Microtissues for *in vitro* toxicity assessment: Cost effective and *in vivo* relevant toxicology tools

Conventional two-dimensional (2D) cell models (adherent cells grown on cell culture plates or cells in suspension) are limited in their abilities to accurately predict clinical toxicity since they lack the fundamental complexity of *in vivo* tissue environments. As a result efforts are being directed towards more sophisticated multicellular three-dimensional (3D) cell models with improved *in vitro* to *in vivo* correlation.

By Dr Stephanie Ravenscroft, Dr Caroline Bauch, Dr Laura Hinton and Dr Paul Walker

Conventional 2D cell cultures comprising a monolayer or suspension of cells have been at the forefront of *in vitro* toxicology advancements since the first immortalised cell line, HeLa, was established in 1951. Thus commenced an era of rapid and exciting developments in cell line identification and characterisation running in parallel to increases in sophistication of analysis techniques and their scalability. The field of *in vitro* toxicology subsequently established its role in preclinical drug safety assessment.

Despite enabling these advancements, 2D models lack the mature complexity of human organ tissue. A cells natural morphology *in vivo* is three-dimensional with cell types differing in their size, shape and cellular interactions enabling the creation of a unique tissue specific microenvironment. The aim of 3D cell culture models is ultimately to reflect this physiology and thus improve *in vitro* to *in vivo* translations. As a result of intense activity multiple 3D cell culture formats have arisen such as encapsulation of cells in collagen gels, micropattern plates (eg Hepregen's HepatoPac), biomaterial scaffolds (eg NanoFiber Solutions) or reconstituted tissues growing on transwell membrane plates (eg MatTek). However, while useful, these models do not always recapitulate the direct 3D cell-cell adhesions required to fulfil a cells *in vivo* phenotype.

One popular form of 3D culturing is the self-organisation of human derived cells into organotypic microtissues. Microtissues can allow scaffold-free direct cell-cell contacts with simultaneous extracellular matrix (ECM) interactions, often with cost-effective cell usage. Microtissue formation derives from tumour spheroid technology. Spheroids are 'spherical, heterogeneous aggregates of proliferating, quiescent and necrotic cells in culture that retain 3D architecture'[1]. While microtissues are considered spherical multicellular aggregates engineered to recapitulate the smallest functional unit of a tissue or organ. During self-organisation, cells produce their own

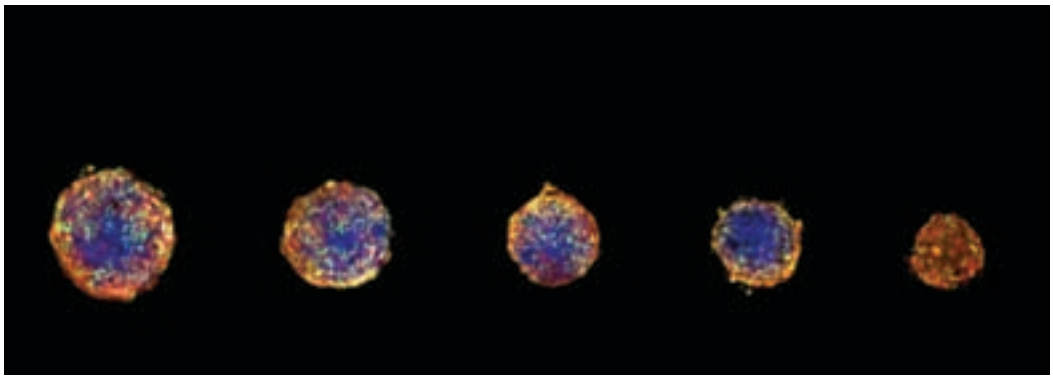

Figure 1
Representative 3D confocal high content screening (HCS) images of a known hepatotoxin, acetaminophen, labelled with Syto11 (green) to detect DNA structure, monochlorobimane (mBCL) (Blue) to detect GSH content, dihydroethidium (DHE) (yellow) to detect reactive oxygen species (ROS) formation and MitoTracker deep red (Red) to detect mitochondrial function

