essential.
- Adverse Outcome Pathways (AOP) describe the sequence of biological events known as key events (KEs) that are required to result in a pathological event and are therefore considered to be useful mechanistic tools for the development of novel endpoint targets for human and environmental risk assessment.
- Understanding AOPs permits the identification of possible mechanistic biomarkers that have the potential to be integrated into advanced *in vitro* testing systems, to improve predictive toxicology.

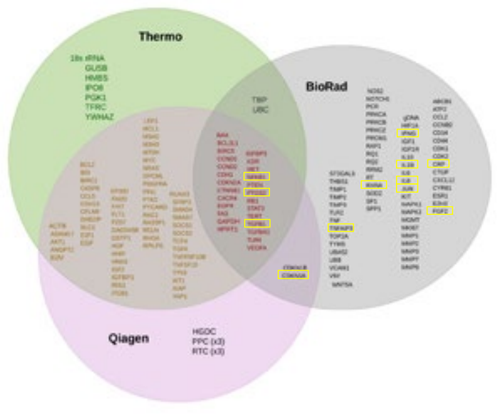

Figure 1. PTGS to Hepatocellular Carcinoma Gene Array Comparison. A cross gene analysis was performed of the genes on pre-defined liver cancer PCR array plates from three companies (Qiagen, Thermo Scientific, and Bio-Rad). Pathway based similarity analysis confirmed that the Bio-Rad plate demonstrated closer overlap (17s genes) with predictive toxicogenomics space (PTGS) liver specific components (which cover 299 genes) when compared to the Thermo Scientific and Qiagen plates (10 and 9 genes respectively).

Aim:

The aim of this study was to develop a novel panel of biomarkers to detect KEs or Molecular Initiating Events (MIEs) that are indicative of AOP. This will allow us to develop the current understanding of human liver AOPs in relation to ENM exposure scenarios and to develop a more targeted *in vitro* testing approach to predict adverse outcomes.

Methods:

- A cross gene analysis was performed of different PCR arrays and genes associated with Liver AOP specific key events (figure 1 & table 1).
- HepG2 3D liver spheroids (figure 2) were used as an effective screening approach for adverse effects to human health following exposure to ENM. The 3D HepG2 cell line based liver model was developed to support both short and long term ENM exposure regimes.
- 3D HepG2 liver spheroids were exposed to either TiO_2 or Ag for up to 120 hours prior to RNA being extracted (figure 3).
- Reverse transcription real-time polymerase chain reaction (RT-qPCR), using a predefined hepatocellular carcinoma PCR array, was used to allow for the study of multiple molecular pathways at a time.

Table 1. A summary of the key genes listed throughout all Liver AOP specific key events. All genes in red are included in the Bio-Rad Hepatocarcinoma PCR Array.

Key Genes listed throughout all Liver AOP specific key events	
ADM	NFKB1
BNIP3	NFKB2
CDKN1A	NFKBIA
CEBPB	NFKBIE
CFLAR	PTGS2
CXCL2 – CXCL12	PDGFA
CXCL3	RELB
FGF2	RXRA
ICAM1	SERPINE1
IER3	SMAD3
IFNG	SMURF2
IL1B	TGFB2 – TGFB1
IL8	TNFAIP3
JUN	TGM2
MAP2K3 – MAPK1 / MAPK3	TNIP1
MAPK9	

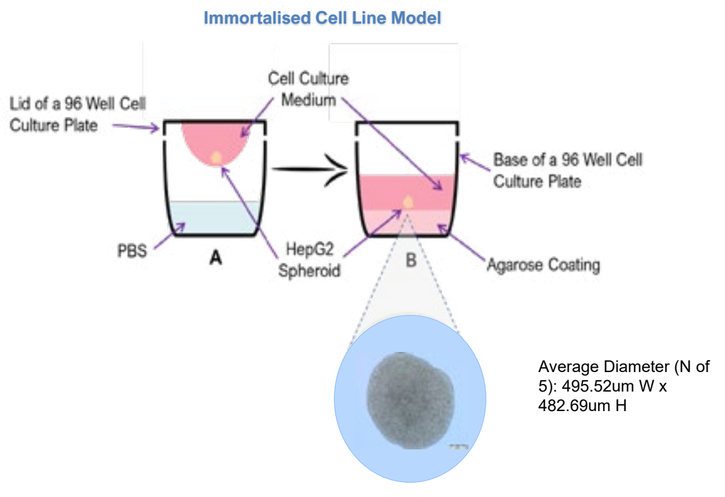

Figure 2. Schematic diagram displaying the formation of 3D HepG2 spheroid model using the hanging drop method initially (A) before transferring into agarose coated well plates (B).

