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Cell-free DNA in a three-dimensional spheroid cell culture model: A preliminary study

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ABSTRACT

Background: Investigating the biological functions of cell-free DNA (cfDNA) is limited by the interference of vast numbers of putative sources and causes of DNA release into circulation. Utilization of three-dimensional (3D) spheroid cell cultures, models with characteristics closer to the *in vivo* state, may be of significant benefit for cfDNA research.
Methods: CfDNA was isolated from the growth medium of C3A spheroid cultures in rotating bioreactors during both normal growth and treatment with acetaminophen. Spheroid growth was monitored via planimetry, lactate dehydrogenase activity and glucose consumption and was related to isolated cfDNA characteristics.
Results: Changes in spheroid growth and stability were effectively mirrored by cfDNA characteristics. CfDNA characteristics correlated with that of previous two-dimensional (2D) cell culture and human plasma research.
Conclusions: 3D spheroid cultures can serve as effective, simplified *in vivo*-simulating "closed-circuit" models since putative sources of cfDNA are limited to only the targeted cells. In addition, cfDNA can also serve as an alternative or auxiliary marker for tracking spheroid growth, development and culture stability.
Biological significance: 3D cell cultures can be used to translate "closed-circuit" *in vitro* model research into data that is relevant for *in vivo* studies and clinical applications. In turn, the utilization of cfDNA during 3D culture research can optimize sample collection without affecting the stability of the growth environment. Combining 3D culture and cfDNA research could, therefore, optimize both research fields.

1. Introduction

Since the discovery of cell-free DNA (cfDNA) in human plasma and other biological fluids (Fleischhacker and Schmidt, 2007; Peters and Pretorius, 2011), investigation of these peculiar cfDNA fragments has become a fast-growing research field due to its immense potential as a non-invasive diagnostic and prognostic marker for both disease and normal physiological conditions. Intercellular and inter-organ messaging functions have also been proposed for cfDNA in the form of newly synthesized, spontaneously released DNA/RNA-lipoprotein complexes (Aucamp et al., 2016; Gahan, 2006; Gahan and Stroun, 2010). Apart from the active release of cfDNA (Gahan and Stroun, 2010; Van der Vaart and Pretorius, 2007), practically any physiological source or process that can result in the release of DNA into biological fluids can contribute to cfDNA content (refer to Thierry et al. (2016) as an example of the multiple putative cfDNA sources in cancer). From an *in vivo* perspective this multitude of putative cfDNA sources results in background noise and false results. This interference, in concurrence with a lack of knowledge regarding the biological functions of cfDNA, are factors that prevent the effective translation of cfDNA research into clinical practice.

The study of the biological characteristics and functions of cfDNA through the utilization of *in vitro* cell cultures has been proposed. Our previous research showed that an osteosarcoma cell culture model has a distinct pattern of apoptotic and actively released DNA levels (Bronkhorst et al., 2016). However, two-dimensional (2D) cell cultures have their own set of difficulties regarding cfDNA research, one being whether the obtained information sufficiently reflects that of *in vivo* conditions. To date it has been well acknowledged that 2D cell cultures are not representative of the cellular environment found in organisms

Abbreviations: cfDNA, cell-free DNA; 2D, two-dimensional; 3D, three-dimensional; LDH, lactate dehydrogenase; CE, capillary electrophoresis; APAP, acetaminophen