completely native ECM, facilitating extensive cellular contacts and thus promoting tissue-specific functions and integrated cellular responses to environmental stimuli with natural oxygen, osmotic and nutrient diffusion gradients. Multiple methods for microtissue or spheroid formation exist. The 'hanging drop' technique is a widely-used option relying on the aggregation potential of the cells within a suspended droplet of cell media. Traditionally performed using inverted petri dishes, multiple providers (InSphero AG, 3D Biomatrix) now supply 96- and 384-well plates for high throughput, uniform microtissue formation, utilising this technique. Using the same principles Corning® developed 96- and 384-well round bottom plates coated with an ultra-low attachment (ULA) formulation to prevent cell attachment and promote cell aggregation. Both of these plate types, hanging drop or ULA, permit high throughput biochemical analysis of microtissues or spheroids for toxicology assessment. However, due to the thin clear bottomed black walled wells, the ULA plates have allowed the advancement of 3D high content imaging of the microtissues or spheroids resulting in the possibility of performing complex multiparametric endpoint measurements on a single population of microtissues or spheroids.

Microtissue models and their *in vivo* relevance

In drug safety assessment mammalian multi-cellular microtissues are some of the most exciting *in vitro* 3D models available. Their development and use has been greatly advanced by improved primary cell isolation techniques, stem cell technology and deepened understanding of media supplements. Hence, organotypic microtissue models such as liver, cardiac and kidney now exist and are providing *in vitro* toxicology with more human physiologically relevant tools.

Hepatotoxicity

The liver is the largest solid organ in the human body and plays a critical role in dietary and pharmaceutical metabolism, blood glucose regulation, blood clotting, serum proteins synthesis and bile production. Hepatocytes are the main parenchymal cell within the liver, accounting for 60% of the total cell population. Pharmaceuticals can alter the activity of metabolising enzymes within hepatocytes leading to negative *in vivo* consequences such as drug-drug interactions, reduced efficacy and/or toxicity. Culturing of hepatocyte cells in 2D, however, results in loss of these hepatic-specific phenotypes over time and therefore often results in poor predictions of chemically induced hepatotoxicity *in vitro*. The remaining cell populations within hepatic tissue comprise non-parenchymal cells including Kupffer and endothelial cells. An alternative mechanism of hepatotoxicity is the generation of an immune response during which Kupffer cells play a key role. Therefore the development of organotypic *in vitro* liver models has aimed to incorporate both parenchymal and non-parenchymal cells within a 3D architecture.

Human liver microtissues (hLiMTs) comprising human cryopreserved primary hepatocytes and human cryopreserved primary non-parenchymal cells including Kupffer and endothelial cells have been developed, characterised and used in hepatotoxicity studies. Alternatively, the terminally differentiated hepatic cells, HepaRG, which are derived from a human hepatic progenitor cell line have also been used to form human relevant hepatic microtissues[2]. Both models display stable size and ATP content over five weeks in culture[3]. This prolonged life span permits their use in long term toxicity experiments to allow better reflection of a clinical repeat dose strategy. The multicellular aspect of hLiMTs allows them to replicate an inflammatory response in the presence of stimuli. This has been shown by the increased release of inflammatory marker IL-6

following exposure to lipopolysaccharide found on bacterial membranes[4]. Cytochrome P450 activity has been shown to be increased in hepatic microtissues and maintained for longer than in hepatocytes in 2D. Other liver characteristics such as albumin production and bile canaliculi structure also show marked improvements over conventional 2D hepatic models. Gunness et al[2] displayed enhanced albumin, urea, glucose, lactate and pyruvate production up to 21 days in HepaRG microtissues compared to monolayer HepaRG cells. MRP2 transporter activity was also highlighted by Gunness et al[2], while Messner et al[4] have highlighted the presence of BSEP and MDR1 transporters in hLiMTs suggesting the potential use of microtissues for hepatobiliary drug transport studies as well as hepatic cholestasis profiling.