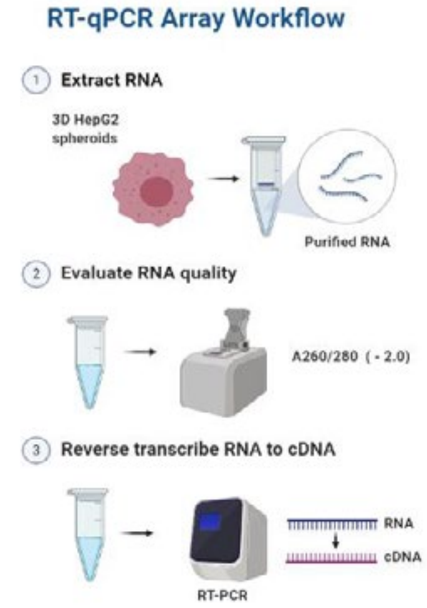

Figure 3: Schematic Diagram of RT-qPCR Workflow (Created with BioRender.com).

Results:

Table 2: Changes in the 3D liver spheroids gene expression profile following 24hr exposure to AFB1, Ag and TiO_2. Red cells indicate upregulation & yellow cells indicate down regulation. *FC, fold change, SEM, standard error of the mean*.

	AFB1		AG 1.0		AG 5.0		TiO2 1.0		TiO2 5.0	
	FC	SEM	FC	SEM	FC	SEM	FC	SEM	FC	SEM
ATF2					7.188	18.214				
BCL2L1					4.671	15.081				
CCND2	19.356	16.725			8.109	26.991				
CD44					21.927	54.750				
CDH1					4.244	12.176				
CDKN1A					20.500	51.187				
CDKN2A					6.707	13.194				
CTNNB1					4.667	15.179				
FGF2					15.692	39.182				
HIF1A					7.720	16.937				
IGF1							10.231	12.594		
IGFBP3					5.307	15.228	4.786	4.088	6.213	2.995
IL8					6.878	22.261				
JUN					7.613	20.998				
KIT			5.532	2.061	24.170	60.349				
MAPK1					7.027	17.459				
MET					6.111	17.388				
MMP1					11.189	27.938				
MMP2	-4.572	0.189			24.392	60.904				
MMP7					4.198	10.482	-4.882	0.072		
NFKB1					7.738	22.703				
NOTCH1	6.225	5.379								
PRKCB					9.437	23.563				
PTEN					20.385	48.127				
RRM2					4.676	8.399				
RXRA					-4.415	0.970				
SP1					8.443	20.487				
TGFB1					4.393	17.217	5.069	3.818	4.264	3.599
THBS1					4.019	11.357				
VCAM1	5.673	4.902			8.665	21.635				
WNT5A	4.491	3.880			11.550	25.678	5.923	2.518		

Table 3: Changes in the 3D liver spheroids gene expression profile following 120hr exposure to AFB1, Ag and TiO_2. Red cells indicate upregulation and yellow cells indicate down regulation. FC, fold change, SEM, standard error of the mean.