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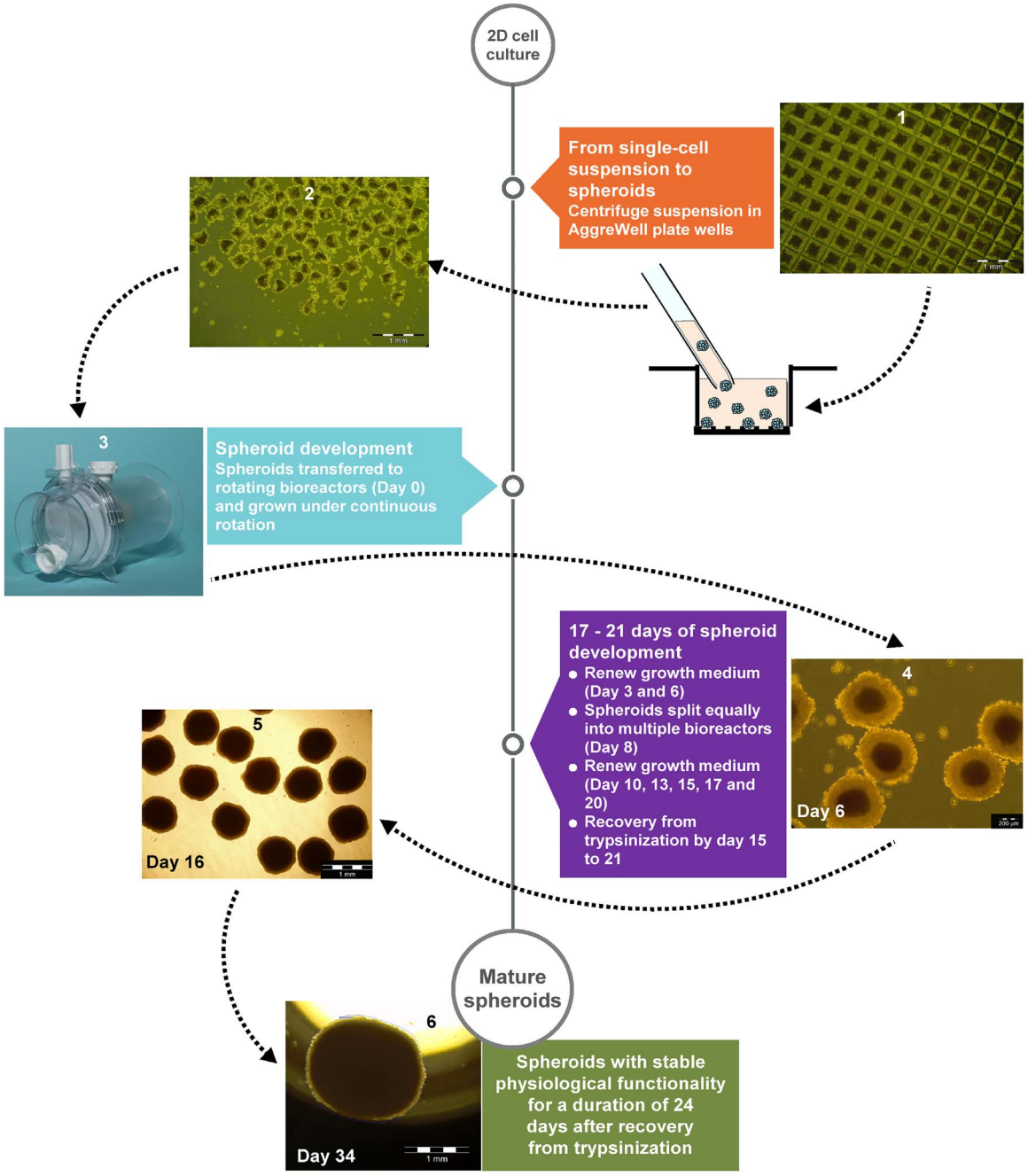

Fig 1. The development of HepG2/C3A spheroids via the rotating bioreactor technique (Wrzesinski and Fey, 2013; Wrzesinski et al., 2014). (1) AggreWell™400 with cells after centrifugation of the single cell suspension and 24 h of incubation; (2) Spheroids collected from the AggreWell™400; (3) Rotating bioreactor (MC2 Biotek) (Fey and Wrzesinski, 2012a); (4) Light microscopy of 6 day old HepG2/C3A spheroids; (5) Light microscopy of 16 day old HepG2/C3A spheroids; (6) Light microscopy of a HepG2/C3A spheroid 14 days after development (34 days old).

as they cannot effectively simulate tissue-specific architecture, mechanical and biochemical cues and cell–cell communication (Edmondson et al., 2014; Pampaloni et al., 2007). The use of living organisms, on the other hand, has its own unique scientific, ethical and social challenges (Edmondson et al., 2014). 2D cultures serve as "closed-circuit" models that promote the restriction of putative DNA sources to only that of the cell type in question. One will no longer have a "closed-circuit" model when using living models and, therefore, no control over the targeted physiological environment, resulting in background noise due to unforeseen biological influences. Moreover, the use of living organisms over *in vitro* methods also contravenes the 3Rs principle (Flecknell, 2002).

Comparison of the size profile of cfDNA from 2D cell cultures (Bronkhorst et al., 2016) to that of plasma samples (Applied-Biosystems, 2015) showed striking similarities, as both samples contained nucleosomal fragment patterns, as well as DNA fragments with a size of 2 000 bp, which was determined to not be of apoptotic or necrotic origin (Bronkhorst et al., 2016). This observation bridges the gap between the utilization of cell cultures and biological fluids in researching the biological functions of cfDNA. To further benefit from the simple, highly flexible, morally acceptable and less invasive approach of cell cultures, while maintaining the physiologically relevant cellular behavior of living organism models, the utilization of three-dimensional (3D) cell cultures has been investigated.