HepaRG spheroids and hLiMT have been shown to predict hepatotoxicity with improved *in vivo* relevance. For example, acetaminophen, a known hepatotoxin, is often only determined to be toxic at concentrations far beyond relevant human concentrations using 2D systems. Both liver microtissue models (hLiMTs and HepaRG), however, are able to detect this toxicity at far lower concentrations following a repeat dosage scheme. **Figure 1** displays representative images of HepaRG microtissues exposed to increasing concentrations of acetaminophen and the resultant decrease in microtissue size and health. The minimal effective concentration (MEC) is 240µM, much closer to the maximum clinical concentration (Cmax) (165µM). The alternative 2D HepaRG model showed no sensitivity to acetaminophen (MEC >10,000µM). hLiMTs also show this enhanced sensitivity[3,4]. These findings further highlight the advantages of microtissue models as their enhanced longevity enables repeat dosing schedules.

An alternative cell line of relevance to hepatotoxicity that readily form spheroids are human hepatoma HepG2 cells. Ramaiahgari et al[5] developed and characterised HepG2 spheroids, concluding that HepG2

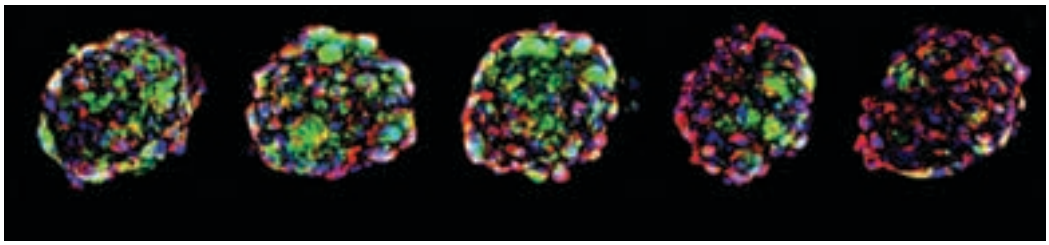

Figure 2: Representative 3D confocal HCS images of Isoproterenol calcium dyshomeostasis in spontaneously beating cardiac MT's labelled with Hoechst (blue) to detect DNA structure, Fluo-4 AM (green) to detect calcium dyshomeostasis and TMRE (red) to detect mitochondrial disruption

cells form functionally differentiated spheroids with good albumin production, cytochrome P450 activity, hepatocyte-like polarisation and hepatobiliary transport activity. Several known hepatotoxins were tested in 2D and 3D with or without repeat dosing. The results concluded that 3D HepG2 spheroids with repeat dosing correlates closest with *in vivo* findings. Together with HepaRG microtissues and hLiMTs this provides a broad range of options for studying hepatotoxicity *in vitro*.

Cardiotoxicity

Cardiovascular toxicity is the leading cause of drug attrition at the clinical level of drug development suggesting our pre-clinical models lack human *in vivo* relevance. The *in vivo* heart must respond rapidly to a wide range of cues ranging from neuronal to hormonal signals as well as ion flux and load pressure variations. The development of a representative *in vitro* model is therefore highly complicated.

Myocardial tissue is composed of 30% cardiomyocytes, the fundamental work unit of the heart. The remaining 70% of the myocardial cell population are non-cardiomyocytes predominantly fibroblasts, followed closely with microvascular endothelial cells[6]. In the myocardial tissue these three cell types are situated in close proximity to one another facilitating their dense network of communications. Fibroblasts are able to manipulate the extracellular matrix (ECM) and undergo dedifferentiation in response to cardiotoxins. This can ultimately lead to cardiac fibrosis influencing the stiffness of the myocardium and overall cardiac output. Microvascular endothelial cells form the dense microvascular network within the myocardium which enables the supply of oxygen and nutrients to service the high metabolic demands of the cardiomyocytes. Damage to the microvasculature through drug therapy can therefore ultimately lead to reduced cardiac efficiency.