	AFB1		AG 1.0		AG 5.0		TiO2 1.0		TiO2 5.0	
	FC	SEM	FC	SEM	FC	SEM	FC	SEM	FC	SEM
ATF2	-6.854	0.317								
BAX	-44.296	0.096			-9.180	0.000				
BCL2L1	-30.163	0.123			-6.559	0.000				
BIRC5	-24.153	0.137								
CCNB2	-16.108	0.167								
CCND1	-73.252	0.070	4.768	0.282						
CD14	-5.406	0.512					7.216	19.300		
CD44			4.521	0.121	5.191	0.000	14.511	30.115		
CDK1	-6.590	0.290								
CDK2	-86.185	0.042								
CDKN1A			6.816	0.151	-6.189	0.000				
CDKN1B	-12.161	0.248								
CTGF	-18.864	0.151								
CTNNB1	-15.417	0.224								
CRP			5.255	2.870						
CXCR4					-11.604	0.000	25.072	52.032		
CYR61	-8.491	0.339			-4.341	0.000	18.138	37.642		
EGFR	-22.285	0.160								
ESR1			30.683	16.755					20.954	13.415
EZH2	-19.873	0.155								
FAS	-8.417	0.325								
FGF2					7.581	0.000	17.988	37.331		
HIF1A	-6.730	0.233								
IGF1	9.057	0.000			4.577	0.000				
IGF1R	-9.734	0.309								
IGFBP3	-40.846	0.083			-6.029	0.000				
IL10			7.190	0.452					-8.149	0.397
IL8	-8.956	0.331								
IL6							6.930	14.383		
JUN	-10.978	0.182								
MAPK1	-6.850	0.289								
MET	-14.698	0.223								
MGMT	-14.800	0.228								
MKI67	-27.950	0.109								
MMP1							21.515	44.651		
MMP3	-9.248	0.226	6.243	0.128					-4.552	0.265
MMP7					4.917	0.000				
NFKB1	-10.111	0.296								
NOTCH1	-8.676	0.000	46.647	0.012						
PRKCB					67.286	0.000				
PRKCZ	-7.364	0.357								
PROM1	-22.098	0.147								
PTEN	-9.277	0.281								
RAF1	-37.832	0.089			-5.991	0.000				
RB1	-31.684	0.086								
RRM2					6.625	0.000	5.196	8.692		
RXRA	-190.567	0.028								
SOD2	-21.750	0.149								
SP1	-11.755	0.208								
SPP1	-5.623	0.352								
ST3GAL5					-10.107	0.000				
STAT3	-43.198	0.352								
TERT							12.723	26.405		
TGFB1					-5.281	0.000				
TGFBR2	-25.607	0.148	11.751	0.151	-11.347	0.000			-5.941	0.431
TIMP1	-22.320	0.147								
TIMP2	-79.553	0.059								
TIMP3	-46.668	0.077			-4.077	0.000				
TNF			4.640	2.534						
TNFAIP3	-7.812	0.377								
TOP2A	-10.934	0.298								
TLR2	0.244	0.000								
TYMS	-13.108	0.199								
UBA52	-47.167	0.073								
UBB	-22.496	0.151			-5.829	0.000				
UBC	-71.676	0.065			-5.881	0.000				
VEGFA	-48.828	0.083								
VCAM1							8.141	16.895		
VIM									-4.736	0.135
WNT5A	-4.918	0.330								

Table 4. Key genes highlighted from gene expression qPCR arrays under different treatment conditions.

24 Hours	Ag	TiO_2
	RXRA	IGFBP3
	/	TGFB1
	/	WNT5A
120 Hours	Ag	TiO_2
	IL10	IL10
	CD44	/
	CDNK1A	/
	ESR1	ESR1
	MMP3	MMP3
	TGFBR2	TGFBR2

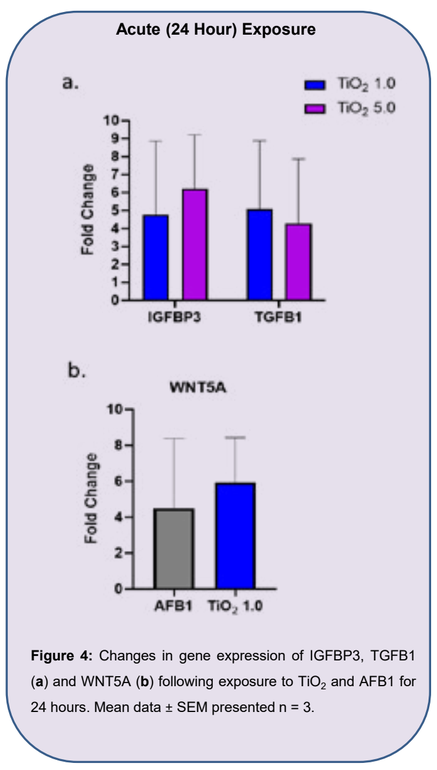

Figure 4: Changes in gene expression of IGFBP3, TGFB1 (**a**) and WNT5A (**b**) following exposure to TiO_2 and AFB1 for 24 hours. Mean data ± SEM presented n = 3.