Current *in vitro* cardiotoxicity assessments predominantly focus on cardiomyocytes alone in a restrictive 2D format, a long way from the architectural complexity of the myocardial tissue. With advancements in stem cell technology came the advent of stem cell-derived cardiomyocytes and as a result cardiac microtissue models have begun to emerge. Beauchamp et al[7] have developed and characterised cardiac microtissues formed from induced pluripotent stem cell-derived human cardiomyocytes. They found myofibrils, the contractile units of a cardiomyocyte, are present and aligned along the curvature of the outside of the microtissue often with continual linearity from one cell to the next allowing the microtissue to synchronise its spontaneous beat. This organisation is a feature of myocardial tissue *in vivo* and yet absent from standard 2D monolayer cultures of stem cell derived cardiomyocytes. Calcium homeostasis, an important highly controlled communication mechanism within mature cardiac tissue is often the target of drug-induced cardiac toxicity, therefore replicating this feature *in vitro* is critical to accurate drug safety assessment. The calcium modulator, caffeine was used to show the massive release and subsequent reuptake of calcium by the sarcoplasmic reticula in cardiac microtissues. Again this critical feature is often lacking in 2D cardiac culture systems.

Single cell-type cardiac microtissues have improved our ability to replicate *in vivo* phenotypes, however, one major drawback of these models is the lack of a multicellular microenvironment. Cyprotex has recently developed and launched a cardiac microtissue comprising stem cell-derived cardiomyocytes, cardiac microvascular endothelial cells and cardiac fibroblasts. This model has been used to demonstrate the accurate prediction of structural cardiotoxicity using high content screening (HCS) with a luminescence readout of cellular ATP content. Pointon et al[8] failed to detect the structural cardiac toxicity of isoproterenol and

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cyclophosphamide in their HCS assay based upon a monolayer of stem cell-derived cardiomyocytes. Using a similar high content approach Cyprotex in-house microtissues displayed a loss in calcium homeostasis with isoproterenol (**Figure 2**). These data provide further evidence of the benefit of cardiac microtissues in cardiac toxicity assessment and the potential for the accurate safety profiling of novel pharmaceuticals.

Nephrotoxicity

Drug-induced nephrotoxicity (DIN) is a major concern to drug discovery programmes leading to drug failures, withdrawals, or limiting therapeutic usage, especially for aminoglycoside antibiotics such as gentamicin. Some drugs and/or their metabolites may have the appropriate charge and size for filtration at the glomerulus gaining entry into the renal tubular epithelial cells via pinocytosis or endocytosis[9]. Other drugs are transported via peritubular capillaries and gain entry into renal tubular epithelial cells at the basolateral surface, where they are taken up by organic anion transporters (OATs) and organic cation transporters (OCTs) and eventually are effluxed into tubular lumens[10]. The proximal tubule is one of the main sites of reabsorption, as such DIN is often caused by accumulation of drugs in the renal cortex with resulting tubular damage and tubular cell cytotoxicity. Tubular fluid flows down the loop of Henle from the proximal tubule, where water is reabsorbed further increasing the tubular concentration of drug to potentially toxic levels. Tubular cells in the collecting duct and loop of Henle are at further risk for nephrotoxicity as they are highly metabolically active due to the presence of cytochrome P450s and other enzyme systems, therefore nephrotoxicity may be mechanistically linked to reactive oxygen species (ROS) as well as direct effects of drug metabolites[9].

To recapitulate this complex structure and function of the kidney *in vitro* is challenging. Current methods to detect potential DIN using *in vitro* high-throughput cytotoxicity screens have primarily relied upon using 2D monolayers of either primary kidney cells or kidney cell lines derived from proximal tubules, such as HK-2. Cellular responses indicative of DIN are often increased levels of ROS, oxidative stress, unfolded protein response (ER stress) and/or changes in cellular energy levels. Interference of drugs with transporters is another effect of nephrotoxicants and the uptake of proteins such as albumin can be inhibited. The use of primary kidney cells is preferred over immortilised cell lines as the latter lack many of the characteristics of their primary analogues often required for toxicity prediction with any clinical relevance. In addition, the lack of longevity seen with 2D *in vitro* models limits nephrotoxicity determination and is driving the desire to develop models that are more stable in culture.

In contrast to liver and cardiac 3D *in vitro* modelling which has been heavily influenced by microtissue formation, 3D kidney models began utilising transwell membrane culture systems and only recently expanded into microtissue development. Proximal tubular cells grown on transwell membrane culture systems allow the formation of epithelial barriers as they occur *in vivo* and recapitulate the apical and basolateral uptake of compounds *in vitro*. This approach allows the *in vitro* profiling of a drug's potential kidney transport. DesRocher et al[11] used immortilised human renal cortical epithelial cells in a transwell dish. This model permitted long-term culture of human-derived kidney cells with *in vivo*-like epithelial barriers. The authors concluded that 3D culturing in a transwell format improved *in vitro* to *in vivo* correlation due to enhanced sensitivity compared with conventional 2D kidney cell culture. Wilmes et al[12] determined the nephrotoxicity of cisplatin is associated with transporter-mediated accumulation of cisplatin and formation and accumulation of cisplatin metabolites in a human renal proximal tubule cell line which ultimately affected several cellular pathways (eg Nrf2, p53 signalling). This study was conducted using a 14-day repeat dosing regime. Prange et al[13] demonstrated the successful formation and characterisation of two kidney microtissues utilising either immortilised human kidney cell line HK-2 or primary human renal proximal tubular epithelial cells (HRPTEpiC) in combination with fibroblasts. The human primary kidney microtissues displayed enhanced expression of epithelial differentiation markers (AQP1, megalin and cubilin). Microtissues were also found to functionally uptake albumin and respond with increased sensitivity to known nephrotoxins, gentamicin and cadmium. Cyprotex recently developed multicellular kidney microtissues alongside HEK293 (immortilised human embryonic kidney cells) spheroids. Both models were sensitive to a panel of nephrotoxins including diclofenac which elicited induced oxidative stress as detected using confocal high content screening (**Figure 3**). Primary human kidney 3D cell models permit the *in vitro* replication of clinical repeat exposure strategies in a model with improved *in vivo* relevance and yet reduced cell usage costs.