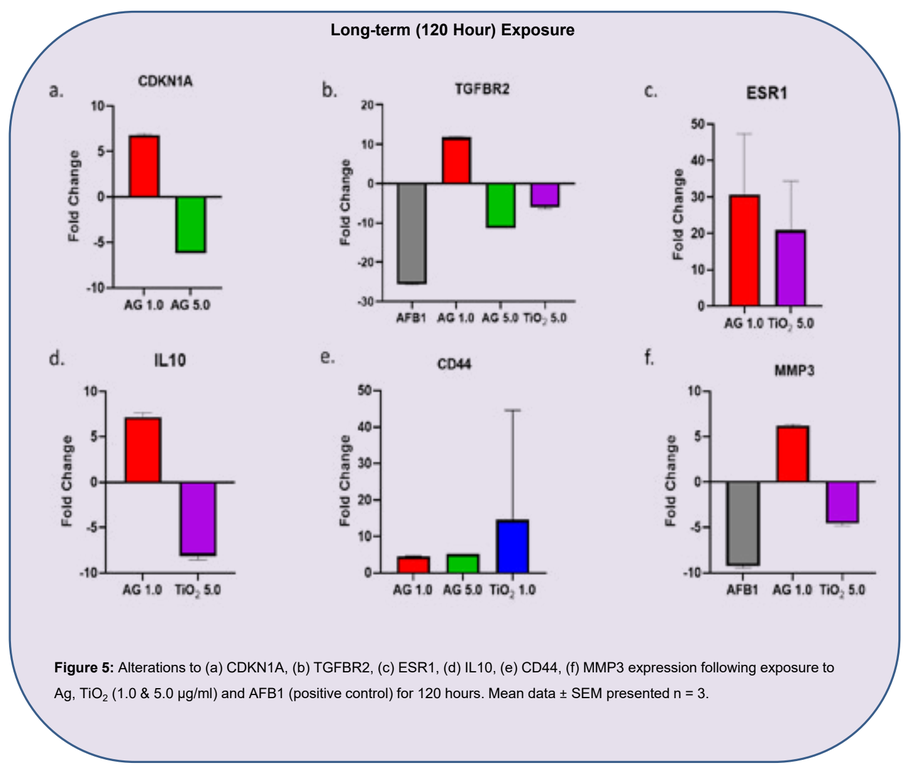

Figure 5: Alterations to (a) CDKN1A, (b) TGFBR2, (c) ESR1, (d) IL10, (e) CD44, (f) MMP3 expression following exposure to Ag, TiO_2 (1.0 & 5.0 µg/ml) and AFB1 (positive control) for 120 hours. Mean data ± SEM presented n = 3.

Summary of key findings:

- Exposure of Ag and TiO_2 to 3D HepG2 liver spheroids results in transcriptional alternations in genes known to be important in driving hepatocellular carcinoma. These changes appear to differ depending on the whether it is an acute or chronic exposure period (table 2 & 3).
- Key genes from each time point following the PCR array analysis have been highlighted (figure 4 & 5). Interestingly, the genes highlighted after 24hr exposures have been linked to both liver fibrosis and inflammation which known are precursors liver carcinogenesis.
- Due to little overlap between genes highlighted at 24 and 120hr, the transcriptional changes are time specific (table 4). At 24hr, shorter term changes are most likely related to early stage changes such as inflammation and fibrosis; while longer exposures are associated with transcriptional changes that are more relevant in carcinogenesis.
- By using the qPCR liver cancer arrays, we have been able to highlight a number of genes (table 4) that should be explored further to better understand their role in liver associated carcinogenesis following long-term ENM exposure.

Acknowledgments:

Thanks go to the *in vitro* toxicology group at Swansea University and all WP4 members of PATROLS

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

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