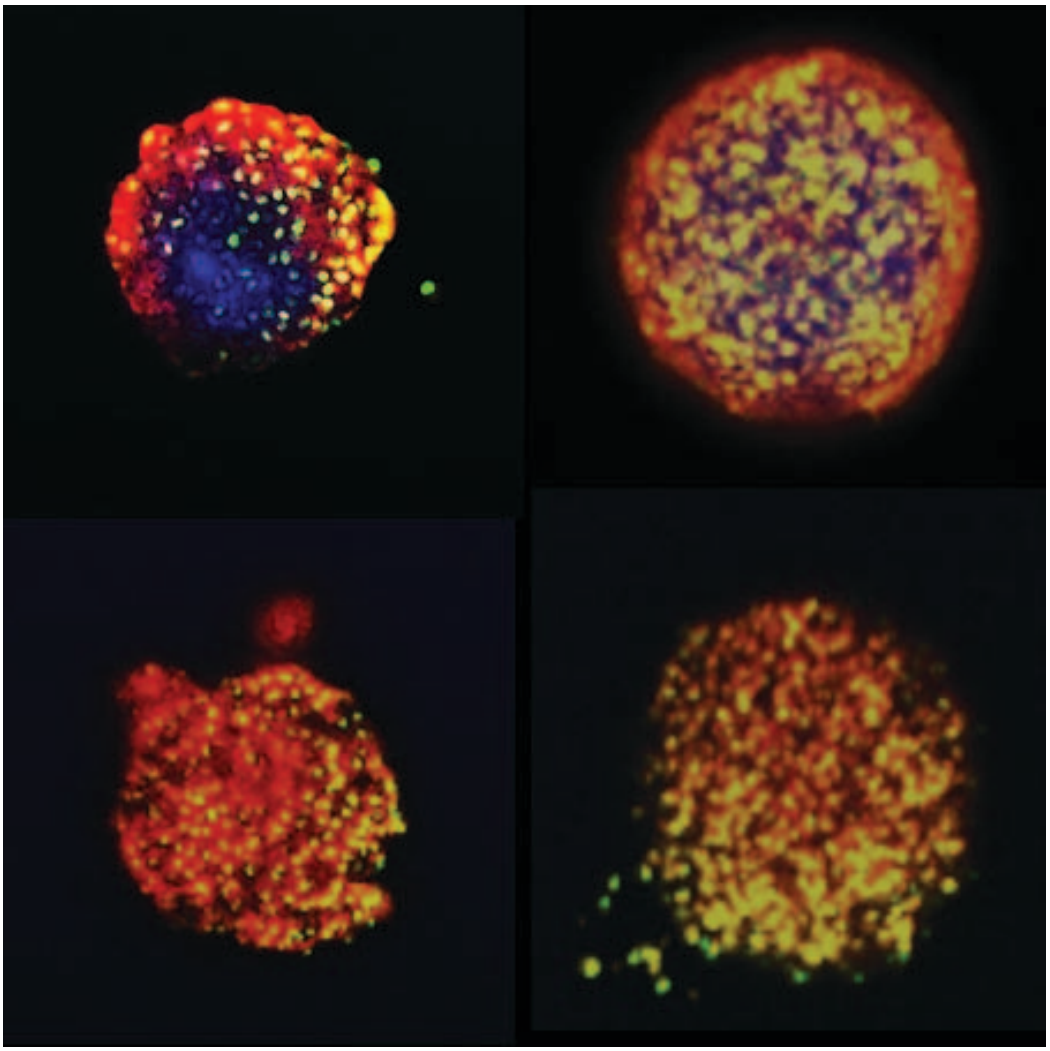

Figure 3: Representative 3D confocal HCS images of DMSO control and diclofenac (100μM) treated kidney spheroids. Cellular parameters assessed showed depletion in glutathione content (blue), increase in mitochondrial response (yellow). No changes in reactive oxygen species were detected (red)

Discussion

Recent and continued developments in 3D culturing of various cell types in co-culture are resulting in a 3D microtissue portfolio with a variety of options for drug safety assessment. Microtissues and spheroids are not only characteristically more *in vivo* relevant models but they also minimise cell usage allowing traditionally costly primary or stem cell-derived models to migrate to earlier in the safety assessment pipeline. Typically, a 96-well plate of microtissues would require 10-15x less cells than required in a 2D format, a dramatic cost reduction when using costly primary cells or iPSCs. These cost-effective organotypic models represent early *in vitro* screening tools capable of improving *in vitro* to *in vivo* translation.

In the pipeline over the coming months and years it can be expected that various other organ types will be the target of microtissue development including, but not limited to, brain, bone and lung. In parallel to these advancements 'Organs-On-Chip' technology is predicted to add another dimension to our current 3D capabilities. Organs-On-Chip are composed of a clear, flexible polymer about the size of a computer memory stick which contain hollow microfluidic channels. The ultimate aim of this technology is to link each human 3D organ model within one system to allow complete *in vitro* human toxicity profiling of a novel agent.

With the expansion of 3D analysis techniques such as the improvements in high content confocal imaging alongside the continual refinement of existing microtissue models and the addition of other organ types and technologies, this field is

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predicted to become a key aspect of the safety assessment pipeline. DDW

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